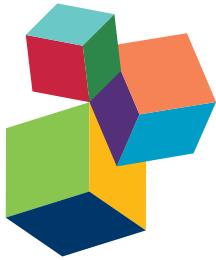


RECENT ADVANCES IN FLOWERING TIME CONTROL

EDITED BY: Christian Jung, Klaus Pillen, Dorothee Staiger,
George Coupland and Maria von Korff

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RECENT ADVANCES IN FLOWERING TIME CONTROL

Topic Editors:

Christian Jung, Christian-Albrechts-University of Kiel, Germany

Klaus Pillen, Martin Luther University Halle-Wittenberg, Germany

Dorothee Staiger, Bielefeld University, Germany

George Coupland, Max Planck Institute for Plant Breeding Research, Germany

Maria von Korff, Cluster of Excellence in Plant Sciences (CEPLAS), Heinrich-Heine-University Düsseldorf, Germany



Field trial in Northern Germany with oilseed rape (*Brassica napus* L.). The genotypes differ by their flowering time which is an important character in oilseed rape cultivation.

Cover photo by Christian Jung

The onset of flowering is an important step during the lifetime of a flowering plant. During the past two decades, there has been enormous progress in our understanding of how internal and external (environmental) cues control the transition to reproductive growth in plants. Many flowering time regulators have been identified from the model plant *Arabidopsis thaliana*. Most

of them are assembled in regulatory pathways, which converge to central integrators which trigger the transition of the vegetative into an inflorescence meristem. For crop cultivation, the time of flowering is of upmost importance, because it is a major yield determinant. Phenotypic variation for this trait is largely controlled by genes, which were often modified during domestication or crop improvement. Understanding the genetic basis of flowering time regulation offers new opportunities for selection in plant breeding and for genome editing and genetic modification of crop species.

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Editorial: Recent Advances in Flowering Time Control

Christian Jung^{1*}, Klaus Pillen², Dorothee Staiger³, George Coupland⁴ and Maria von Korff^{4,5}

¹ Plant Breeding Institute, Christian-Albrechts-University of Kiel, Kiel, Germany, ² Plant Breeding Institute, Martin Luther University of Halle-Wittenberg, Halle, Germany, ³ Molecular Cell Physiology, Faculty of Biology, Bielefeld University, Bielefeld, Germany, ⁴ Department of Plant Developmental Biology, Max Planck Institute for Plant Breeding Research, Cologne, Germany, ⁵ Cluster of Excellence in Plant Sciences, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

Keywords: floral transition, crop plants, *Arabidopsis*, phenological development, yield, evolution

Editorial on the Research Topic

Recent Advances in Flowering Time Control

The phenological development of plants can be broadly divided into 4 stages, embryo/juvenile, adult (all vegetative stages), reproductive (the generative stage), and senescent. This research topic focusses on the transition from vegetative growth to reproductive development, commonly referred to as floral transition. Plants have coordinated the seasonal timing of flowering and reproduction with the prevailing environmental conditions. In agriculture, flowering is a prerequisite for crop production whenever seeds or fruits are harvested. In contrast, avoidance of flowering is necessary for harvesting vegetative parts of a plant such as tubers or roots. Late flowering also severely hampers breeding success due to long generation times. Thus, flowering time regulation is of utmost importance for genetic improvement of crops.

In the past decades, we have gained increasing knowledge of flowering time regulation in model species such as *Arabidopsis thaliana* (Blümel et al., 2015). Genes coordinately regulating floral transition have been grouped into different pathways that have recently been illustrated in a WIKIPATHWAYS web interface (<http://wikipathways.org//index.php?query=flowering&title=Special%3ASearchPathways&doSearch=1&sa=Search>). Several of these pathways are activated by different environmental factors such as low temperature, day length, light intensity, or stress. Conservation of major flowering-time regulators and regulatory pathways between different species as well as increased availability of genome sequences and improvements in computational biology offer a unique opportunity to study flowering time genes across species. In general terms, the central elements that perceive day-length signals to control floral transition are conserved across the plant kingdom. CONSTANS (CO)-like genes and PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN (PEBP) encoding genes play major roles in these pathways and were first identified by genetic analysis in *Arabidopsis*. CO-like sequences seem to exist in all plants. Recent evidence indicates that CO of *Arabidopsis* arose from a family-specific duplication and similar events might have occurred independently in many plant families (Simon et al., 2015). In contrast, plants from the Amaranthaceae family are lacking a true CO ortholog (Dally et al., 2014).

This research topic is focused on flowering time control in cultivated species. It contains nine review, perspective, and opinion articles and 14 original research articles which cover a large range of organisms from model species to crops.

New components have been added to the network of flowering time regulators mostly working upstream of key regulator elements, e.g., GATA transcription factors, small RNAs, in particular microRNAs (miRNAs) or sugar molecules. The GATA transcription factors *GATA*, *NITRATE-INDUCIBLE*, *CARBON-METABOLISM INVOLVED* (*GCN*), and *GCN-like* (*GNL*) previously identified as growth regulators mediating control by several phytohormones have

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Neelima Roy Sinha,
University of California, Davis, USA

*Correspondence:

Christian Jung
c.jung@plantbreeding.uni-kiel.de

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emerged as repressors of flowering, acting via *SOC1* (Richter et al., 2013; Behringer and Schwechheimer). During the juvenile to adult phase transition, a gradual decrease in miR156 and a reciprocal increase in miR172 ultimately leads to the activation of *FLOWERING LOCUS T* (*FT*) (Wang et al., 2009a; Wu et al., 2009). To facilitate the genome-wide analysis of small RNA-seq data, the DARIO tool developed for animals has been adapted for use in plants (plantDARIO) (Patra et al.).

The research topic also reflects the immense technical progress from the past years. Initially, flowering time regulators from crops were mainly cloned due to their sequence homology with known flowering time genes, mainly from *Arabidopsis* although the *INDETERMINATE* flowering gene was cloned from maize by transposon tagging (Colasanti et al., 1998). Later, new genes were identified from crops using flowering time QTLs (quantitative trait loci) by map-based cloning approaches. Now, whole genome or candidate gene association mapping and transcriptome analysis have become important strategies (Schiessl et al.). For instance, whole transcriptome analysis revealed the circadian clock homolog of *EARLY FLOWERING 3* (*ELF3*) and mapping-by-sequencing applied on exome-capture data from phenotypic bulks identified *PHYTOCHROME C* as important components of photoperiodic flowering in barley (Faure et al., 2012; Pankin et al., 2014). In addition, the wild barley nested association mapping population HEB-25 was used to associate major flowering time genes with phenological development in different field environments (Maurer et al., 2015, 2016). Furthermore, several flowering time genes like *Ppd-H1* (*PRR37*) and *HvSDW1* (*GA20ox2*) were linked to both, developmental and yield-related traits. In a salinity tolerance study with HEB-25, the wild barley allele at the *HvCEN* locus (*Antirrhinum CENTRORADIALIS*, *TFL1*-like) promoted flowering and maturity, resulting in a higher harvest index and a higher yield under salt stress in the field (Saade et al., 2016). These findings indicate that searching for allelic variants of known flowering time genes, also taken from related wild species, may substantially support future plant breeding efforts to increase plant performance under optimal cultivation conditions as well as under stress.

Flowering time regulation is strongly conserved among the Brassicas to which *Arabidopsis* also belongs. Two articles (Guo et al.; Schiessl et al.) describe flowering time genes from oilseed rape where the vernalization pathway with its central element *FLOWERING LOCUS C* (*FLC*) is essentially the same as in *Arabidopsis*. In contrast, an *FLC* homolog from beet was proven not to be a major regulator of vernalization response in biennial beets Vogt et al. New sequence variation has been induced in rapeseed by EMS mutagenesis which gave rise to plants with altered flowering time in spite of the polyploid nature of this species. Mutations within a single gene can have a big impact on flowering time even if there are several paralogs of an *Arabidopsis* flowering time gene present in the rapeseed genome.

The research topic demonstrates that the range of model species has been constantly increased to allow a broader range of flowering-related traits to be studied. *Arabis alpina* and *Brachypodium distachyon* serve as models for perennials (Wang et al., 2009b) and for grasses Woods et al., respectively. A recent overview on flowering regulation in grass species is given

in this research topic (Fjellheim et al.). The authors discuss molecular pathways that control seasonal flowering responses in the *Pooideae* sub-family and how variations in flowering time gene activities contributed to the adaptation to different environments. Refined flowering time regulatory pathways have been identified from rice (Shrestha et al., 2014) and barley/wheat (Chen et al., 2014) (Mulki and von Korff, 2016). Loscos et al. show that natural allelic variation in copy number of the florigen *HvFT1* is present in European spring barley cultivars lacking a vernalization requirement to initiate flowering (Loscos et al., 2014). However, no clear relationship between *HvFT1* copy number and expression was observed in a set of diverse spring barley genotypes.

Some articles from this research topic highlight multiple functions of flowering time genes beyond floral transition. These genes impact multiple developmental processes and they are regulators of yield components and stress tolerance (Kazan and Lyons, 2016). In this respect, members of the PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN (PEBP) gene family, such as *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*) of *Arabidopsis* attracted the highest attention. As highlighted for tomato (Lifschitz et al.) and for rice (Izawa et al., 2016) fine tuning of the *SFT/SP* (which are true orthologs of *FT* and *TFL1*) ratio is an important process for patterning plant architecture. Lifschitz et al. explain how the ratios between *FT*-like and *TFL1*-like genes control the patterning of the shoot systems across many different plants. In tomato, an increase of the florigen protein *SINGLE FLOWER TRUSS* (*SFT*, *FT*-like), relative to the anti-florigen protein *SELF PRUNING* (*SP*, *TFL1*-like) induces growth arrest and termination of meristems across the tomato shoot, while high relative levels of *SP* promote the formation of an indeterminate vegetative inflorescence. Consequently, *SFT/SP* ratios determine the number of flowers and eventually, tomato fruits per inflorescence. Naturally occurring mutations have been selected in both gene families to adapt crops to different environments and to increase productivity. *TFL1* mutants have been frequently used in breeding (e.g., tomato, soybean, roses, and barley). Likewise, mutations of *FT*-like genes were selected in sunflower, sugar beet, rice, potato, and wheat. Moreover, single point mutations within *FT*- and *TFL1* orthologs can drastically alter their function from floral inducers to floral repressors, as demonstrated for sugarcane Coelho et al. and beet (Pin et al., 2010). We propose that selecting for *FT/TFL1* sequence variations in crop plants may pave the way to further improvements in elite crop productivity.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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GIGANTEA – an emerging story

Priyanka Mishra and Kishore C. Panigrahi*

Plant Science Lab, School of Biological Sciences, National Institute of Science Education and Research, Bhubaneshwar, India

Edited by:

George Coupland, Max Planck Society, Germany

Reviewed by:

Damon Lisch, Purdue University, USA
Fabio Fornara, University of Milan, Italy

***Correspondence:**

Kishore C. Panigrahi, Plant Science Lab, School of Biological Sciences, National Institute of Science Education and Research, IOP campus, Sachivalaya Marg, P.O. Sainik School, Bhubaneshwar 751005, Orissa, India
e-mail: panigrahi@niser.ac.in

GIGANTEA (GI) is a plant specific nuclear protein and functions in diverse physiological processes such as flowering time regulation, light signaling, hypocotyl elongation, control of circadian rhythm, sucrose signaling, starch accumulation, chlorophyll accumulation, transpiration, herbicide tolerance, cold tolerance, drought tolerance, and *miRNA* processing. It has been five decades since its discovery but the biochemical function of GI and its different domains are still unclear. Although it is known that both *GI* transcript and GI protein are clock controlled, the regulation of its abundance and functions at the molecular level are still some of the unexplored areas of intensive research. Since GI has many important pleiotropic functions as described above scattered through literature, it is worthwhile and about time to encapsulate the available information in a concise review. Therefore, in this review, we are making an attempt to summarize (i) the various interconnected roles that GI possibly plays in the fine-tuning of plant development, and (ii) the known mutations of *GI* that have been instrumental in understanding its role in distinct physiological processes.

Keywords: GIGANTEA, flowering time regulation, circadian clock control, GI mutants, light signaling

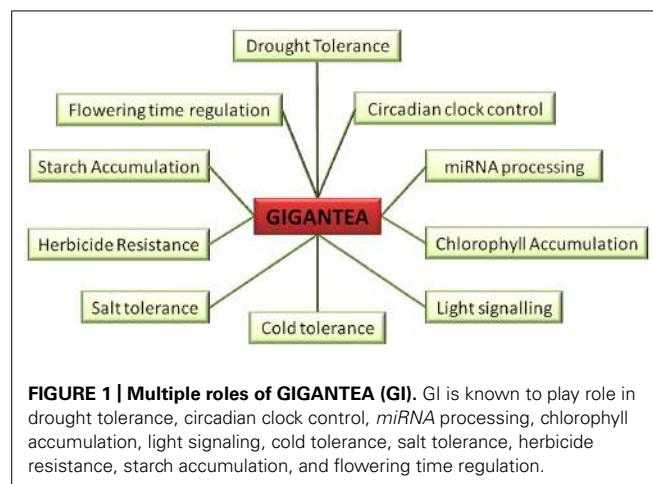
INTRODUCTION

GIGANTEA (GI), the unique plant specific nuclear protein, although identified way back (Rédei, 1962) as a late flowering mutant (*gi*) in *Arabidopsis thaliana* (*At*), its precise biochemical roles are far from being understood (de Montaigu et al., 2010). The genomic organization of *GI* was evident after it was fine-mapped to chromosome 1 and subsequently, the *GI* cDNA was isolated (Fowler et al., 1999). The genomic locus of *GI* of *At* consists of 14 exons and encodes for a protein of 1173 amino acids (Fowler et al., 1999; Park et al., 1999). *GI* expression is ubiquitous and is detected throughout various stages of plant development indicative of its involvement in several functions summarized in Figure 1 (Fowler et al., 1999; Park et al., 1999). It is interesting to note the ubiquitous expression of *GI* that reflect upon its pleiotropic roles in multitude of responses ranging from breaking of seed dormancy, hypocotyl elongation, initiating the circadian rhythm in seeds to the setting of fruits in the adult plant. Many of the above listed responses integrate information from the light input and external temperature, making it an interesting but complicated area of plant science.

Experiments aimed at understanding the abundance of the transcript and the protein are typically carried out in controlled cabinets, where the subjective time of the diurnal cycle are referred as the Zeitgeber time (ZT). Both the *GI* transcript and GI protein are under the control of diurnal regulation. Under long day (LD) growth cycle of 16 h light and 8 h dark (16 hL/8 hD), the *GI* mRNA peaks at ZT 10 and shows a trough at ZT 0, while under short day (SD) cycle of 8 hL/16 hD, *GI* transcript level peaks at ZT 8 (Fowler et al., 1999). The GI protein abundance also follows a similar pattern to its transcript accumulation (David et al., 2006). The regulation of *GI* is important for the control of circadian clock and several genes such as *FLAVIN-BINDING*, *KELCH REPEAT*, *F BOX 1 (FKF1)*, a blue light photoreceptor, and *CYCLING DOF FACTORs (CDFs)*, which are involved in the transcription of a

flowering time regulator *CONSTANS* (CO; Fornara et al., 2009). In addition, the diurnal regulation of the protein might also play an important role in the diurnal control of stomatal opening (Ando et al., 2013).

In order to assign a function to *GI*, it was of interest to enumerate its precise sub-cellular localization. Therefore, N-terminal GFP fusion of *GI* was constructed and transiently transfected in onion epidermal cells. The fluorescence microscopy of the fusion protein for the first time demonstrated that *GI* is predominantly localized to the nuclei and forms nuclear bodies (Huq et al., 2000). Later, the *GI* protein was also found to be localized in the nucleus of different cell types of transgenic *At* plants over-expressing *GI:GFP* (Mizoguchi et al., 2005). Four clusters of basic amino acids resembling the nuclear localization signal (NLS) in the *GI* sequence explained its nuclear abundance (Huq et al., 2000). *GI* has been shown to form nuclear bodies of diverse numbers, size, and shape (Kim et al., 2013c). To understand the molecular composition of *GI* nuclear bodies, attempts were made to evaluate the co-localization of *GI* with marker proteins of known sub-nuclear compartments such as heterochromatin bundles, nucleoli, spliceosome, and Cajal bodies. This piece of work demonstrated that *GI* did not localize to any of the above known nuclear compartments (Kim et al., 2013c). This suggested that *GI* might not have role in processes such as biogenesis of rRNA and snRNP, pre-mRNA splicing, and protein degradation. Since these co-localization studies were carried out in *Arabidopsis* mesophyll protoplasts using a transient over-expression method, it does not mimic the exact physiological environment. Furthermore, the association and dissociation rate of proteins to nuclear bodies has been shown to be affected by specific post-translational modifications. The spatio-temporal mis-localization of proteins can also affect its post-translational modifications. With so many complexities involved, stable transgenic lines expressing fluorescent tagged marker proteins and *GI* under their native promoters



would be an impressive feat to achieve in order to understand the molecular composition of the GI complexes. Understanding the molecular composition of GI nuclear bodies (NBs) at different diurnal time-points would be a valuable asset.

The formation of GI nuclear bodies is light dependent since, the sequestration of GI into NBs is facilitated by EARLY FLOWERING 4 (ELF4) during the day, thus inhibiting the *CO* transcription. Likewise, EARLY FLOWERING 3 (ELF3) promotes the interaction of GI and CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) to form NBs which degrade GI in *planta* (Yu et al., 2008). The dynamic association of GI with heterogenous nuclear bodies during the light to dark transition needs to be evaluated. In other words, the question still remains, if GI associates and dissociates in a light dependent manner on a core complex within the nuclei based on its differential post-translational modification status.

Although studies showed the presence of GI predominantly in the nuclei, *in silico* analysis predicted the presence of 11 trans-membrane domains in *AtGI* which argues in favor of a possible membrane localization (Fowler et al., 1999). Furthermore, membrane localized GI possibly has a role in the regulation of ion channels during salt stress and stomatal opening as seen in phototropins (Stoelzle et al., 2003). Purified recombinant GI from *Escherichia coli* when subjected to electron microscopic study, revealed a tetrameric arrangement *in vitro*. However, its quaternary structure *in vivo* is still unclear (Black et al., 2011). This multimeric organization of a protein would not only offer more epitopes for interactions with diverse regulators but also would offer additional layers of control on its stability.

ALLELES OF GIGANTEA WITH DISTINCT PHENOTYPES

The *gi* mutants were described as late flowering mutants for the first time (Rédei, 1962). There are several *gi* mutants described in literature such as *gi-1*, *gi-2*, *gi-3*, *gi-4*, *gi-5*, *gi-6*, *gi-11*, *gi-12*, *gi-100*, *gi-200*, *gi-201*, *gi-596*, and *gi-611* (summarized in **Table 1**; **Figure 2**). Some of the *gi* mutants were shown to influence the activity of the circadian clock, while others alter diverse responses (Park et al., 1999). The *gi-1* allele, lacking the C-terminal part of GI, was responsible for shortening the period of the clock, while the *gi-2* allele, lacking both the C-terminal and the central region

of GI, lengthened the period. While the *gi-1* mutation shortened the period of *CAB2* expression, the *gi-2* mutation lengthened the period of *CAB2* expression (Park et al., 1999). This suggests that the central region of the protein or the terminal half of the protein most probably fine-tunes the period length of the circadian clock.

The *gi-2* mutant at higher temperature of about 28°C showed longer hypocotyl and flowered earlier in comparison to the plants grown at temperatures of 18 and 22°C (Araki and Komeda, 1993). Even though higher temperature were shown to regulate flowering (at 18, 22, 28°C) and hypocotyl elongation (at 22, 28°C) in *gi-2* mutant, it was almost equally sensitive toward vernalization as in WT. Vernalization is the exposure of plants to prolonged cold temperature that leads to earlier flowering cue in *Arabidopsis*. This implies that probably GI regulates flowering using a vernalization-independent pathway (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991; Araki and Komeda, 1993).

The alleles of *GI* are the result of random mutagenesis or T-DNA insertion which have aided in understanding its various functions. Alleles such as *gi-1*, *gi-2*, *gi-3*, and *gi-6* introduce premature stop codon whereas *gi-4* and *gi-5* most probably alter the C-terminus of the protein due to frame-shift mutations (Fowler et al., 1999). No *GI* expression was detected in the *gi-11* and *gi-201* alleles carrying T-DNA insertion (Richardson et al., 1998; Martin-Tryon et al., 2007). The *gi-100* mutation, originally identified in a red light screen, also contained a T-DNA insertion, but produced a truncated transcript of about 2 kb due to the absence of the 3' end of *GI* (Huq et al., 2000). The transcript level in *gi-1*, *gi-2*, and *gi-3* is lower compared to that of *gi-4*, *gi-5*, *gi-6*, and *gi-100*, which show similar or higher levels compared to their respective WT (Fowler et al., 1999; Huq et al., 2000). The role of GI in blue light dependent hypocotyl elongation was revealed using the *gi-200* allele, consisting of a substitution of the serine 932 (Martin-Tryon et al., 2007).

Various deletions in *GI* sequences and its phenotypes are summarized in **Table 1**. After analyzing the data depicted in **Table 1**, it is evident that any deletion in *GI* mostly causes defects in the flowering time, circadian clock, and control of hypocotyl elongation. In the *gi-4* mutant, improper splicing leads to a loss of 90 amino acids from the C-terminus causing late flowering. This deletion also causes the over-expression of its own transcript suggesting that the C-terminal 90 amino acids are required for its auto-regulation and flowering time (Fowler et al., 1999). The abundance of the *gi-4* transcript could be due to increased stability or decreased decay which needs to be verified. Since GI stimulates *CO* transcription, this C-terminal domain of GI might be acting as an enhancer of *CO* transcription or involved in the recruitment of activators to the *CO* promoter.

The seeds of Wassilewskija (Ws) ecotype expressing *CAB:LUC* were mutagenized and screened for altered period length. Two novel alleles *gi-596* and *gi-611* were identified in this screen (Gould et al., 2006). In the *gi-596* allele, mutation caused by the substitution of the serine residue at 191 position to phenylalanine (S191F) did not affect the flowering-time although the period length of the circadian clock is lengthened and longer hypocotyl was observed under both red and blue light conditions. This suggests that the

Table 1 | List of known *Gi* alleles and their phenotypes.

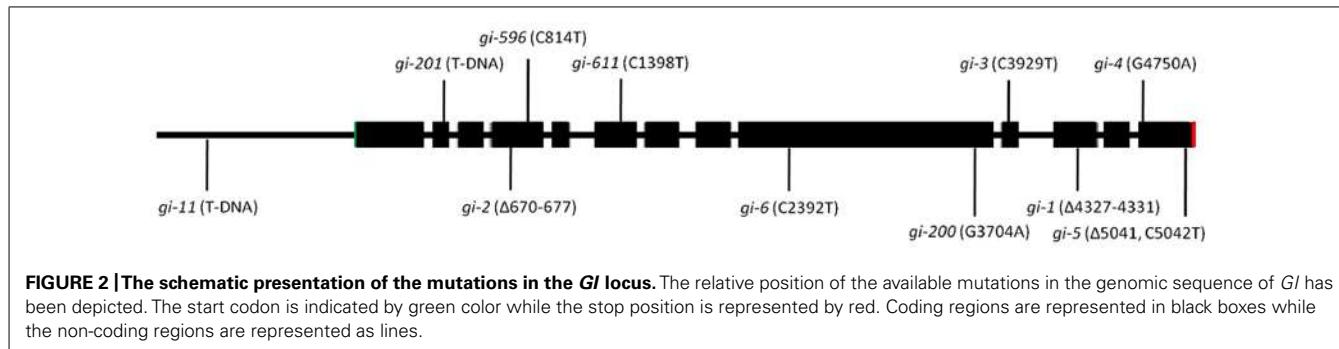
| No. | Allele name background site of mutation | Phenotypes | | | | | | Key reference |
|-----|----------------------------------------------------------------------------------------|-----------------------------------------|-------------------------------------|-----------------------------------------------|------------------------|------------------------------------|-------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| | | Flowering time | Starch content | Circadian rhythm | Herbicide tolerance | Hypocotyl length | Cold tolerance | |
| 1 | gi-11 Ws | Late ^c | NA | NA | NA | NA | NA | TAIR; ^c Fowler et al. (1999) |
| | T-DNA insertion 407 bp upstream of start codon. | | | | | | | |
| | 3–4 kb of the genomic sequence, removing the 5' half of the gene and upstream sequence | | | | | | | |
| 2 | gi-201 Col-0 | Late ^f | NA | Damped, altered circadian rhythm ^f | NA | Longer in RL and BL ^f | NA | f Martin-Tryon et al. (2007) |
| | T-DNA insertion in second exon (66 amino acid, aa) | | | | | | | |
| 3 | gi-2 Col-0 | Late ^{a,c} | High ^b | Short decreased period length ^d | NA | Longer in RL and BL ^{e,f} | NA | TAIR; ^a Araki and Komeda (1993), ^b Eilmert et al. (1995), ^c Fowler et al. (1999), ^d Park et al. (1999), ^e Huq et al. (2000), f Martin-Tryon et al. (2007), ^f Gould et al. (2006) |
| | Δ (670–677) bp premature stop codon (144 aa) | | | | | | | |
| 4 | gi-596 Ws | Not Affected ⁱ | Long circadian period ^j | NA | NA | NA | NA | TAIR; ⁱ Gould et al. (2006) |
| | S191F (C814T) | | | | | | | |
| 5 | gi-611 Ws | Early in short photoperiod ^j | Short circadian period ^j | NA | NA | NA | NA | TAIR; ^j Kurepa et al. (1998a), ⁱ Gould et al. (1999) |
| | L281F (C1398T) | | | | | | | |
| 6 | gi-6 Ler-0 | Late ^c | NA | NA | NA | NA | High | TAIR; ^c Fowler et al. (1999) |
| | Q493Stop codon (C23992T) – 492 aa | | | | | | | |
| 7 | gi-200 Col-0 | NA | NA | Short circadian rhythm ^f | NA | Longer in RL and BL ^f | NA | f Martin-Tryon et al. (2007) |
| | S932N (G3704A) | | | | | | | |

(Continued)

Table 1 | Continued

| No. | Allele name background site of mutation | Phenotypes | | | | | | Key reference |
|-----|-----------------------------------------------------------------------------------------------------------------|-----------------------|-------------------|---------------------------------------------|----------------------------------------------------------------------|------------------------------------|-----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| | | Flowering time | Starch content | Circadian rhythm | Herbicide tolerance | Hypocotyl length | Cold tolerance | |
| 8 | gi-3 Ler-0 Q964Stopcodon (C3929T) – 963 aa | Late ^{a,c,h} | High ^b | LHY, CCA1 expression lowered ^h | Tolerant toward paraquat, H ₂ O ₂ ^g | Longer in RL and BL ^{f,h} | Tolerant ⁱ | High ^g TAIR; ^a Araki and Komeda (1993), ^b Eimert et al. (1995), ^c Kurepa et al. (1998a), ^d Fowler et al. (1999), ^e Cao et al. (2005), ^f Mizoguchi et al. (2005), ^g Martin-Tryon et al. (2007) |
| 9 | gi-1 Col-0 Δ (4327–4331) bp | Late ^{a,c} | High ^b | Short; decreased period length ^d | NA | Longer in RL and BL ^{e,f} | NA | NA TAIR; ^a Araki and Komeda (1993), ^b Eimert et al. (1995), ^c Fowler et al. (1999), ^d Park et al. (1999), ^e Huq et al. (2000), ^f Martin-Tryon et al. (2007) |
| 10 | gi-4 Ler-0 G4750A (G-splice acceptor site) – improper splicing. Premature stop codon (1083 aa). | Late ^c | NA | NA | Longer in RL and BL ^f | Longer in RL and BL ^f | Tolerant ⁱ | High ^g TAIR; ^a Kurepa et al. (1998a), ^c Fowler et al. (1999), ⁱ Cao et al. (2005), ^f Martin-Tryon et al. (2007) |
| 11 | gi-5 Ler-0 C5042T and Δ5041 bp deletion | Late ^c | NA | NA | NA | NA | NA | High ^g TAIR; ^a Kurepa et al. (1998a), ^c Fowler et al. (1999) |
| *12 | gi-12 Col-0 T-DNA insertion in GI coding region | Late ^c | NA | NA | NA | NA | NA | cFowler et al. (1999) |
| *13 | gi-100 Col-0 3' end unexpressed – ~2 kb transcript produced | Late ^e | NA | NA | NA | Longer in RL ^e | NA | NA eHuq et al. (2000) |

Various reported alleles of GI based on the site of mutation and the corresponding phenotypes are depicted in the table below. In the case of GI alleles represented by *, the precise location of the mutations are not known and as such, arranged based on the time of discovery.



serine 191 residue might have an important role in photoreceptor signaling. On the contrary, the mutation in *gi-611* allele was mapped to the lysine 281. This allele showed significantly early flowering in SDs suggesting that this lysine in WT is involved in decelerating the flowering time (Gould et al., 2006). Since the Ws ecotype is a natural null for the high light sensor Phytochrome D, the phenotype observed could be a combinatorial effect of the lack of this photoreceptor and the respective mutations in *GI* allele (Aukerman et al., 1997). It would be interesting to evaluate if these alleles in Col background would show the similar light dependent effect to rule out the involvement and interaction of PHYD in this process. Both the positions, Lys281 and Ser191 are conserved in the Col-0 and Ler-0 ecotypes and thus, the role of these residues could be confirmed by the expression of the respective *GI* alleles containing the substitutions in these ecotypes to determine the importance of these mutated residues.

TRANSCRIPTIONAL REGULATION OF GIGANTEA

Defects in the circadian clock components have been found to affect the *GI* transcription. CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), a core component of the circadian clock, reduces the *GI* expression by binding to CCA1-binding site on *GI* promoter (Lu et al., 2012). *GI* transcript, thus accumulates toward the middle of the day, when CCA1 expression is repressed by TIMING OF CAB EXPRESSION 1 (TOC1). The rhythmicity of *GI* transcript level is lost in *elf3* mutant in continuous light (LL) suggesting that ELF3 might also regulate the *GI* mRNA abundance (Fowler et al., 1999). Since CCA1 and ELF3 have been proposed to physically interact to control flowering time and hypocotyl elongation, it would be interesting to investigate the coordinated involvement of these two proteins in the regulation of *GI* transcription. Clock proteins, such as, LIGHT-REGULATED WD 1 and 2 (LWD1 and LWD2) also affect the *GI* expression pattern, since in *lwd1lwd2* double mutant *GI* transcript is most abundant at ZT 6 instead of ZT 10 (Wu et al., 2008). The two proteins being very similar (~90% identity) possess functional redundancy, evident from single mutants being phenotypically similar to WT. Another clock associated gene, TIME FOR COFFEE (*TIC*) is also known to regulate the rhythmicity of *GI* in *Arabidopsis*. In *tic* mutants, *GI* transcript level is lower and the peak is shifted ~4 h earlier than in WT plants (Hall et al., 2003). Since in both the *lwd1lwd2* and *tic* mutants the *GI* expression is shifted to ZT6, it suggests that the activities of both the proteins might be required for the repression of the *GI* transcription in the morning. pseudo-response regulators (PRRs),

namely, PRR5, PRR7, and PRR9 also have been proposed to regulate *GI* expression and therefore, flowering time via the CO-FT module (Nakamichi et al., 2007; Kawamura et al., 2008). Epistatic analysis and mutant combinations between *LWD1/2*, *PRRs*, and *TIC* would be beneficial to explain the additive roles of these genes products and the genetic hierarchy of the genes regulating the inhibition of *GI* expression. The expression of *GI* at the wrong time of the diurnal cycle is known to cause flowering time defects in *At* (Fornara et al., 2009). These mutants might behave as late flowering due to the untimely expression of *GI*. Although a lot is known from the transcript analysis, the work at the protein level is far from being understood due to the unavailability of a *GI* anti-serum that could detect the endogenous *GI* protein. The detailed post-translational regulation of *GI* is explained in the Section “Post-Translational Regulation of GIGANTEA.”

Several studies have demonstrated that light quality and quantity influence *GI* transcription, although systematic studies involving changes in the light fluence and wavelength to evaluate *GI* expression is yet to be carried out. In *Arabidopsis*, upon transition to night, *GI* mRNA level decreases with a half-life of about 1 h irrespective of the photoperiod (Fowler et al., 1999). A significant light dependent down-regulation is also detected in the legume *Medicago truncatula* suggesting a similar mechanism might coordinate light sensing with transcriptional activity (Paltiel et al., 2006). *GI* mRNA accumulation pattern in both *Arabidopsis* and *M. truncatula* showed a secondary peak at ZT 2 under SDs as well as LDs (Paltiel et al., 2006). This peak could be the result of an acute response to light. A similar peak of *GI* mRNA at ZT 2 has also been documented in plants grown under blue light. The role of blue light in the regulation of this early secondary peak of *GI* needs to be thoroughly examined using mutants that are affected in blue light signaling. This would clarify if the peak at ZT 2 is due to photoreceptors or secondary signaling components involved in blue light signaling. The peak expression of *GI* is delayed by approximately 4 h in plants grown in low red:far-red (R:FR) light conditions in comparison to plants grown in white light condition (Wollenberg et al., 2008). This indicated that the photoreceptors and their activity are fine-tuning the timing and quantity of the *GI* transcript. The accumulation of the *GI* protein in the morning around ZT 3–4 and its consequence in plant development has not been studied yet, that needs to be evaluated in depth.

Besides light, temperature too has been found to regulate *GI* expression. Warmer temperature of 28°C up-regulates *GI* mRNA

level as compared to the cooler temperatures of 12°C at dawn (Paltiel et al., 2006). The night time repression of *GI* transcription was shown to be temperature dependent and regulated by evening complex (EC) night time repressor constituted of ELF3, ELF4, and LUX ARRHYTHMO (LUX; Nusinow et al., 2011; Mizuno et al., 2014). The EC night time repressor was revealed to bind to the *GI* promoter through LUX binding site (LBS).

GIGANTEA has been proposed to regulate its own expression, since the mutants, *gi-1* and *gi-2*, have lower expression of the *GI* alleles, approximately 40 and 20% of the WT transcript, respectively (Fowler et al., 1999; Park et al., 1999). But this auto-regulatory role of *GI* transcription is contradictory, since, *gi-4* and *gi-6* lines show ~30% higher expression of *GI* compared to its WT (Fowler et al., 1999). This effect could be either due to the difference in the ecotypes or differential regulation of the transcript stability. Another question worth investigating would be the abundance of the mutant proteins produced in each mutant, which would require a functional *GI* antiserum. The positive or negative auto-regulatory role suggests that mutations at different residues in the coding sequence can influence the abundance of transcriptional enhancers or repressors, affecting *GI* expression.

POST-TRANSLATIONAL REGULATION OF GIGANTEA

Over-expression of *GI* leads to the constitutive accumulation of the *GI* transcript throughout the photoperiod. Despite its constant expression level, *GI* protein follows a cyclic pattern of accumulation in both LDs and SDs. This is suggestive of the degradation of the *GI* protein (David et al., 2006). *GI* was found to be ubiquitinated upon dusk, a pre-requisite for its degradation via the 26S proteasome mechanism (David et al., 2006). In the dark phase, nuclear *GI* abundance has been shown to be regulated by the E3 ubiquitin ligase activity of COP1 and ELF3 (Yu et al., 2008). The interaction between COP1 and *GI* is ELF3 dependent, where ELF3 serves as an adaptor protein (Yu et al., 2008). The shuttling of COP1 between the nucleus and the cytoplasm is regulated by light (von Arnim and Deng, 1994). COP1 being nuclear localized in the night phase makes it competent for COP1–ELF3 mediated degradation of *GI* through 26S proteasome.

Upon heat shock *GI* is SUMOylated (López-Torrejón et al., 2013). It has been proposed that SUMOylation prevents the degradation of *GI*, thus enhancing its abundance. *GI* accumulation has been correlated with earlier flowering under heat stress. The identification of SUMOylation and ubiquitination sites in *GI* that alter its stability and degradation could be of pivotal importance in manipulating flowering time of crop plants. Current knowledge on the transcriptional and post-translational regulation of *GI* is presented schematically in **Figure 3**.

ROLES OF GIGANTEA

GIGANTEA plays multiple roles throughout plant development. Its functions in processes such as light signaling, circadian clock regulation, flowering time control, chlorophyll accumulation, sugar metabolism, and stress tolerance have been discussed below.

LIGHT SIGNALING

Photoreceptors such as phytochromes, cryptochromes, UV-light receptor, and phototropins help plants to sense variations in the

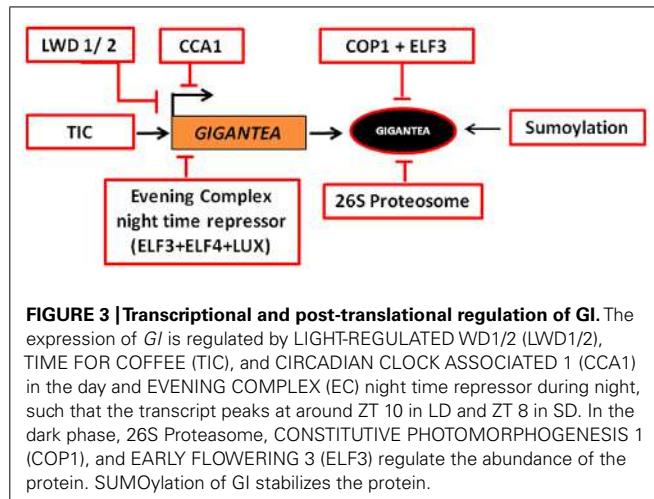


FIGURE 3 | Transcriptional and post-translational regulation of *GI*. The expression of *GI* is regulated by LIGHT-REGULATED WD1/2 (LWD1/2), TIME FOR COFFEE (TIC), and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) in the day and EVENING COMPLEX (EC) night time repressor during night, such that the transcript peaks at around ZT 10 in LD and ZT 8 in SD. In the dark phase, 26S Proteasome, CONSTITUTIVE PHOTOMORPHOREGULATION 1 (COP1), and EARLY FLOWERING 3 (ELF3) regulate the abundance of the protein. SUMOylation of *GI* stabilizes the protein.

light quality, quantity, and direction. The red and far-red light photoreceptors, phytochromes, are encoded by a multigene family, PhyA–E in *Arabidopsis*. While PhyA is the far-red light receptor, PhyB–E function as red light receptors with PhyB playing a predominant role. They mediate very-low-fluence responses (VLFRs), low-fluence responses (LFRs), and the high-irradiance responses (HIRs), with reference to the photon flux density (Casal et al., 1998). Like *phyB-9* mutant, *gi-100* also shows elongated hypocotyl when grown under saturated red light (Huq et al., 2000). Neither the genes nor the proteins abundance of PhyA and PhyB are influenced in *gi-100* (Huq et al., 2000). Therefore, *GI* was suggested to function downstream of PhyA and PhyB. Mutation in *GI* leads to decreased VLFR under FR light suggesting its role in PhyA signaling (Oliverio et al., 2007). The *gi* mutants showed reduced seed germination and cotyledon unfolding in FR light conditions. These phenotypes are rescued by over-expression of *GI*. This suggested that *GI* might have a positive role specifically in PhyA mediated VLFR. *GI* also has a role in regulating flowering in low R:FR ratio which might be attributed to PhyA signaling (Wollenberg et al., 2008). Both PhyA and PhyB form NBs like *GI*. It would be interesting to determine if Phys and *GI* are present in the same sub-nuclear complexes and the localization of *GI* in the NBs alters the Phy-mediated functions.

The *gi* mutants showed longer hypocotyl in comparison to WT under blue light (Martin-Tryon et al., 2007). Earlier, it had been suggested that *GI* may be either a positive regulator of TOC1 or act parallel to it for the regulation of hypocotyl elongation. Since only *gi* not *toc1* mutants show the longer hypocotyl in blue light, it can be inferred that *GI* does not regulate TOC1 for hypocotyl elongation (Martin-Tryon et al., 2007).

CIRCADIAN CLOCK CONTROL

The circadian clock controls many processes depending on the length of the day and night cycle in an organism. In plants, the rhythmic expressions of various genes are influenced by the circadian clock, thereby regulating functions such as elongation of hypocotyl, petioles and inflorescence stem, movement of cotyledon and leaf, and flowering time. CCA1, LATE ELONGATED HYPOCOTYL (LHY), and TOC1 are the core components of

circadian oscillator in plants (Somers, 1999). In 2005, the clock was proposed to be an interlocking network of proteins working in a feedback loop (Locke et al., 2005). According to the new model of clock, while the morning elements LHY and CCA1 repress TOC1 transcription, the evening element TOC1 down-regulates LHY/CCA1 accumulation, differing with the earlier observations (Alabadí et al., 2001; Gendron et al., 2012; Huang et al., 2012).

To understand the circadian rhythm in *Arabidopsis*, the ESPRESSO Quantitative Trait Loci (QTL) was generated from the cross between Ler and Cvi ecotypes (Swarup et al., 1999). Ler and Cvi ecotypes were suggested to comprise of an even distribution of alleles involved in the shortening and lengthening of period, since the progeny of their cross generated lines which had period length both longer and shorter than the parents. GI was identified as one of the genes that could be responsible for regulating the rhythms of cotyledon movement (Park et al., 1999; Swarup et al., 1999). The *gi* mutants have diverse circadian periods than WT concluding that GI has a role in period length regulation. Mutation in *GI* affects CHLOROPHYLL A/B-BINDING PROTEIN 2 (CAB2) gene expression which is also under the control of circadian clock (Park et al., 1999).

Soon after a day of imbibition of seeds, GI is required for initiating the rhythmicity of the circadian clock (Salomé et al., 2008). Mutations in the *GI* locus affect the *CCA1* and *LHY* gene expression in both LDs and SDs conditions (Fowler et al., 1999). A recent study proposed that both the nuclear and cytosolic GI are required to positively and negatively regulate *LHY* expression, respectively, that fine-tunes the clock function (Kim et al., 2013b). Over-expression or mutations of *CCA1* and *LHY* disrupted the *GI* expression (Fowler et al., 1999). Accordingly, the double mutant of *LHY* and *CCA1* showed early abundance of *GI* transcript (Mizoguchi et al., 2002, 2005). It suggests that *GI* operates in a feedback loop as a component to maintain the rhythmicity and period length of the clock.

The established *LHY/CCA1*-TOC1 module of the clock could not explain the experimental data like the time difference of about 12 h between *LHY/CCA1* abundance in morning and *TOC1* accumulation in evening (Alabadí et al., 2001; Locke et al., 2005). It was therefore proposed that *LHY/CCA1*-TOC1 module comprises of other components. One of the components was predicted to be *GI*, whose expression followed the same pattern as predicted by the *in silico* analysis and was subsequently experimentally confirmed (Locke et al., 2006). Further work suggested that *GI* alone would not be able to regulate the observed time difference (Kawamura et al., 2008). TOC1 in turn is regulated by *GI* along with ZEITLUPE (ZTL), an F-box protein (Kim et al., 2011). ZTL is a blue light photoreceptor which is stabilized by its interaction with *GI* and Heat Shock Protein 90 (HSP90). Together the ZTL-GI complex control TOC1 level (Kim et al., 2007).

Temperature compensation is an important characteristic of the circadian clock to maintain the rhythm over a range of environmental temperature. *GI* was recognized as a candidate regulating temperature compensation effect, especially at higher temperatures (Edwards et al., 2005; Gould et al., 2006). Since fluctuations in the temperature regulate the abundance of *GI* transcript, it

could be plausible that *GI* and temperature sensing mechanism crosstalk and feedback each other.

Arabidopsis thaliana dawn and dusk, *GI* regulates the clock rhythm along with ELF4 (Kim et al., 2012). *GI* was also required for iron-deficiency induced long circadian clock rhythm (Chen et al., 2013). Reduced depolymerization of actin filament caused the period of the circadian clock to shorten, as evident from the shortened period of *GI* expression (Tóth et al., 2012). Since *GI* expression is under the control of the circadian clock, *GI* accumulation pattern has been exploited to screen for novel clock mutants (Onai et al., 2004). Many components that mediate between *GI* and the clock are still to be unraveled. The role of *GI* in the regulation of the clock documented till date is summarized in Figure 4.

PHOTOPERIODIC FLOWERING-TIME REGULATION

GIGANTEA is a major mediator between the circadian clock and the master regulator of photoperiodic flowering time control, CO. *GI* upregulates *CO* transcription, thereby accelerating time required to flower. Koornneef et al. (1998) showed that a novel mutant, *gi-3*, is epistatic to *CO* and *FLOWERING LOCUS T* (*FT*) way back. Mutation in *GI* led to a decrease in the accumulation of *CO* mRNA without affecting its cycling phase compared to its WT that led to delayed flowering (Suárez-López et al., 2001). Mutants in the *GI* locus or over-expressors of *GI* did not discriminate day-length for flowering. Accordingly, *gi* mutants were later flowering and over-expressors were earlier in both LDs and SDs (Rédei, 1962; Araki and Komeda, 1993; Mizoguchi et al., 2005).

During dawn, *CO* transcription is repressed by the combinatorial activity of the DOF transcription repressors bound to the *CO* promoter. In LDs, the expression of *FKF1* and *GI* coincide at ZT10. Therefore, toward the middle of the day the accumulation of *GI* along with *FKF1* forms a complex competent to degrade the DOF factors. This elevates the *CO* transcription, thereby leading to *FT* expression (Imaizumi et al., 2003, 2005; Sawa et al., 2007). While in SDs, since *FKF1* accumulates 3 h after *GI* peaks, it does not allow the formation of the degradation complex, therefore leading to a low abundance of *CO* transcript. This photoperiod

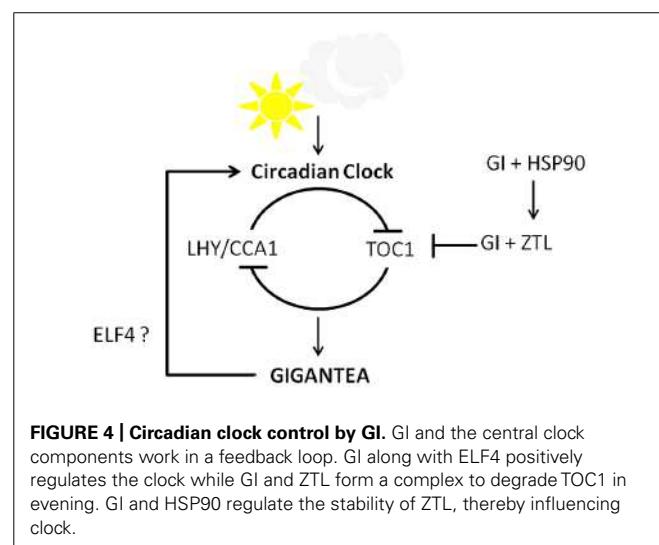


FIGURE 4 | Circadian clock control by *GI*. *GI* and the central clock components work in a feedback loop. *GI* along with ELF4 positively regulates the clock while *GI* and ZTL form a complex to degrade TOC1 in evening. *GI* and HSP90 regulate the stability of ZTL, thereby influencing clock.

pathway where GI regulates *FT* expression in a CO-dependent manner is schematically depicted in **Figure 5**. GI regulates the abundance of FKF1, which is involved in the proteasomal degradation of proteins (Fornara et al., 2009). Post-transcriptionally, GI also controls the sub-cellular level of CYCLING DOF FACTOR 2 (*CDF2*; Fornara et al., 2009). FKF1 belongs to a family of F-Box proteins containing two other candidates – LOV KELCH Protein 2 (LKP2) and ZTL. The blue light dependent interaction between GI and FKF1 is mediated by the LOV (Light, Oxygen, or Voltage) domain of FKF1 and the amino-terminal of GI *in vivo* (Sawa et al., 2007). The *gi-100* mutant is later flowering than the F-Box triple mutant *fkl1 ztl-4 lkp2-1*. This might be due to the presence of GI in *fkl1 ztl-4 lkp2-1*, which down-regulates the abundance of *CDF* transcripts, or the presence of an additional layer of control by GI bypassing the triple F-Box module.

There are at least two independent mechanisms through which GI regulates *FT* expression independent of CO. While the first mechanism involves microRNA, the second mechanism is through the binding of GI to the *FT* promoter. The microRNA based control involves *miRNA172*, which is positively regulated in the presence of GI. The *miR172* inhibits the expression of *TARGET OF EAT1* (*TOE1*), an APETALA 2 (AP2)-related transcriptional repressor of *FT* (Jung et al., 2007). In the recent past, expression of *GI* specifically in the mesophyll or vascular tissue was carried out. This rescued the late-flowering phenotype of *gi-2* under both day length conditions and two different temperatures of 16 and 23°C (Sawa and Kay, 2011). The expression of *GI* in mesophyll and vascular tissue was done using tissue specific promoters *LIGHT-HARVESTING COMPLEX B2.1* (*pLhCB2.1*) and *SUCROSE TRANSPORTER 2* (*pSUC2*), respectively. While expression pattern of *GI* under the control of *pLhCB2.1* is altered and peaked at ZT 0, *GI* expressed under the phloem specific promoter led to the over-expression of the transcript with peak at ZT 10. The *FT* transcript level was up-regulated without an increase in

CO mRNA in both day-length conditions. GI was shown to binds to the *FT* transcriptional repressors such as SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO 1 (TEM1), and TEMPRANILLO 2 (TEM2), and their specific target regions within the *FT* promoter in the mesophyll, thereby relieving the repression and promoting *FT* transcription (Sawa and Kay, 2011). The degradation of the *FT* transcriptional repressors or the unavailability of their binding sites on the *FT* promoters due to the presence of GI could lead to the abundance of the *FT* transcript. *FT* expressed in the vascular tissue normally triggers flowering. Since *GI* expressed in mesophyll accelerated flowering, elevating the *FT* level in vasculature, the signal from mesophyll GI most likely induces CO transcription in vasculature. Alternatively, the GI could be transported to the vascular tissues and induce the photoperiod module which needs to be investigated.

Expression of 35S::*GI*:GFP in *gi-3* plants complemented the late flowering phenotype of *gi-3*. On the contrary, expressing the 35S::GFP:*GI* in *gi-3* caused later flowering compared to the background lines indicating that the N-terminal fusion of GI might be either non-functional or might not be imported into the nucleus. In the transgenic line expressing C-terminal fusion, the fusion protein was localized to the nucleus and formed NBs (Mizoguchi et al., 2005). In an independent study, transgenic plants expressing glucocorticoid receptor (GR) fusion of GI flowered with ~20 leaves less when treated with dexamethasone, compared to its untreated control which flowered with ~55 leaves under LDs (Günl et al., 2009). In 15 day old seedlings, the induction of flowering time genes like CO and *FT* took place ~28 h after dexamethasone treatment causing early flowering. This indicates that cytoplasmic retention of GI most probably delays time to flower. Mutation in *GI* is epistatic to mutation in *ELF4* and together regulate CO expression (Kim et al., 2012). Recent studies showed that *ELF4* sequesters GI into nuclear bodies, thereby preventing GI to associate with the CO promoter (Kim et al., 2013b). It would be interesting to know the nature of the GI nuclear bodies and the components there

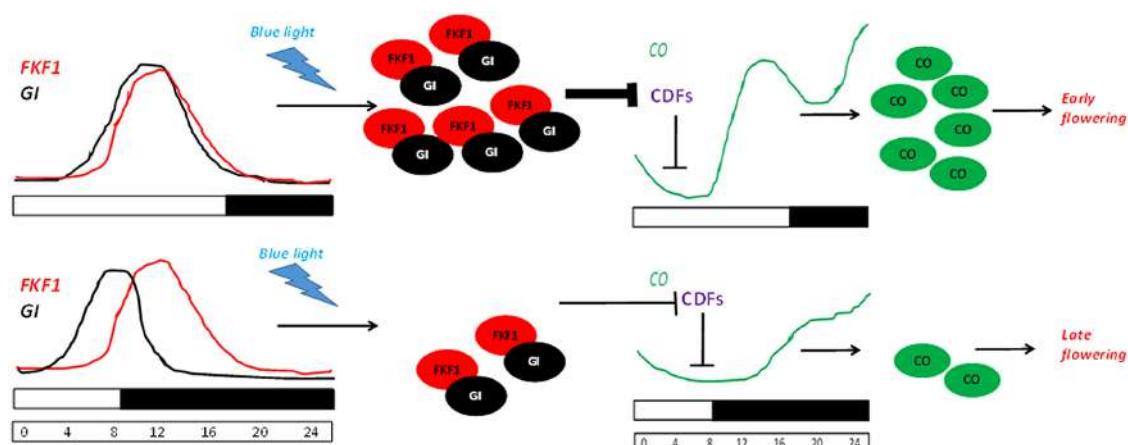


FIGURE 5 | GI-FKF1 complex regulates the photoperiodic flowering time.

In LD, the peak expression of *GI* and *FLAVIN-BINDING KELCH REPEAT F-BOX 1* (*FKF1*) coincide, leading to the accumulation of GI-FKF1 complex. The GI-FKF1 complex regulates the degradation of Cycling DOF factors

(CDFs) bound to the CONSTANS (CO) promoter. In the absence of the inhibitor CO transcription occurs resulting in the accumulation of CO protein that promotes flowering. While in SDs, less abundance of GI-FKF1 complex does not degrade CDFs repressing CO transcription.

in, using biochemical approach followed by mass spectrometric analysis.

GIGANTEA interacts with N-terminal tetracopeptide domains of SPINDLY (SPY), a plant O-linked β -N-acetylglucosamine transferase, and antagonizes its activity, thereby, promoting flowering (Tseng et al., 2004). Acetylglucosamine transferases have role in the addition of acetylglucosamine residues to proteins, which often competes with phosphorylation. This suggests that sugar modification may function as an important event in flowering time regulation. The known pathways through which GI regulates flowering are summarized in the **Figure 6**.

PLEOTROPIC FUNCTIONS OF GIGANTEA

Besides flowering time, circadian clock, and light signaling regulation, GI has been implicated in other processes such as, sucrose signaling (Dalchau et al., 2011), starch accumulation (Eimert et al., 1995), and stress tolerance (Kurepa et al., 1998a; Fowler and Thomashow, 2002; Kim et al., 2013a; Riboni et al., 2013). The control of cotyledon movement, transpiration, and hypocotyl elongation responses have been shown to be attributed to the concerted activity of SPY and GI (Sothern et al., 2002; Tseng et al., 2004). The precise nature of this interaction is still unclear. However, GI functions antagonistically to SPY. The interaction of GI with SPY and ELF4 independently regulates hypocotyl length, where mutation in ELF4 and SPY are epistatic to *gi*-2.

GIGANTEA has been demonstrated to play a role between sucrose signaling and the circadian clock while grown in DD (Dalchau et al., 2011). Plants entrained in LD when shifted to DD, maintained the rhythmic GI expression exclusively in the presence of sucrose suggesting light independent control of GI rhythmicity. Although contradictory evidence on role of sucrose on GI expression has been reported, sucrose seems to affect the GI expression through SENSITIVE TO FREEZING6 (SFR6) locus (Knight et al., 2008; Usadel et al., 2008). More precise experiments are required

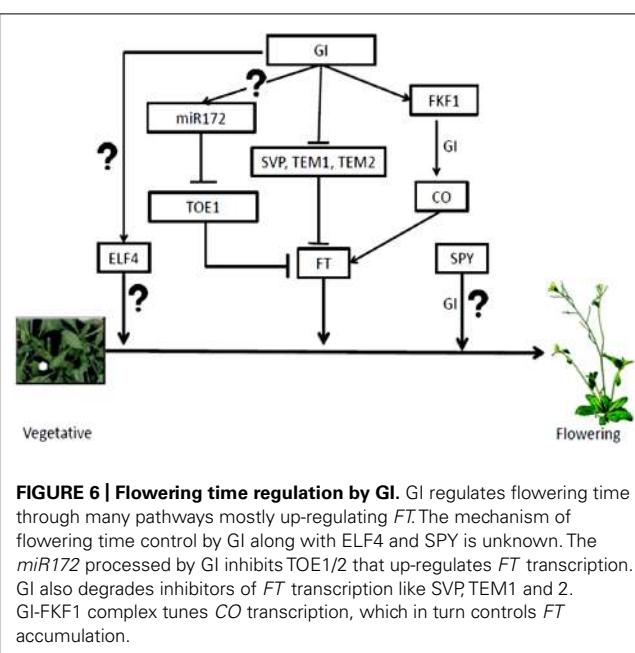
to unravel this mechanism. In the leaves of *Arabidopsis*, starch accumulation is elevated in the *gi* mutants (Eimert et al., 1995). On the contrary, presence of multiple copies of GI led to starch accumulation in the progeny of a cross between *A. thaliana* and *A. arenosa*, suggesting the antagonistic role of GI in these plants (Ni et al., 2009).

The *gi*-3 mutants showed higher tolerance capacity to redox cycling agent, paraquat, and H₂O₂ (Kurepa et al., 1998a). Tolerance against paraquat is counteracted by the exogenously applied polyamines such as spermidine, spermine, and putrescine (Kurepa et al., 1998b). Paraquat treatment upregulated endogenous levels of putrescine in *gi*-3 and WT. Since exogenous application of polyamines is effective for the resistance, the mechanism of the transporters during this stress needs attention. Oxidative stress due to herbicide imazethapyr has been shown to increase GI abundance and cause earlier flowering by ~4 days (Qian et al., 2014). The mechanism behind higher tolerance to oxidative stress mediated by GI is still unclear.

Kurepa et al. (1998a) showed that *gi* mutants, *gi*-3, *gi*-4, *gi*-5, and *gi*-6, have more chlorophyll accumulation in comparison to WT in presence of paraquat. Even treatment with nitric oxide (NO) reduces the GI mRNA abundance and increases the chlorophyll content (He et al., 2004). In both the cases above, lower abundance of functional GI can be correlated to higher accumulation of chlorophyll. The role of GI in regulating the chlorophyll content needs to be studied in mutants and over-expressors of GI. Chlorophyll accumulation in allotetraploid, obtained by a cross between *A. thaliana* and *A. arenosa*, is higher than the WT individuals (Ni et al., 2009). The starch and chlorophyll accumulation in allotetraploids is exactly opposite in comparison to that seen in *A. thaliana*. The reverse trend might be due to post-transcriptional silencing posed by the presence of multiple homologous sequences of GI transcript, essentially a co-suppression phenomenon.

Dynamin, a GTPase having role in vesicle recycling during endocytosis, was found to interact with TAP tagged GI in rice (Abe et al., 2008). Although mutation in *dynamin* gene did not have any effect on the flowering time, it showed aerial rosette phenotype in *Arabidopsis*. In *Arabidopsis*, GI has been found to be involved in setting of fruits (Brock et al., 2007). No significant association of the GI haplogroup was detected with days to flower, petiole length, and inflorescence height. A significant association was observed between one haplogroup with fruit set, producing 14% more fruit than other haplogroups. Such studies in the crop plants could help in increasing the yield.

GIGANTEA mRNA levels increases about five- to eightfold in the cold treated *Arabidopsis* plants suggesting that GI is a cold-responsive gene (Fowler and Thomashow, 2002). The flowering time of *gi* mutants was further delayed when exposed to low temperature compared to WT (Cao et al., 2005). C-repeat Binding proteins (CBFs) have been known to regulate various genes responsive to cold and are implicated in cold stress tolerance. On the contrary, Cao et al. (2005), it was revealed that GI regulates cold acclimation through CBF-independent pathway. The ability to tolerate and acclimatize toward cold is reduced in *gi* mutants suggesting the protective role of GI in cold tolerance.



Recently, the role of GI under salt stress was documented (Kim et al., 2013a). Although, salt stress did not affect the *GI* expression, it affected the *GI* protein stability in *pGI::GI-HA* transgenics (Cao et al., 2005; Kim et al., 2013a). It seems plausible that there is a mechanism at the post-translational level that regulates *GI* abundance. *GI* also regulated the activities of the proteins involved in the salt stress tolerance. It interacts with Salt Overly Sensitive 2 (SOS2) directly and inhibits the activity of SOS1, a Na^+/H^+ antiporter. Therefore, *GI* is a negative regulator of salt tolerance and is degraded during salt stress. According to a recent model, plants under salt stress would flower later than when grown in normal growth conditions reasoned for the degradation of *GI* (Park et al., 2013).

In *At* and other plants, the tolerance to higher salinity, enhanced cold, and sustained drought were manifested by the increase of sub-cellular level of abscisic acid (ABA). Recent reports indicated that *GI* has role in ABA-dependent drought escape tolerance. It suggests that the *GI* regulation of salt and cold stress tolerance could very likely be ABA-mediated (Riboni et al., 2013). Drought stress up-regulates *GI* transcription and in turn, increases the abundance of *miR172E* variant (Han et al., 2013). WRKY DNA binding protein 44 (WRKY44) was found to be suppressed by *GI* in drought stress and interact with TOE1. *GI-miR172-WRKY44* were proposed to be in the same pathway possibly associated with drought stress tolerance. On the same line of thinking, the light dependent *GI*-mediated stomatal opening response could be ABA mediated (Ando et al., 2013). *GI* also has a role in wall in-growth deposition in phloem parenchyma transfer cells in *A. thaliana* in response to high light and cold stress (Edwards et al., 2010; Chinnappa et al., 2013).

ROLE OF GIGANTEA HOMOLOGS

GIGANTEA homologs in prokaryotes, fungi, mosses, or animals have not been reported as yet (Holm et al., 2010). *GI* homolog has been shown to be absent in the green unicellular alga *Ostreococcus tauri* (Corellou et al., 2009). Evolution of *GI* has been correlated with the evolution of higher plants from liverwort onward, although being absent in mosses. The evolution of *GI* can be proposed to have taken place alongside the origin of land plants. The role of *GI* in light signaling, circadian clock control, and flowering time regulation seems to be conserved across the plant kingdom, as inferred from the various studies to understand the role of *GI* homologs in *Arabidopsis*. *GI* homologs from the non-flowering and flowering plants have been summarized below.

The *GI*-FKF1 interaction and function has been recently shown to be conserved in the liverwort *Marchantia polymorpha* (Kubota et al., 2014). The LOV domain of FKF1, which has been found to be required for the interaction with *GI*, contains a conserved cysteine residue in *AtGI* and *MpGI* important for its blue light dependent functions (Sawa et al., 2007). *GI*-FKF1 module has been proposed to be important for the transition of plants from water to land and the evolution of vascular system. The *Marchantia polymorpha* ortholog of *GI*, *MpGI*, has been shown to partially rescue the late flowering phenotype of *Arabidopsis gi* mutant suggesting the functional conservation of *GI* across the plant kingdom. The FKF1 homologs have been shown to be present in *A. thaliana* (*AtFKF1*, *AtZTL*, and *AtLKP2*), *Oryza sativa* (*OsFKF1*, *OsZTL1*, and *OsZTL2*), *Glycine max* (*GmFKF1*, *GmZTL1*, and *GmZTL2*), *Triticum aestivum* (*TaFKF1* and *TaZTL*), *Allium cepa* (*AcFKF1* and *AcZTL*), *Mesembryanthemum crystallinum* (*McFKF1* and *McZTL*), and *Selaginella moellendorffii* (*SmFKF1*; Kubota et al., 2014). The *GI* counterparts in the above mentioned species are also conserved. This shows that *GI*-FKF1 module has been conserved since the primitive time and thus might be have been important in shaping the development of higher plant. This light perceiving module needs to be studied in detail to understand the evolution of various functions and residues along with putative domains required to carry out these functions in plants. The conserved interaction of *GI* with FKF1 has been shown to be conserved in soybean.

GYMNOSPERM

Norway Spruce (Picea abies)

GIGANTEA ortholog of *Picea abies*, *PaGI* and *AtGI* share 58% identity and 72% similarity. Natural variations in *GI* have been correlated to clinal variations in the different populations of close relative of the Scandinavian Norway spruce (Chen et al., 2014). Over-expression of *PaGI* in WT *Arabidopsis* did not show any phenotypical changes (Karlgren et al., 2013). However, when *PaGI* was over-expressed in *gi-2* mutant, it partially rescued the late flowering phenotype and flowered at the same time as WT plants suggesting that *PaGI* and *AtGI* are functionally conserved to large extent. The strength of the over-expression has neither been verified at the gene expression level nor the protein accumulation level and therefore needs to be confirmed.

ANGIOSPERMS (MONOCOTS)

Barley (Hordeum vulgare)

GIGANTEA homolog in Barley was identified using BLAST searches and later confirmed by Southern hybridization analyses. Only one homolog was detected in barley. Barley *GI* (*HvGI*) has ~94 and ~79% similarity with *OsGI* and *AtGI*, respectively (Dunford et al., 2005). Barley, being a long-day plant, its *GI* expression followed the pattern documented for *AtGI*. Characteristically, in SDs, the peak of expression was noticed about 6–9 h after dawn whereas, in LDs, the peak is shifted to 15 h after dawn (Dunford et al., 2005). The mutation in *HvELF3* (*mat-a.8*), a 4 bp deletion causing frame shift and premature stop codon, was found in the barley cultivar Mari (Zakhrebekova et al., 2012). This mutation led to the up-regulation of *HvGI* transcription and was found to be the reason for early flowering phenotype in this cultivar. Although, post-translational interaction between *AtELF3* and *AtGI* is known, no evidence is there in *Arabidopsis* suggesting the transcriptional regulation of *GI* by *ELF3*.

Duckweed (Lemma gibba)

The *AtGI* homolog of *L. gibba*, *LgGIH1*, a LD plant, plays a pivotal role in its circadian clock control, since the *LgGIH1* knockdown resulted in the arrhythmic gene expression phenotype in plants (Serikawa et al., 2008). Earlier reports suggested that *AtGI* and *LgGIH1* followed similar expression pattern in both LD and LL conditions (Miwa et al., 2006). The function of *GI* and *ELF3* homologs are shown to be conserved between *Arabidopsis* and *L. gibba*.

Maize (*Zea mays*)

Maize is a SD plant, which has two diurnally regulated GI homologs called *gigantea* of *Z. mays* 1a and 1b (*gi1* and 2) due to tetraploidy events and genome evolution expressed in leaves (Gaut and Doebley, 1997; Swigonová et al., 2004; Miller et al., 2008; Hayes et al., 2010; Khan et al., 2010; Schnable et al., 2011). Among the two homologs, the *gigantea1* transcript was highly expressed. Mutation in *gi1* caused early flowering in LD but had lesser effects in SD. The *gi1* mutation also increases plant height and alters the timing of the vegetative phase (Bendix et al., 2013). The early flowering phenotype of *gi1* mutant was due to the conserved pathway involving the up-regulation of CO-like flowering regulatory gene called *CO* of *Z. mays*1 (*conz1*) and *FT*-like floral activator gene named *Z. mays centroradialis8* (*zcn8*).

Purple False Brome (*Brachypodium distachyon*)

GIGANTEA ortholog of *B. distachyon* (*BdGI*) is rhythmically regulated by the circadian clock and up-regulated by both cold and dark (Hong et al., 2010). *BdGI* was identified by BLAST search followed by Southern hybridization analysis. The *BdGI* transcript level was found to be oscillating in both SD and LD conditions, like *AtGI*. While the lowest transcript level in both SD and LD was at ZT 0, the peak in SD was at ZT 8 and in LD was at ZT 12. *BdGI* shares 65% identity with *AtGI*. *BdGI*, like *AtGI*, is a nuclear localizing protein and interacts with COP1 and ZTL proteins as evident from the yeast two-hybrid assays. *BdGI* complements the late flowering phenotype of *Arabidopsis gi-2* mutant suggesting the conserved function of GI in monocots and dicots. While *PhyC* does not show a pronounced effect in the LD model *Arabidopsis*, it causes late flowering in this temperate grass (Woods et al., 2014). In *phyC* mutants, *GI* expression is almost undetectable. The low *GI* expression could explain the lower abundance of the homologs of *CO* and *FT*. The delayed flowering phenotype suggests that the photoperiodic flowering pathway through *GI* is conserved in grasses as in *Arabidopsis*.

Rice (*Oryza sativa*)

Rice and *Arabidopsis* *GI* share 67% similarity and the NLS are quite conserved between *OsGI* and *AtGI* (Hayama et al., 2002). *GI* expression pattern was similar in both rice and *At* (Hayama et al., 2002) and similarly, *OsGI* acts as a positive regulator of *Hd1* (*CO* homolog of rice; Hayama et al., 2003). It controls the rhythm of nearly 27000 genes in rice (Izawa et al., 2011). When *gi* mutants are grown in field conditions, sucrose, and starch content increases, chlorophyll content decreases, stomatal conductance increases, panicle, and spikelet number increases and fertility was reduced. *OsGI* was shown to be involved in ETR2 (ethylene receptor)-dependent late flowering phenotype and starch accumulation thus, regulating the developmental transition based on the availability of energy (Wuriyanghan et al., 2009).

Tulip (*Liriodendron tulipifera*)

GIGANTEA ortholog was shown to be closer to eudicot *GI* sequence than the monocot sequences (Liang et al., 2010).

Wheat (*Triticum aestivum L.*)

Wheat is a LD plant and has been shown to have an ortholog of *AtGI*, referred as *TaGI1* (Zhao et al., 2005). *TaGI1* has ~81 and

63% identity with *OsGI* and *AtGI*, respectively. The *TaGI1* follows rhythmic pattern of expression similar to that of *Arabidopsis* and over-expression of *TaGI1* complements late flowering phenotype of *gi-2* mutant *Arabidopsis*. *TaGI* was also associated with “earliness phenotype” of wheat which helps in its adaptation and increase in yield in varied environmental conditions (Rousset et al., 2011).

ANGIOSPERMS (DICOTS)

Common Ice Plant (*Mesembryanthemum crystallinum*)

A crassulacean acid metabolism plant, *Mesembryanthemum crystallinum*, also showed a rhythmic expression of the orthologs of *GI*, *McGI* (Boxall et al., 2005). The ortholog was identified using BLAST search and later isolated and sequenced. *McGI* expression peaks at ZT 9 similar to *AtGI*.

Morning Glory (*Pharbitis nil*)

PnGI protein shares 70 and 67% identity with *AtGI* and *OsGI* protein, respectively (Higuchi et al., 2011). *PnGI* mRNA is also circadian regulated like the other *GI* orthologs. Over-expression of *PnGI* led to altered period length affecting the expression pattern of downstream genes. *Pharbitis nil* is a SD plant, and like *OsGI*, *PnGI* inhibits the expression of *PnFT* (*FT* homolog of morning glory).

Pea (*Pisum sativum*)

LATE BLOOMER 1 (*LATE1*) is the *AtGI* ortholog in pea, a LD plant, and follows a rhythmic pattern of expression as seen in *Arabidopsis* (Hecht et al., 2007). *LATE1* was shown to be regulating the pea homologs of *Arabidopsis* circadian clock genes. Apart from its role in flowering time and circadian clock regulation, *LATE1* has been implicated in *Phy-B* dependent seed de-etiolation in red light. *LATE1* was found to regulate circadian clock gene expression in constant light and dark (Liew et al., 2009). In LD and SD, *LATE1* was shown to control a mobile signal that regulates the flowering time.

Radish (*Raphanus sativa*)

In another instance, expression of antisense *AtGI* gene, under the constitutive 35S promoter, led to delayed bolting in LDs, proving that *GI* has an important role in photoperiodic flowering time control in this plant (Curtis et al., 2002). The bolting and flowering time was delayed by 17 and 18 days, respectively, with respect to WT plants.

Soybean (*Glycine max*)

Glycine max, a SD plant, has two *GI* orthologs – *GmGIa* and *GmGIb* (Watanabe et al., 2011). Both the *GmGI* sequences have nearly 70–91% identity to eudicot and monocot genes. Like *OsGI*, *GmGI* regulated *GmFT* paralogs. *GmGI* has been shown to have role in soybean seed maturity. *GmGI* loss of function leads to early flowering as in the model SD rice plant. Interestingly, a recent study in soybean suggested that there are three *AtGI* homologs in the soybean genome unlike previously suggested two orthologs *GmGIa* and *GmGIb* (Li et al., 2013). The third form is a result of alternative splice form of *GmGIa*, resulting in *GmGIα* and *GmGIβ*. The *GI* orthologs were diurnally regulated and differentially expressed in different tissues adding up to a more complex

regulation. GmGI proteins have the conserved NLS and localize to nucleus. GmGI proteins have been shown to interact with orthologs of FKF1 in soybean suggesting that function most likely is conserved.

Tomato (*Solanum lycopersicum*)

Tomato is a day neutral plant. GI was shown to be up-regulated and inhibit tomato seed germination thereby promoting seed dormancy under FR condition in the presence of functional PhyA (Auge et al., 2009). On the contrary, in *Arabidopsis*, loss of function of GI led to elevated dormancy (Penfield and Hall, 2009). In other members of the Solanaceae such as potato and tobacco, photoperiodic control of GI was also shown to be operational (Rutitzky et al., 2009).

The conserved diurnal regulation of GI in different plants described above suggests the prevalence of an important transcriptional machinery as well as the GI promoter. The availability of GI antiserum would help to understand the regulation of GI in these crop plants. The localization and the stability of GI in most of these plants are still to be addressed. While few of the interaction with proteins such as orthologs of ELF3, COP1, ZTL, and FKF1 are shown to be conserved, the function of these complexes needs to be disclosed in various species.

CONCLUSION AND PERSPECTIVE

GIGANTEA seems to be a very important plant protein involved in various processes, from developmental regulation to metabolic flux. Despite its pivotal roles, it is surprising that GI null mutants are not lethal. Being a large protein, it might satisfy to function in several pathways summarized, yet to be fully understood. It would be a great challenge to understand and connect the functional roles of GI at different developmental stages. Although GI is a multifunctional protein, the role of its various functional domains are still in darkness. A functional antiserum against a conserved domain of GI that would detect the endogenous level of protein across species and in multiple mutational background would be very useful. The lack of such an antiserum possesses a serious bottleneck delaying the understanding of its abundance, regulation at the protein level and regulatory functions like the GI-FKF1 module across the plant kingdom. Despite this problem, several elegant experiments have been published where researchers have attempted to understand its role using transgenic plants expressing tagged versions of GI. Although time-consuming, these are the impressive feats that place GI mechanistically in a network of photoperiod control pathway.

The role of GI in flowering time regulation, circadian clock control, and light signaling is still being pursued. But less-known functions such as sucrose signaling, chlorophyll accumulation, oxidative stress resistance demand more attention. More functions of GI are beginning to be documented. Recently, the emerging role of GI in salt tolerance has been demonstrated, which indicates that we are still not saturated in understanding the various functions GI. It would be interesting to understand how GI regulates so many functions before going into the complex cross talk between them it can fine tune. The lower plant moss *Physcomitrella patens* does not have a GI ortholog but still carries out most of the developmental aspects except flowering. It is very interesting to note that they do

have CO-like genes, therefore the evolution of GI function is still an interesting area and demands further attention (Zobell et al., 2005).

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B-GATA transcription factors – insights into their structure, regulation, and role in plant development

Carina Behringer and Claus Schwechheimer*

Department of Plant Systems Biology, Technische Universität München, Freising, Germany

Edited by:

Dorothee Staiger, Bielefeld University, Germany

Reviewed by:

Wolfgang Lukowitz, University of Georgia, USA

Yi-Hsuan Chiang, University of California, Davis, USA

***Correspondence:**

Claus Schwechheimer, Department of Plant Systems Biology, Technische Universität München, Emil-Ramann-Straße 4, 85354 Freising, Germany
e-mail: claus.schwechheimer@wzw.tum.de

GATA transcription factors are evolutionarily conserved transcriptional regulators that recognize promoter elements with a G-A-T-A core sequence. In comparison to animal genomes, the GATA transcription factor family in plants is comparatively large with approximately 30 members. Here, we review the current knowledge on B-GATAs, one of four GATA factor subfamilies from *Arabidopsis thaliana*. We show that B-GATAs can be subdivided based on structural features and their biological function into family members with a C-terminal LLM- (leucine–leucine–methionine) domain or an N-terminal HAN-(HANABA TARANU) domain. The paralogous GNC (GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED) and CGA1/GNL (CYTOKININ-INDUCED GATA1/GNC-LIKE) are introduced as LLM-domain containing B-GATAs from *Arabidopsis* that control germination, greening, senescence, and flowering time downstream from several growth regulatory signals. *Arabidopsis* HAN and its monocot-specific paralogs from rice (NECK LEAF1), maize (TASSEL SHEATH1), and barley (THIRD OUTER GLUME) are HAN-domain-containing B-GATAs with a predominant role in embryo development and floral development. We also review GATA23, a regulator of lateral root initiation from *Arabidopsis* that is closely related to GNC and GNL but has a degenerate LLM-domain that is seemingly specific for the *Brassicaceae* family. The *Brassicaceae*-specific GATA23 and the monocot-specific HAN-domain GATAs provide evidence that neofunctionalization of B-GATAs was used during plant evolution to expand the functional repertoire of these transcription factors.

Keywords: GATA, HAN-domain, LLM-domain, B-GATA, HANABA TARANU, GNC, GNL, CGA1

B-GATA TRANSCRIPTION FACTORS

GATA factors are evolutionarily conserved transcription regulators that were named after their DNA-binding preference to the consensus sequence W-GATA-R [W, thymidine (T) or an adenine (A); R, guanidine (G) or adenosine (A); Reyes et al., 2004]. All GATA transcription factors from *Arabidopsis* have a type IV zinc finger with the consensus C-X₂-C-X_{17–20}-C-X₂-C (C, cysteine; X, any residue) followed by a highly basic amino acid stretch (Reyes et al., 2004). While the zinc finger engages in hydrophobic interactions with the minor groove of the target DNA, the basic stretch interacts with the negatively charged phosphate backbone. Whereas all *Arabidopsis* GATAs have only one DNA-binding domain, several GATA transcription factors from rice, similarly to their animal counterparts, contain more than one zinc finger (Reyes et al., 2004).

The interest in GATA transcription factors from plants was originally instigated by the observation that GATA motifs are enriched in promoters of light-regulated genes and of genes controlled by the circadian clock (Arguello-Astorga and Herrera-Estrella, 1998). The interest in GATAs was further stimulated by the fact that the GATA factor AreA from the fungus *Aspergillus nidulans* is a key regulator of nitrogen signaling, which suggested that studies of plant GATAs may also lead to advances in understanding nitrogen signaling in plants (Daniel-Vedele and Caboche, 1993; Scazzocchio, 2000). In spite of this long-standing interest, only recently the identification and availability of mutants and overexpressors

has allowed determining the function of these GATA factors in a biologically relevant context. Although in several cases functional redundancy between different GATA genes has rendered the identification of their biological functions difficult, it is now apparent that GATAs play a key role in a wide array of biological processes.

The knowledge about the identity of GATA factors from *Arabidopsis* and rice allowed subdividing the approximately 30 plant GATA factors into four conserved and distinct classes; class A through class D (Reyes et al., 2004). This classification was based on several criteria such as sequence conservation within the DNA-binding domain, the presence and absence of additional recognizable protein domains as well as the exon-intron structures of the respective genes. The focus of this review is on class B GATAs (B-GATAs), which can be subdivided into at least two functional subfamilies based on the presence of conserved domains. Whereas some B-GATAs contain a conserved LLM- (leucine–leucine–methionine) domain at their very C-terminus with an invariant L–L–M motif (Behringer et al., 2014) others contain a conserved HAN domain, which was first described in the *Arabidopsis* B-GATA HAN (HANABA TARANU; **Figure 1A**). LLM- and HAN-domain containing B-GATAs can be identified in all sequenced dicot and monocot species suggesting that they existed before the monocot-dicot divergence (**Figure 1B**; Behringer et al., 2014). Several members of the *Arabidopsis* B-GATA family have already been intensively studied: first, the paralogous GNC (GATA,

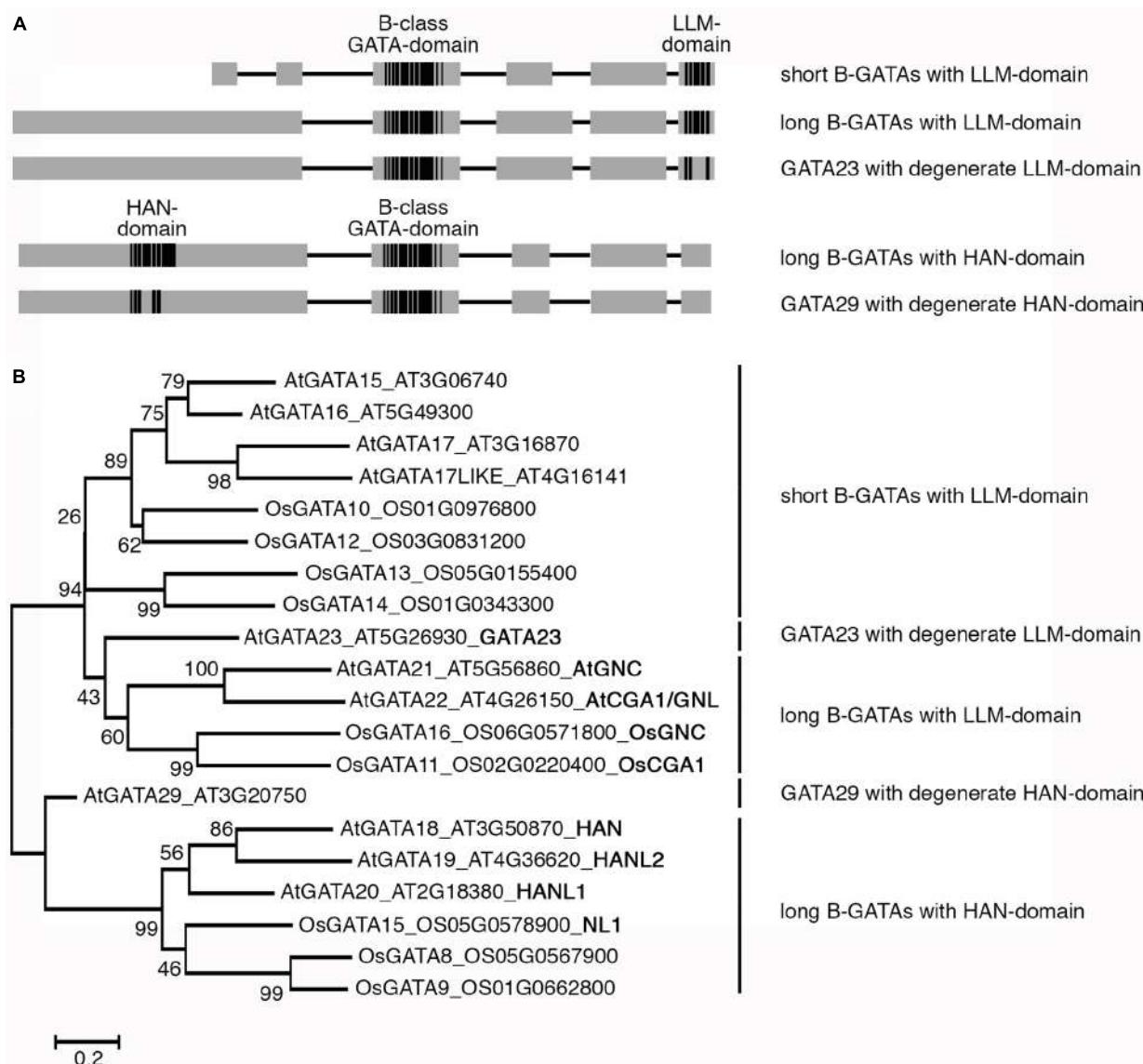


FIGURE 1 | Overview of B-class GATA transcription factors from *Arabidopsis thaliana* and rice (*Oryza sativa*). (A) Schematic representation of B-class GATAs with their B-class GATA DNA-binding domain, the C-terminal LLM- (leucine–leucine–methionine) domain and the N-terminal HAN- (HANABA TARANU) domain. In *Arabidopsis*, B-GATAs with a degenerate HAN- or LLM-domain can be found as specified in subsequent Figures. Boxes represent protein regions with sequence similarity (gray) or high sequence conservation (black), lines represent protein regions with restricted sequence conservation. The schemes are not drawn to scale but reflect the presence of long and short proteins and the respective positions of the LLM- or

HAN-domain. **(B)** Phylogenetic tree of B-GATAs from *A. thaliana* and rice (*O. sativa*). Where available, trivial names as introduced in the main text are provided (bold). The phylogenetic tree was generated using the Geneious R Software based on a MUSCLE alignment in MEGA6.06 using the following settings: Gap penalty, gap open –2.9, gap extend 0, hydrophobicity multiplier 1.2; iterations, maximum iterations 8; clustering method, all iterations UPGMB and minimum diagonal length (lambda) 24. The Neighbor Joining tree was generated with the bootstrap method (1000 replications) using the Jones-Taylor-Thornton model using default settings. Bootstrap values are indicated by each node. Bar = 0.2 amino acid substitutions per site.

NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED) and CGA1/GNL (CYTOKININ-INDUCED GATA1/GNC-LIKE; hitherto GNL), representative B-GATAs with an LLM-domain; second, HAN and HANL (HAN-LIKE) proteins from *Arabidopsis* and monocots, B-GATAs with a HAN-domain. Furthermore, there are *Brassicaceae*-specific as well as monocot-specific B-GATAs that together provide evidence that the neofunctionalization of B-GATAs was used during plant evolution to expand their functional

repertoire (Figure 1). In this review, we will summarize the current knowledge about B-GATAs, their structure, their regulation, and their role in plant development.

GNC AND GNL – GROWTH REGULATORS DOWNSTREAM FROM MULTIPLE PHYTOHORMONE PATHWAYS

GNC and its paralog GNL (GNC-LIKE) had first been noted based on their transcriptionally regulation by nitrate (Wang et al.,

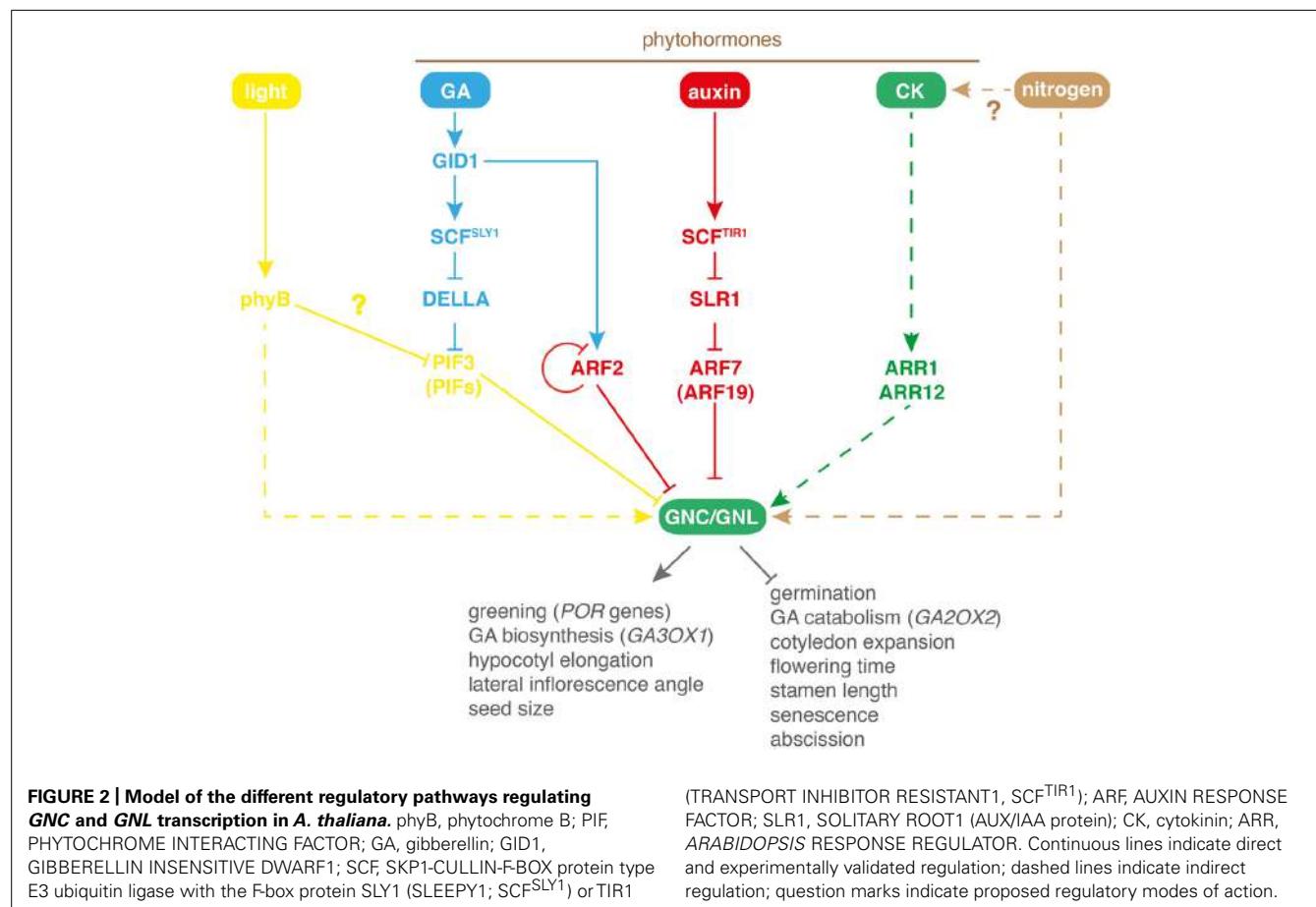
2003; Price et al., 2004; Scheible et al., 2004; Bi et al., 2005; Kiba et al., 2005). GNC was subsequently identified as a gene required for proper chlorophyll accumulation and was designated *GATA*, *NITRATE-INDUCIBLE*, *CARBON-METABOLISM INVOLVED* based on the transcriptional regulation by nitrate and the misregulation of genes involved in carbon metabolism in the *gnc* mutant (Bi et al., 2005; **Figures 2 and 3**). GNL had initially been designated CGA1 based on its strong transcriptional regulation by cytokinin (CK) and light (Naito et al., 2007; **Figures 2 and 3**). Subsequent studies could then show that both B-GATAs, GNC, and GNL, contribute to the control of greening and also play a role in the regulation of plant development downstream of the hormones gibberellin (GA) and auxin (Richter et al., 2010, 2013b; **Figure 2**). Thus, these B-GATAs are under the control of multiple signaling pathways including nitrogen availability, several phytohormones as well as light (**Figure 2**). Common to at least some of these input pathways is that they modulate the greening of the plant, which is the most prominent phenotype not only in the loss-of-function mutant but also in the overexpressors of *GNC* and *GNL* (**Figure 4**). *GNC* and *GNL* were also identified as direct targets of the floral homeotic regulatory APETALA3 and PISTILLATA but the functional significance of this regulation remains to be explored (Mara and Irish, 2008).

Arabidopsis has six LLM-domain B-GATAs that can be subdivided into short and long family members. Comparative

analyses suggest that the presence or absence of the LLM-domain correlates with functional differences between these B-GATAs but not protein length (**Figure 3**; Behringer et al., 2014).

REGULATION OF *GNC* AND *GNL* TRANSCRIPTION BY GIBBERELLIN

Gibberellin signaling is mediated by interactions between GA and the GIBBERELLIN INSENSITIVE DWARF1 (GID1) GA receptors (Schwechheimer, 2014). GA-binding triggers the proteasomal degradation of DELLA proteins, negative regulators of GA signaling, via the E3 ubiquitin ligase *SCF^{SLY1}* (SKP1 – CULLIN – F-BOX PROTEIN with the F-box protein SLEEPY1 [SLY1]) or related complexes (Dill et al., 2004). DELLA proteins interfere with the activities of other proteins, mainly transcription factors such as PHYTOCHROME INTERACTING FACTORS (PIFs). The GA-induced degradation of DELLA proteins relieves – in the case of the PIFs – their repressive interactions and allows PIFs to bind DNA (de Lucas et al., 2008; Feng et al., 2008). Studies on the role of *GNC* and *GNL* in GA signaling were instigated by the observation that their transcription is repressed by GA signaling. This transcriptional regulation of the two B-GATAs could be explained by the DELLA-dependent control of PIFs, notably PIF3, which directly binds to *GNC* and *GNL* promoter elements (**Figure 2**; Richter et al., 2010). Since PIF activity is not only negatively regulated by DELLA interactions but also by light, the previously reported light-induced transcription of *GNL* may be



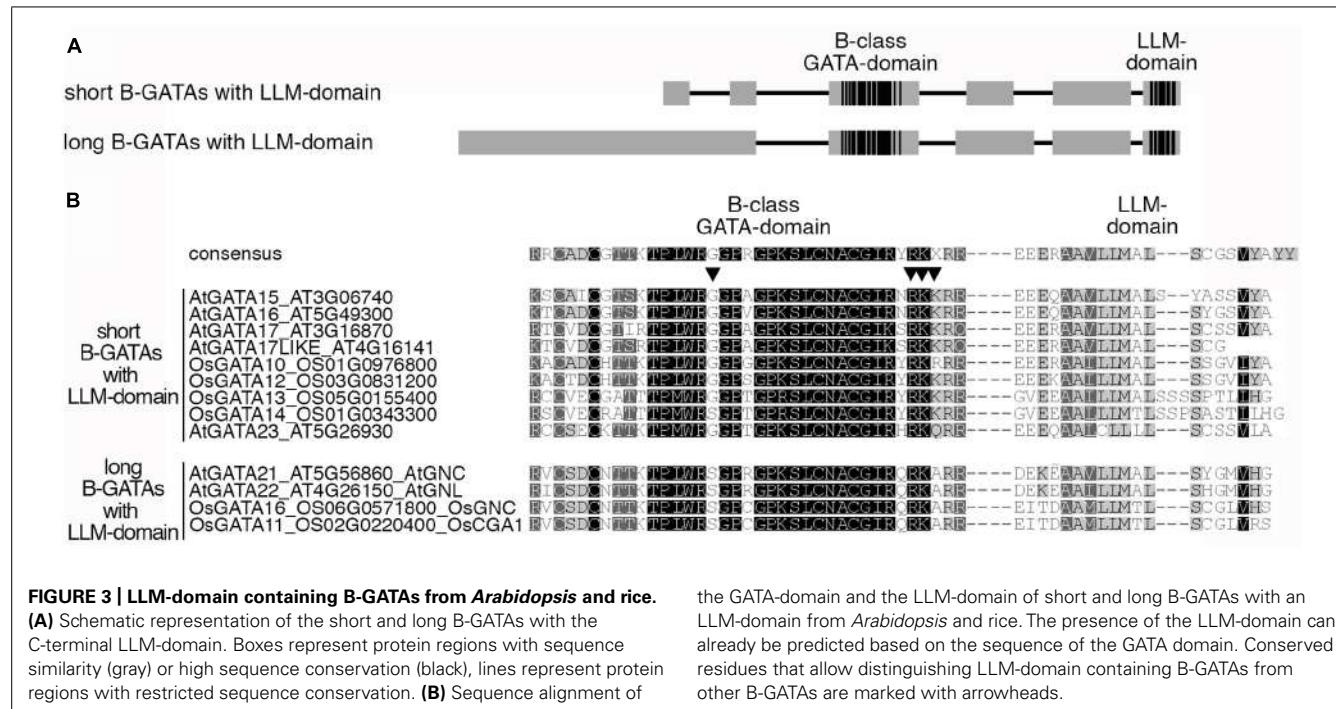


FIGURE 3 | LLM-domain containing B-GATAs from *Arabidopsis* and rice.

(A) Schematic representation of the short and long B-GATAs with the C-terminal LLM-domain. Boxes represent protein regions with sequence similarity (gray) or high sequence conservation (black), lines represent protein regions with restricted sequence conservation. (B) Sequence alignment of

the GATA-domain and the LLM-domain of short and long B-GATAs with an LLM-domain from *Arabidopsis* and rice. The presence of the LLM-domain can already be predicted based on the sequence of the GATA domain. Conserved residues that allow distinguishing LLM-domain containing B-GATAs from other B-GATAs are marked with arrowheads.

explained by the regulation of *GNC* by PIFs (Naito et al., 2007; **Figure 2**).

Mutants and overexpressors of *GNC* and *GNC* have a number of phenotypes that can be explained by defects in GA signaling in that they promote greening and hypocotyl elongation but repress germination and flowering (**Figure 4**). When compared to mutants with a strong GA pathway defect, the contribution of *GNC* and *GNC* to plant growth regulation is comparatively subtle. For example, the strong flowering time delay of the *ga1* mutant is only partially suppressed in *ga1 gnc gnl* (Richter et al., 2010, 2013a). In qualitative terms, this suppression is comparable to the suppression of the *ga1* phenotype by *DELLA* gene mutants from *Arabidopsis*. There, the loss of individual members of the five member *DELLA* gene family only partially suppresses *ga1* phenotypes, whereas the loss of multiple *DELLA* genes results in a strong genetic suppression (Cheng et al., 2004; Cao et al., 2005). Taking into account that there are six presumably functionally redundant LLM-domain containing B-GATAs in *Arabidopsis* (**Figure 3**; Behringer et al., 2014), it could be envisioned that a stronger suppression of *ga1* can be achieved when all six LLM-domain B-GATAs are mutated in *ga1*.

REGULATION OF *GNC* AND *GNC* BY AUXIN AND CROSS-TALK WITH GA SIGNALING

GNC and *GNC* are also transcriptionally repressed by AUXIN RESPONSE FACTOR2 (ARF2; Richter et al., 2013b). ARF2 belongs to the family of ARF transcription factors that have been subdivided into ARF+ that can bind the auxin-labile AUX/IAA repressors and ARF- that do not engage in such repressive interactions (Vernoux et al., 2011). AUX/IAA repressor abundance is negatively regulated by auxin through a specific SFC-type E3 ubiquitin ligase (Dharmasiri et al., 2005a,b). This regulation, however,

only affects the activity of ARF+ and not that of ARF- such as ARF2.

arf2 mutants share a number of phenotypes with *GNC* and *GNC* overexpression lines such as seed size, chlorophyll biosynthesis, stamen length, floral organ abscission, and senescence (Ellis et al., 2005; Okushima et al., 2005; Richter et al., 2013b; **Figure 4**). Interestingly, these *arf2* phenotypes are partially or fully suppressed in the presence of *gnc* and *gnc* loss-of-function mutants (Richter et al., 2013b). Thus, *arf2* mutant phenotypes may be explained by increased *GNC* or *GNC* transcript levels in *arf2* and the repressive activities of the GATAs in this mutant background. Indeed, *GNC* and *GNC* transcription is elevated in *arf2* mutants and ARF2 directly binds to the *GNC* and *GNC* promoters (Richter et al., 2013b).

Although ARF2 is an auxin regulation-independent ARF-, the transcriptional repression of *GNC* and *GNC* can be modulated by auxin. This suggested that also auxin-responsive ARF+ and AUX/IAAs may regulate *GNC* and *GNC* expression. Indeed, loss-of-function mutants of the ARF+ proteins ARF7 and its paralog ARF19 as well as gain-of-function mutants of their interacting AUX/IAA SLR1 (SOLITARY ROOT1) are phenotypically similar to *GNC* and *GNC* overexpressors. In line with a direct activity of ARF7 on the B-GATA promoters, an auxin-modulated binding of ARF7 to the *GNC* and *GNC* promoters could be demonstrated. Thus, *GNC* and *GNC* transcription is under the control of auxin- and AUX/IAA-independent (ARF2) as well as auxin- and AUX/IAA-dependent (ARF7) transcription factors (Richter et al., 2013b; **Figure 2**).

The observation that the two phytohormones, GA and auxin, repress the transcription of *GNC* and *GNC* suggested that modulation of the expression of the two GATAs would allow for a transcriptional cross-talk between these two pathways. In fact,

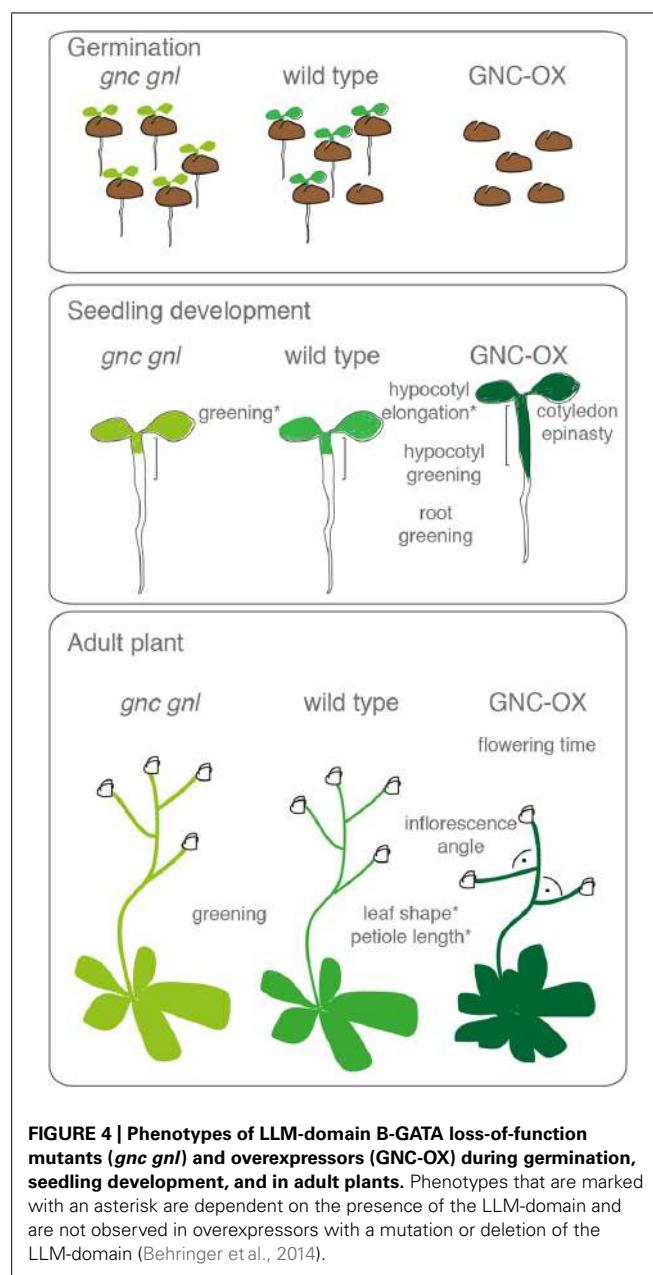


FIGURE 4 | Phenotypes of LLM-domain B-GATA loss-of-function mutants (*gnc gnl*) and overexpressors (GNC-OX) during germination, seedling development, and in adult plants. Phenotypes that are marked with an asterisk are dependent on the presence of the LLM-domain and are not observed in overexpressors with a mutation or deletion of the LLM-domain (Behringer et al., 2014).

several phenotypes of the *arf2* mutant could be suppressed by GA treatments or in the presence of a *spy* (*spindly*) mutation, which phenotypically mimics the phenotypes of plants with constitutively active GA signaling (Richter et al., 2013b). Thus, GA and auxin signaling converge on the transcriptional regulation of *GNC* and *GNL* and these two signals control at least in part the same growth responses (Figure 2).

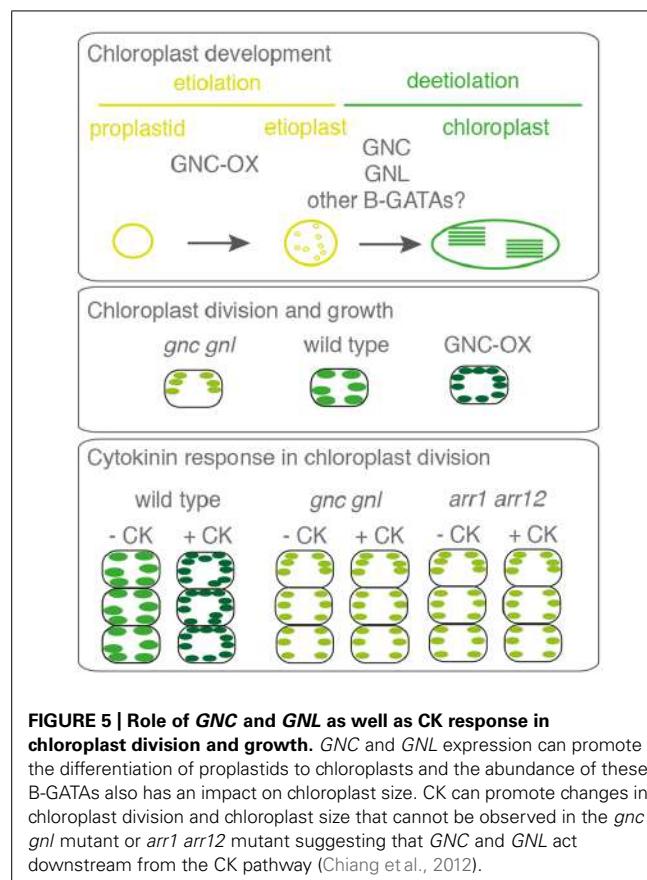
The analysis of this GA-auxin cross-talk also resulted in the identification of two feedback regulatory mechanisms that contribute to the regulation of *GNC* and *GNL* expression (Richter et al., 2013b). First, ARF2 autoregulates its own transcription and thereby negatively feeds back on its own transcription as well as *GNC* and *GNL* regulation, and second, GA promotes ARF2 abundance by controlling ARF2 translation or by controlling the

stability of a *de novo* synthesized and unknown GA-responsive protein involved in regulating ARF2 abundance (Richter et al., 2013b).

GNC AND GNL PROMOTE GREENING DOWNSTREAM FROM CYTOKININ

GNC was isolated based on the greening defect of its loss-of-function mutant (Bi et al., 2005). Although such a greening defect is not visible (but quantifiable) in the *gnl* mutant, it is enhanced in the *gnc gnl* double mutant. *GNC* and *GNL* thus redundantly regulate greening, possibly together with other LLM-domain containing B-GATAs (Figure 4; Richter et al., 2010; Behringer et al., 2014). The greening phenotype of *GNC* and *GNL* overexpression lines correlates with the increased expression of the chloroplast localized *GLUTAMATE SYNTHASE*, *HEMA*, *GENOMES UNCOUPLED4*, and *PROTOCHLOROPHYLLIDE OXYDOREDUCTASE* genes as well as that of *PDV2* (*PLASTID DIVISION2*; Richter et al., 2010; Hudson et al., 2011). At the same time, overexpression of *GNC*, *GNL*, or that of other B-GATAs induces a strong greening phenotype in tissues that normally do not contain significant numbers of chloroplasts such as the lower hypocotyl, the upper part of the root, and epidermal cells of cotyledons, and the hypocotyl (Richter et al., 2010; Chiang et al., 2012; Behringer et al., 2014). The role of the LLM-domain containing B-GATAs in the control of greening is conserved across species since the overexpression of LLM-domain containing B-GATAs from barley, tomato, or rice induces similar phenotypes when tested in *Arabidopsis* or rice, respectively (Hudson et al., 2011; Behringer et al., 2014). Taken together, LLM-domain containing B-GATAs are at least in some tissues sufficient to strongly promote greening.

B-GATAs may control greening by promoting chlorophyll biosynthesis, chloroplast formation, or chloroplast size. In this regard, it is important to note that CK, which induces *GNC* and *GNL* expression, can promote greening in multiple developmental contexts (Kiba et al., 2005; Figure 5). CK induces chloroplast division by activating the expression of the chloroplast division regulators *PDV1* and *PDV2* in a manner that is dependent on the CK-induced regulator *CRF2* (CYTOKININ RESPONSE FACTOR2; Okazaki et al., 2009). Although this increase in chloroplast division correlates with a reduction of chloroplast size, CK-treated plants have elevated chlorophyll levels (Okazaki et al., 2009). Furthermore, CK can promote greening ectopically in tissue that normally does not contain many chloroplasts including the upper part of the root (Kobayashi et al., 2012). Along these lines, the strong greening phenotype of the *GNC* overexpressors can be explained by an increased number of chloroplasts that is accompanied by the reduction of chloroplast size as it is typical for CK-treated seedlings (Chiang et al., 2012; Figure 5). Although the number of chloroplasts is not reduced, *gnc gnl* mutants have smaller chloroplasts in the hypocotyls and reduced chlorophyll levels in seedlings (Richter et al., 2010; Chiang et al., 2012). Additionally, ectopic expression of *GNC* promotes the differentiation of etioplasts from proplastids in dark-grown seedlings, which also can be correlated with an accelerated greening when etiolated seedlings are exposed to light (Chiang et al., 2012). CK treatment induces the expression of *GNL* but is less efficient in inducing the expression of *GNC* (Naito et al., 2007; Chiang et al., 2012).



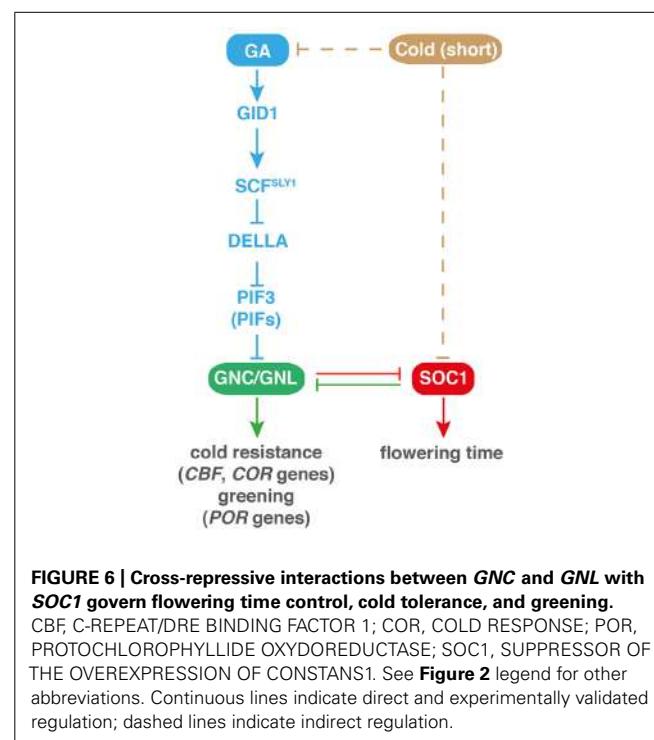
The type-B response regulators ARABIDOPSIS RESPONSE REGULATOR1 (ARR1) and ARR12 are important for this regulation since CK-induced gene expression of GNL is strongly compromised in *arr1 arr12* mutants where CK effects on chloroplast division are also compromised (Argyros et al., 2008; Chiang et al., 2012; Figure 5). Furthermore, *gnc gnl* mutants exhibit reduced CK sensitivity in chloroplast division (Chiang et al., 2012). Taken together these findings suggest that the greening defect of *gnc gnl* mutants is caused by their reduced CK-responsiveness and that this CK response requires the induction of GNL and possibly other B-GATAs through type-B ARR. This adds LLM-domain containing B-GATAs to the list of transcription factors that can promote greening downstream from CK such as the previously mentioned CRF2 but also GLK2 (GOLDEN LIKE2; Fitter et al., 2002; Okazaki et al., 2009; Kobayashi et al., 2012). Although this has not been studied in detail, it may be that the effects of GA and auxin signaling on greening (Richter et al., 2010; Richter et al., 2013b) are, at least in part, also a consequence of the role of GNC and GNL on chloroplast division as demonstrated for CK signaling.

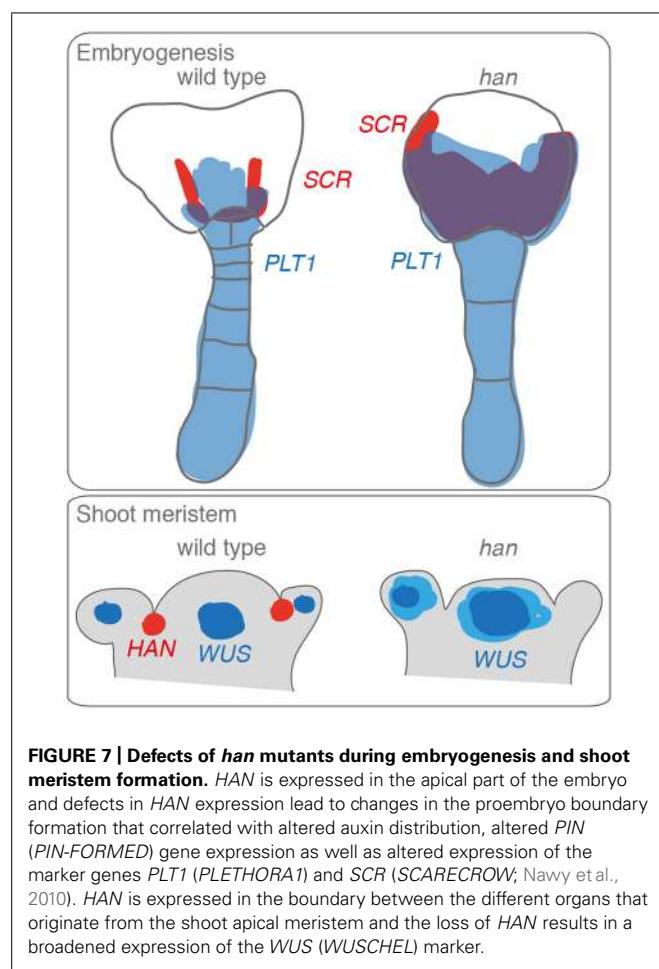
CROSS-REPRESSIVE INTERACTIONS BETWEEN GNC, GNL, AND SOC1 IN THE CONTROL OF FLOWERING TIME, GREENING, AND COLD TOLERANCE

GNC and GNL are flowering repressors. The contribution of these two GATAs to flowering time control can be observed in the GA-deficient late flowering mutant *gal* where loss of GNC and GNL

function promotes the flowering of *gal* by about a month (Richter et al., 2013a). A dedicated analysis has placed these B-GATAs in the network around the flowering time regulator SOC1 (*SUPPRESSOR OF THE OVEREXPRESSION OF CONSTANS1*; Richter et al., 2013a). The MADS-box transcription factor SOC1 is a major regulator of flowering time in *Arabidopsis thaliana*. SOC1 expression is under control of a number of flowering promoting inputs and SOC1 expression is essential for floral induction in long day conditions (Samach et al., 2000; Yoo et al., 2005). In short-day conditions, SOC1 is a major integrator of flowering time stimulation by GA (Blázquez and Weigel, 1999; Moon et al., 2003). Based on the central role proposed for SOC1 in flowering time regulation, SOC1 also qualified as a possible target of flowering time control downstream from GNC and GNL. Indeed, the promoter of SOC1 is recognized by both GATAs and SOC1 expression is strongly downregulated when the GATAs are overexpressed (Figure 6). In turn, when SOC1 expression is uncoupled from GNC and GNL control in a SOC1 overexpression line, the flowering repressive effects of GNC and GNL overexpression are suppressed. Thus, GNC and GNL act upstream of SOC1 in flowering time control.

Curiously, the respective genetic interaction experiments also indicated that there may be an inverse relationship between SOC1 and the GATAs in the control of other B-GATA-regulated responses that are not directly related to flowering time control (Figure 6). In fact, the genetic interaction experiments between SOC1, GNC, and GNL indicated that two other phenotypes of *soc1* mutants, enhanced greening and decreased cold tolerance, are suppressed in the absence of the GNC and GNL regulators (Richter et al., 2013a). Thus, cross-repressive interactions between these B-GATAs and SOC1 govern distinct biological processes.





HAN-DOMAIN CONTAINING B-GATAs REGULATE EMBRYOGENESIS AND FLOWER DEVELOPMENT

HAN (*HANABA TARANU*; Japanese for *floral leaf*; *TARANU*, Japanese for *not enough*) was independently identified in genetic screens as a mutant with altered floral organ identity (Zhao et al., 2004) and altered embryo patterning (Nawy et al., 2010; Figure 7). The HAN-domain, which is specific for this family of B-GATAs was first noted in *HAN* and its HAN-LIKE paralogs from *Arabidopsis* and later used to classify further B-GATAs as monocot-specific HAN-paralogs (Figures 1 and 8; Zhao et al., 2004; Whipple et al., 2010). The biological role of this B-GATA-specific domain is as yet unknown but may serve for interactions with other proteins. Whereas the overexpression of LLM-domain containing B-GATAs gives rise to a number of growth defects, most prominently the accumulation of chlorophyll at the base of the hypocotyl and hypocotyl elongation, *HAN* and *HANL2* overexpressors have different phenotypes, e.g., they accumulate less chlorophyll than the wild type and have normal hypocotyl length (Behringer et al., 2014). Thus, based on these criteria, HAN-domain B-GATAs are functionally distinct from LLM-domain containing B-GATAs.

HAN – A REGULATOR OF EMBRYO DEVELOPMENT

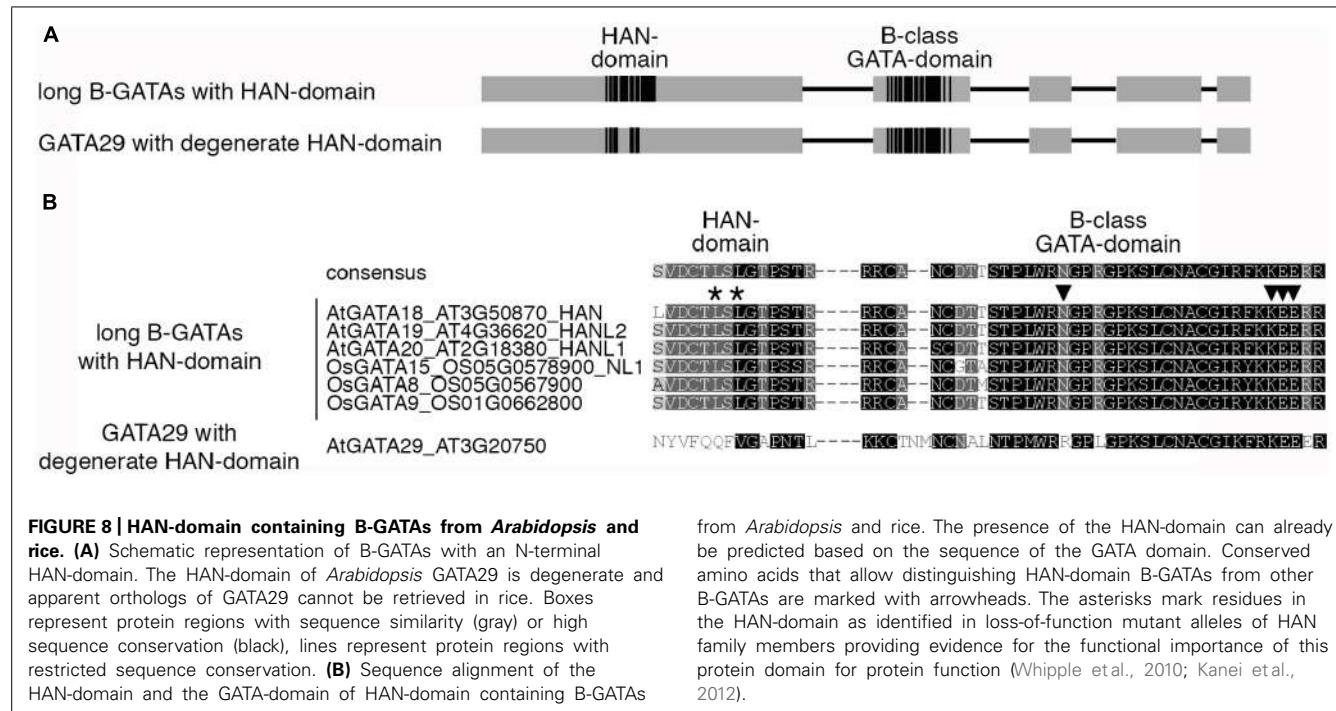
During embryo development, *HAN* is required for the proper positioning of the proembryo boundary (Figure 7). *han* mutant

embryos have several developmental defects, including a vacuolation of the lower tier cells of the embryo and a decrease in suspensor cell divisions (Nawy et al., 2010). The expression domains of embryonic markers for the suspensor and the lower tier are shifted apically in globular stage *han* embryos and this fits, in the case of the suspensor marker-positive cells, to their morphological resemblance to suspensor cells. The apical shift of auxin distribution in *han* mutant embryos and a broadening of the expression of the auxin response marker DR5:GFP (DR5:GREEN FLUORESCENT PROTEIN) are further indications for defects in proembryo boundary positioning in these mutants. Since lower tier cells normally give rise to the root, root formation is impaired in *han* embryos. *han* mutants fail to form an embryonic root and they are unable to undergo an essential cell division of the uppermost suspensor cell, the hypophysis that produces the quiescent center (QC). Interestingly, most *han* mutants can later recover from this defect and produce a root independently of the hypophysis at a later stage of embryo development (Nawy et al., 2010). It is thought that this rescue is the consequence of the coincidental expression of several prerequisites for QC formation: a local auxin maximum and the expression of the root regulators *PLETHORA*, *SHORT ROOT* and *SCARECROW* (Nawy et al., 2010). In addition to root development phenotypes, *han* mutants have also defects in cotyledon growth and initiation. *han* mutants sometimes have up to four cotyledons (Zhao et al., 2004). Ectopic root formation and altered *PLETHORA* expression were also observed in a *han* allele that strongly enhances the phenotype of mutants of the leaf development regulator *ANGUSTIFOLIA3* (Kanei et al., 2012).

Auxin is an actively transported hormone and its distribution within the embryo is mediated by auxin efflux carriers such as PIN1 (PIN-FORMED1) and PIN7 (Friml et al., 2003). In the wild type, auxin initially accumulates in the apical part and shifts to the suspensor preceding hypophyseal cell specification. This shift in auxin distribution correlates with a shift of PIN7 in the suspensor from the apical to the basal plasma membrane and a shift of PIN1 from being non-polarly distributed to being polarly distributed in the provascular cells of the proembryo (Friml et al., 2003). Both, the expression domain of *PIN1* as well as that of *PIN7* is shifted apically in *han* mutants and the ectopic expression of the *PINs*, at least that of *PIN1*, can find its explanation in a possibly direct transcriptional regulation of *PIN1* by *HAN* (Nawy et al., 2010). Thus, the *han* mutant phenotype may have its molecular cause in a misexpression of the *PINs* and consequently altered auxin distribution.

HAN – A FLORAL MORPHOLOGY REGULATOR

HAN was first described based on the *han* mutants with altered shoot meristem morphology (Zhao et al., 2004; Figure 7). When compared to the wild type, *han* loss-of-function mutants have small flat shoot meristems, reduced numbers of floral organs in all four whorls as well as fused sepals. *HAN* is expressed between the meristem and between newly initiated floral organ primordia and in the boundaries between the different floral organ whorls. *HAN* overexpression, on the other side, results in delayed plant growth, disturbed cell divisions, and a loss of meristem activity. Taken together these findings suggest that *HAN* acts as a repressor



of cell proliferation and that loss of this repressive function could lead to the reduced meristem size, which may be the cause for the reduced floral organ numbers and fused floral organs seen in its mutants (Zhao et al., 2004).

HAN expression surrounds the floral meristem cells and *HAN* interacts strongly with the *CLV* (*CLAVATA*) pathway (Zhao et al., 2004). In *Arabidopsis*, shoot meristem size is determined on the one side by the plasma membrane-resident receptor proteins CLV1 and CLV2 that are co-expressed in the outer layer of the shoot meristem as well as their putative peptide ligand CLV3 that is expressed in the underlying tissue layers. Defects in any of the three *CLV* genes results in enlarged shoot and floral meristems and the formation of an increased number of floral organs. The expression of CLV3 is negatively regulated by the homeobox-type transcription factor WUSCHEL (WUS) and CLV3 expression overlies the expression domain of WUS suggesting, in combination with evidence from mutant analyses, that WUS controls meristem size by restricting CLV3 expression as an essential ligand for the CLV1 and CLV2 receptor proteins. A *han* mutation combined with *clv* gene mutations resulted in increased inflorescence fasciation and increased floral abnormalities (Zhao et al., 2004). It has been proposed that the *HAN* gene is required to control WUS expression and reduced WUS expression as well as ectopic WUS expression may have a role in controlling floral meristem growth and repress floral organ primordium initiation (Figure 7). Alternatively, it may be envisioned that defects in nutrient or signal transport hinder meristem growth and floral organ development since *HAN* is also expressed early in provascular cells (Zhao et al., 2004). Interestingly *HAN* is also expressed in the boundaries between different whorls and between different floral organs suggesting that *HAN* could also act as a repressor of cell divisions. In this regard, there are

some interesting parallels to the role proposed for HAN-related B-GATAs in bract suppression in monocots as will be discussed below.

Molecular analysis for transcription factor targets identifies *HAN* as a repression target of JAGGED (Schiessl et al., 2014). Genes acting downstream of *HAN* were also searched for using translational fusions between *HAN* and the glucocorticoid receptor, which allows for the glucocorticoid hormone-induced translocation of the *HAN*-GR fusion protein from the cytoplasm to the nucleus (Zhang et al., 2013). This analysis identified a range of floral development regulators as well as phytohormone-related genes as targets of *HAN* and suggested that *HAN* can act as a transcriptional activator and repressor. Interestingly, amongst the phytohormonal target genes are genes of those phytohormonal pathways that are known to regulate the expression of the LLM-domain B-GATAs GNC and GNL such as the DELLA genes of the GA pathway, ARR genes of the CK pathway, and ARF and AUX/IAA genes of the auxin pathway. Although the chosen experimental approach would have permitted to test for direct transcription targets by blocking *de novo* protein synthesis this possibility was not exploited. In conjunction with the fact that rather long time points (4 h up to 72 h) after glucocorticoid treatment were used for the sampling of the material and that many transcription factor genes were found to be regulated downstream from *HAN* in this experiment argues that the majority of these downstream genes could represent indirect rather than direct targets of *HAN*.

Among the genes that were found to be *HAN*-regulated according to this experiment were also *HANL2*, *GNC* as well as *GNL*. The transcriptional repression of these three genes suggested that their downregulation may be part of a negative feedback mechanism that serves to control B-GATA levels. Indeed, *HAN* was found to

be able to bind to its own promoter as well as the promoter of GNC. Furthermore, genetic interaction studies using mutants of these B-GATAs found that mutant combinations of *han* with *hanl2*, *gnc*, and *gnl* mutations resulted in a strong decrease in the number of petals formed in these mutants, sepal fusion defects, fertility defects, as well as carpel abnormalities (Zhang et al., 2013). Also during embryogenesis, the combination of B-GATA mutations renders the previously described *han* embryogenesis defects more severe and embryos frequently terminate differentiation and form only clusters of cells (Zhang et al., 2013). Although the respective mutant analyses suffer from the weakness that mutations in the Columbia and Landsberg *erecta* backgrounds were combined and some of the observed defects may therefore be the result of these combinations, the genetic interplay between HAN and the other B-GATAs is also supported by the fact that HAN can homodimerize and interact with HANL2 as well as with GNC and GNL in yeast two-hybrid system (Zhang et al., 2013).

MONOCOT-SPECIFIC HAN-PARALOGS

Whereas the formation of bract leaves is blocked in flowers of cultivated rice, maize, or barley, mutants from each of these species are known where the formation of such bract leaves is derepressed. In each case, the respective locus was identified and found to correspond to the *HAN* paralogs genes *NL1* (NECK LEAF1) from rice, *TSH1* (TASSEL SHEATH1) from maize and *TRD* (THIRD OUTER GLUME) from barley (Wang et al., 2009; Whipple et al., 2010; **Figure 1**). In line with the mutant phenotype, it could be shown that the expression of these *HAN*-domain B-GATAs is restricted to a cryptic bract in the zone where the suppression of bract formation is observed in the wild type. Interestingly, these B-GATAs form a monocot-specific subclade of *HAN*-domain B-GATAs indicating that these B-GATAs were recruited for the suppression of bract outgrowth specifically during monocot evolution. The apparent role as a repressor of bract growth also fits to the proposed function for HAN as a repressor of growth and cell cycle activities in the shoot meristem.

GATA23 – A Brassicaceae-SPECIFIC B-GATA WITH A DEGENERATE LLM-DOMAIN

Within both B-GATA subfamilies, there is one family member with a degenerate HAN- or LLM-domain, GATA29, and GATA23, respectively (**Figures 8 and 9**). Whereas there is no information about the role of GATA29, GATA23 has been proposed to act in the root following its identification in a search for genes that are induced during the early steps of lateral root initiation (De Rybel et al., 2010). GATA23 is specifically expressed in xylem pole pericycle cells before their first asymmetric division. Auxin accumulation is the first marker for lateral root founder cells and in line with an early role for GATA23 during lateral root initiation, GATA23 is auxin-induced. Since the expression of GATA23 is impaired in gain-of-function mutants of the AUX/IAA gene *IAA28*, which is defective in lateral root formation, and since IAA28 interacts with several ARFs including the previously introduced ARF+ARF7 and ARF19, a model was proposed, according to which auxin promotes lateral root initiation through degradation of the AUX/IAA IAA28 and subsequent ARF+-mediated GATA23 expression. This model is supported by observations that lateral root initiation is partially

suppressed in plants expressing an GATA23 RNAi construct. The cell type specific expression of GATA23, in turn, correlates with increased lateral root initiations and uncoupling GATA23 expression from auxin control also interferes with the normally regular spacing of lateral roots.

To what degree GATA23 is important for lateral root initiation across species remains to be seen. Phylogenetic analyses have revealed that GATA23 from *Arabidopsis* belongs to a specific clade of B-GATAs with a degenerate LLM-domain that is closely related to GNC and GNL but functionally distinct (**Figures 1 and 9**; Behringer et al., 2014). At present, B-GATAs with the sequence features of GATA23 can only be identified in *Brassicaceae* and thus its function in non-*Brassicaceae* in lateral root initiation cannot be conserved outside of this family. Future research will have to elucidate the apparent functional diversification of these specific B-GATAs.

OUTLOOK

Important advances have been made in understanding the role of B-GATA transcription factors in plant growth and development. Although there is now a comprehensive understanding of how the expression of these B-GATA genes is regulated at the transcriptional level, the knowledge about the identity of their target genes and cell type-specific activities is scarce. Candidate target genes of B-GATAs were genetically validated in a few exceptional cases only and, as yet, high quality transcription target analyses remain to be performed. Such experiments will be key to understand to what extent B-GATAs have overlapping and distinct transcription targets and should permit to delineate further to what extent differences in their expression domains or differences at the protein level contribute to their functional diversification.

Research on the LLM-domain containing B-GATAs has to date largely focused on the signaling events regulating their expression as well as on their role in the control of physiological processes such as greening and flowering. In turn, research on *HAN*-domain B-GATAs mainly focused on their role in the control of development. It can be anticipated that this apparent separation in the biological functions of B-GATAs between physiology and development will become more and more blurred in the future when developmental biologists will start studying LLM-domain B-GATAs and physiologists will study *HAN*-domain B-GATAs.

The fact that B-GATAs are unstable proteins that are turned-over by the 26S proteasome with a half-life of about 30 min is one interesting observation regarding all B-GATAs that requires further exploration (Behringer et al., 2014). It implies that there must be cognate E3 ubiquitin ligases that target these proteins for degradation. The identification of these E3 ligases will allow revealing cellular contexts where B-GATA abundance is differentially controlled and improve our understanding of B-GATA function.

The observation that different members of the plant B-GATA family, namely HAN, GNC, and GNL proteins, can interact with each other, could suggest that also other B-GATAs may act as homo- or heterodimers and may thus engage in interactions that could modulate their DNA-binding specificity or their function as transcriptional activators or repressors (Zhang et al., 2013; Behringer et al., 2014). Mammalian GATA factors interact with other transcription factors designated FRIEND OF GATA (FOG;

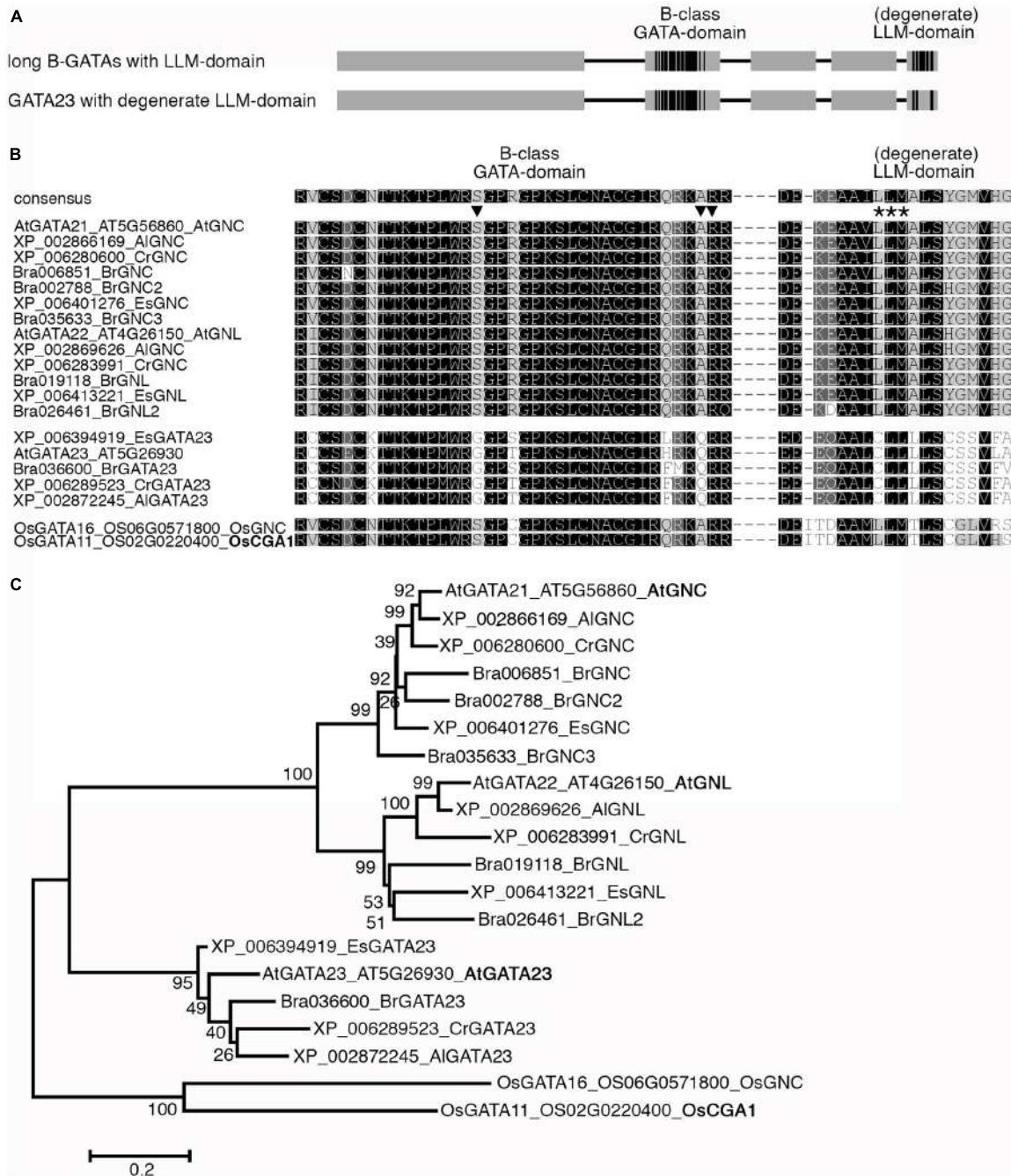


FIGURE 9 | GATA23 is specific for the Brassicaceae. **(A)** Schematic representation of long B-GATAs with the C-terminal LLM-domain and GATA23 with a degenerate LLM-domain. Boxes represent protein regions with sequence similarity (gray) or high sequence conservation (black), lines represent protein regions with restricted sequence conservation. **(B)** Sequence alignment of the GATA-domain and the (degenerate) LLM-domain of the LLM-domain containing B-GATAs AtGNC and AtGNL as representatives for LLM-domain containing B-GATAs as well as AtGATA23 from *A. thaliana* (At) as a B-GATA with a degenerated LLM-domain and their predicted orthologues from other Brassicaceae: *A. lyrata* (Al), *Capsella rubella* (Cr), *Brassica rapa* (Br), *Eutrema salsugineum* (Es). Whereas the core LLM-motif is conserved among the GNC and GNL orthologues from the different Brassicaceae species and rice, it is divergent in the GATA23 B-GATAs. The triangles mark characteristic amino acid residues of the

B-GATA domain that allow predicting the presence of the LLM-domain or a degenerate LLM-domain. Please note the conservation of these residues between the LLM-domain containing B-GATAs from the Brassicaceae and rice whereas the GATA23 orthologues are also divergent in these residues in the DNA-binding domain. **(C)** Phylogenetic tree of the B-GATAs shown in **(B)**. The phylogenetic tree was generated using the Geneious R7 Software based on a MUSCLE alignment in MEGA6.06 using the following settings: Gap penalty, gap open -2.9, gap extend 0, hydrophobicity multiplier 1.2; iterations, maximum iterations 8; clustering method, all iterations UPGMB and minimum diagonal length (lambda) 24. The Neighbor Joining tree was generated with the bootstrap method (1000 replications) using the Jones-Taylor-Thornton model using default settings. Bootstrap values are indicated by each node. Bar = 0.2 amino acid substitutions per site.

Tsang et al., 1997; Fox et al., 1998), but obvious FOG homologues are not encoded by the plant genomes. Thus, this regulatory mechanism is most likely not conserved between animals and plants. It should be noted, however, that GATAs were found as interaction partners in yeast two-hybrid interaction analyses. First, *Arabidopsis* GNC was isolated as an interactor of the transcriptional co-regulator SIN3-LIKE1 (Bowen et al., 2010) and second, the LLM-domain B-GATA AtGATA16 appeared in a screen for proteins interacting with the co-repressor TOPLESS (Causier et al., 2012). Future research will have to reveal the biological significance of these interactions for GATA factor function.

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Arabidopsis MSI1 functions in photoperiodic flowering time control

Yvonne Steinbach^{1*} and Lars Hennig²

¹ Department of Biology, Institute of Agricultural Sciences, ETH Zürich, Zürich, Switzerland

² Department of Plant Biology, Uppsala BioCenter, Swedish University of Agricultural Sciences and Linnean Center for Plant Biology, Uppsala, Sweden

Edited by:

Dorothee Staiger, Bielefeld University, Germany

Reviewed by:

Andreas Erwin Muller, Strube Research, Germany

Chris Helliwell, CSIRO, Australia

***Correspondence:**

Yvonne Steinbach, Department of Biology, Institute of Agricultural Sciences, ETH Zürich, Universitätstr.2, CH-8092 Zürich, Switzerland

e-mail: syvonne@ethz.ch

Appropriate timing of flowering is crucial for crop yield and the reproductive success of plants. Flowering can be induced by a number of molecular pathways that respond to internal and external signals such as photoperiod, vernalization or light quality, ambient temperature and biotic as well as abiotic stresses. The key florigenic signal FLOWERING LOCUS T (FT) is regulated by several flowering activators, such as CONSTANS (CO), and repressors, such as FLOWERING LOCUS C (FLC). Chromatin modifications are essential for regulated gene expression, which often involves the well conserved MULTICOPY SUPPRESSOR OF IRA 1 (MSI1)-like protein family. MSI1-like proteins are ubiquitous partners of various complexes, such as POLYCOMB REPRESSIVE COMPLEX2 or CHROMATIN ASSEMBLY FACTOR 1. In Arabidopsis, one of the functions of MSI1 is to control the switch to flowering. Arabidopsis MSI1 is needed for the correct expression of the floral integrator gene SUPPRESSOR OF CO 1 (SOC1). Here, we show that the histone-binding protein MSI1 acts in the photoperiod pathway to regulate normal expression of CO in long day (LD) photoperiods. Reduced expression of CO in *msi1*-mutants leads to failure of FT and SOC1 activation and to delayed flowering. MSI1 is needed for normal sensitivity of Arabidopsis to photoperiod, because *msi1*-mutants responded less than wild type to an intermittent LD treatment of plants grown in short days. Finally, genetic analysis demonstrated that MSI1 acts upstream of the CO-FT pathway to enable an efficient photoperiodic response and to induce flowering.

Keywords: *Arabidopsis*, *flowering time*, *chromatin*, *MSI1*, *photoperiod*, *FLOWERING LOCUS T (FT)*, *CONSTANS (CO)*

INTRODUCTION

The reproductive success of plants depends on the appropriate time to flower, which is of great agronomic relevance in crops. Flowering can be induced by a number of molecular pathways that respond to internal and external signals. Major genetic pathways controlling flowering time have been characterized based on the phenotype of *Arabidopsis thaliana* flowering time mutants in different growth conditions. These pathways include the photoperiod pathway, which responds to seasonal changes in day length, and the vernalization pathway, which responds to prolonged exposure to cold. The autonomous and gibberellin-pathways mediate the response to endogenous signals. Additionally, light quality, ambient temperature, and biotic as well as abiotic stresses can contribute to floral induction in plants (for review see: Jarillo and Piñeiro, 2011; Srikanth and Schmid, 2011).

The different pathways converge on pathway integrators, a set of genes that strongly promote flowering such as *FLOWERING LOCUS T (FT)*, *SUPPRESSOR OF CONSTANS 1 (SOC1/AGL20)* or the *FT* homolog *TWIN SISTER OF FT (TSF)*. Mutants in these genes have late flowering phenotypes (Kardailsky et al., 1999; Kobayashi et al., 1999; Borner et al., 2000; Lee et al., 2000; Samach et al., 2000; Yamaguchi et al., 2005). The pathway integrators *FT* and *TSF* are antagonistically regulated by the floral repressor *FLOWERING LOCUS C (FLC)* (Michaels and Amasino,

1999) and the floral activator *CONSTANS (CO)* (Yanovsky and Kay, 2002).

The nuclear zinc finger transcription factor CO is the key activator in the photoperiod pathway to promote expression of *FT* and *TSF* (Suarez-Lopez et al., 2001; Valverde et al., 2004). CO protein is stable in the light and rapidly degraded in the dark. CO is regulated by the circadian clock and accumulates diurnally late in the day in long day (LD) conditions. In contrast, CO peaks during the night in SD where protein degradation prevents CO accumulation. *FT* is directly regulated by CO and follows the clock-regulated expression pattern of CO in LD (Suarez-Lopez et al., 2001; Valverde et al., 2004). Regulation of *FT* expression can occur also independently of CO and the photoperiodic pathway such as due to decreased red to far red light ratios in the shade avoidance response (SAR). SAR is mediated through the key regulator of the light-quality pathway phytochrom B by post-transcriptional repression of the *FT*-activator *PHYTOCHROME AND FLOWERING TIME 1 (PFT1)* (Cerdan and Chory, 2003; Halliday and Whitelam, 2003; Bäckström et al., 2007). In several species, such as *Arabidopsis*, tomato, tobacco and rice, the *FT* protein or its homologs are known to move from leaves into the shoot apical meristem (SAM) where it induces the switch to flowering (Corbesier et al., 2007) by inducing the expression of the downstream

targets *SOC1* and *APETALA 1* (*AP1*) (reviewed in Zeevaart, 2008).

The floral integrators *FT*, *SOC1*, and *TSF* are commonly repressed by the potent flowering repressor *FLC*. Vernalization or the autonomous pathway of floral promotion establish low *FLC* levels and thus favor flowering. In contrast to other flowering time pathways, the autonomous pathway does not represent a linear genetic pathway and involves RNA-binding proteins (FCA, FPA, FLK), RNA processing proteins (FY) and chromatin regulators (FVE/MSI4, FLD) (for review see Simpson, 2004).

The autonomous pathway gene *FVE* is needed to establish repressive chromatin at *FLC* and encodes a MULTICOPY SUPPRESSOR OF IRA 1 (MSI1)-like chromatin-adaptor protein (Ausin et al., 2004; Kim et al., 2004; Jeon and Kim, 2011). MSI1-like proteins belong to a subfamily of WD-40 repeat proteins that are subunits in several chromatin remodeling complexes (for review see Hennig et al., 2005). The single Drosophila MSI1-like protein p55 is a core subunit of Polycomb Group Repressive Complex 2 (PRC2), of Chromatin Assembly Factor 1, of histone deacetylase complexes and of other chromatin-associated protein complexes (for review see: Hennig et al., 2005). In contrast to flies, Arabidopsis has five MSI1-like proteins (MSI1-5). While Arabidopsis MSI4 was suggested to act in histone deacetylation (Ausin et al., 2004; Kim et al., 2004; Jeon and Kim, 2011), Arabidopsis MSI1 was shown to be part of Chromatin Assembly Factor 1 and PRC2-like complexes (Kaya et al., 2001; Köhler et al., 2003; Exner et al., 2006; Schönrock et al., 2006a; De Lucia et al., 2008; Derkacheva et al., 2013). MSI1 functions in the FERTILISATION INDEPENDENT SEED (FIS)-PRC2 complex, which silences target genes during gametophyte and early seed development (Köhler et al., 2003; Guitton et al., 2004; Guitton and Berger, 2005); the VERNALIZATION (VRN)-PRC2 complex, which is required for epigenetic repression of *FLC* and acceleration of flowering by extended cold (De Lucia et al., 2008; Derkacheva et al., 2013) and the EMF-complex, which suppresses precocious flowering by repressing *FT* and *AGL19* but which contributes also to repression of *FLC* (Yoshida et al., 2001; Schönrock et al., 2006a; Jiang et al., 2008). Because of the propeller-like structure of the WD40-domain, MSI1 and other MSI1-like proteins can possibly participate in additional chromatin-modifying complexes. Indeed, MSI1 was found to interact with LHP1 connecting plant PRC2 with LHP1 to establish repressive H3K27 methylation marks (Derkacheva et al., 2013). Further, MSI1 interacts with the CUL4-DDB1 complex and the Retinoblastoma-related protein to control imprinting in Arabidopsis (Jullien et al., 2008; Dumbliauskas et al., 2011). Chromatin-based mechanisms have recently emerged as a major means of control for many cellular processes including flowering time. In particular, the importance of chromatin-based regulation for control of *FLC* is well documented (Zografas and Sung, 2012).

Previously, we found that MSI1 represses drought stress responses (Alexandre et al., 2009) and is needed for timely flowering and for normal expression of *SOC1* (Bouveret et al., 2006). Here we demonstrate that MSI1 functions during floral transition by establishing normal expression of the flowering activator *CO* and subsequently of the florigen *FT* and *TSF*. We also show that

delayed up-regulation of gene expression of the floral integrator genes correlates with the delay in flowering in a *msi1*-mutant. Our data suggest that MSI1 is needed for the efficient activation of *CO*, thus allowing full activity of the photoperiodic pathway for floral induction.

MATERIALS AND METHODS

PLANT MATERIAL

Arabidopsis thaliana ecotype Columbia (Col) was used throughout this study. T-DNA insertion lines *phyB* (SALK_022035, Mayfield et al., 2007; Ruckle et al., 2007), *pft1-2* (SALK_129555, Kidd et al., 2009), *flc-6* (SALK_41126, Schönrock et al., 2006a) and *msi1-5* (WiscDs Lox302B08) were obtained from NASC and confirmed by PCR. Seeds of *FRI*-Col, *esd1-10*, *ft-10* and *soc1-2* have been described (Lee and Amasino, 1995; Lee et al., 2000; Yoo et al., 2005; Martin-Trillo et al., 2006) and were kindly provided by J. Jarillo (FRI-Col, *esd1-10*), D. Weigel (*ft-10*), I. Lee (*soc1-2*), B. Ayres (*co-1*). The mutant *co-1* (accession La-0, Redei, 1962) was backcrossed into Col. The line *msi1-tap1* (accession Col) has been described before (Bouveret et al., 2006). Double mutants were identified among progeny of appropriate crosses by PCR with gene-specific primers (**Supplementary Table 1**).

To construct plants that ectopically overexpress *FT* (35S::*FT*), the full-length coding sequence was inserted into the binary destination vector pK7WG2 (Karimi et al., 2002) downstream of the cauliflower mosaic virus (CaMV) 35S promoter and transformed into *msi1-tap1* plants. Transformants were selected on kanamycin plates and genotyped by PCR. Hemizygous T2-generation plants of three independent T1 lines were analyzed for flowering time.

GROWTH CONDITIONS AND FLOWERING TIME

Seeds were sterilized and plants were grown on Murashige and Skoog (MS) basal salt medium (Duchefa, Brussels, Belgium) after stratification at 4°C for 2–3 days. Plants were analyzed on plates or transferred to soil (“Einheitserde,” H. Gilgen optima-Werke, Arlesheim, Switzerland) 10 days after germination. Plants were kept in Conviron growth chambers with mixed cold fluorescent and incandescent light (130 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 21 ± 2°C) under (LD, 16 h light) or short-day (SD, 8 h light) photoperiods or were raised in green houses [LD: 14 h light, 19°C/10 h dark, 14°C; SD: 8 h light, 20°C/16 h dark, 20°C; supplemented with mercury vapor lamps (Sylvania Lighting S.A., Meyrin, Switzerland) to a maximum of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$]. Flowering time was scored as described (Möller-Steinbach et al., 2010).

RNA ISOLATION AND QUANTITATIVE RT-PCR (qRT-PCR)

Total RNA was extracted as previously described (Hennig et al., 2003; Leroy et al., 2007; Alexandre et al., 2009). 1 μg RNA treated with DNase I (Promega, Dübendorf, Switzerland) was transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Nunningen, Switzerland) according to manufacturer’s instructions. qRT-PCR with gene-specific primers (**Supplementary Table 2**) was performed on three technical replicates with the Fast Start Universal Probe Master (Rox) reagent and the Universal Probe Library set (UPL) (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer’s instructions and results were normalized to *PP2A* as described (Exner

et al., 2009). Shown is one of at least two independent biological experiments with similar results.

RESULTS

MSI1 FUNCTIONS INDEPENDENTLY OF LIGHT QUALITY

Previously, we had reported that *MSI1* antisense lines and *msi1* mutants partially complemented with untagged *pMSI1::MSI1* or tagged *pMSI1::MSI1:TAP* constructs were late flowering (Bouveret et al., 2006). This suggests that undisturbed *MSI1* levels are needed for normal flowering promotion. Homozygous *msi1* null mutants are lethal (Köhler et al., 2003; Guitton et al., 2004). Here, we analyzed heterozygous plants of the original *msi1-1* and a novel *msi1-5* allele and found that both flowered later than wild type under LD but not SD (**Supplementary Figure 1**). Similarly, *msi1-1/-* plants partially complemented with a *pMSI1::MSI1:HA* construct were late flowering (**Supplementary Figure 1**). Therefore the dose of *MSI1* is important for flowering time. The observation of a late flowering phenotype for *msi1*-mutants and transgenic lines motivated us to investigate the genetic pathway(s) in which *MSI1* acts to affect flowering. Because the flowering delay was considerably more severe for the *msi1-1/-**pMSI1::MSI1:TAP* (*msi1-tap1*) line than for heterozygous *msi1* mutants, we used the *msi1-tap1* line in subsequent experiments. Unlike heterozygous *msi1* mutants, *msi1-tap1* flowered much later than wild type in SD (Bouveret et al., 2006). This is consistent with the generally milder late flowering phenotype of heterozygous *msi1* mutants. The normal flowering in SD may suggest that a single wild-type *MSI1* allele can largely suffice for normal *MSI1* function in SD. It remains to be tested whether *MSI1* requirements are lower in SD or whether a potential compensatory mechanism can more efficiently up-regulate the remaining *MSI1* allele in SD than in LD.

We then tested a potential function of *MSI1* in the light quality pathway, which functions through phytochromes and independently of the circadian system. *PHYB*, *PHYD*, and *PHYE* repress *FT* expression and therefore flowering, with *PHYB* having the major role in this process (Kim et al., 2008). The *PHYB* target *PFT1* was proposed to directly activate both, *CO* and *FT* expression, while it simultaneously acts as negative regulator of phytochrome signaling by inactivation of *PHYB* protein (Cerdan and Chory, 2003; Wollenberg et al., 2008). Null alleles of *phyB* and *pft1*, which are early and late flowering, respectively, were introduced into the *msi1-tap1* background and flowering time was analyzed in LD. The loss of *PHYB* in the *phyB msi1-tap1* double mutant led to flowering with 15 rosette leaves (RL) in LD, which was intermediate between the *phyB* single mutant (4 RL) and *msi1-tap1* (19 RL, **Table 1**). This result suggests an additive interaction between *MSI1* and *PHYB*. The loss of *PFT1* in the *pft1 msi1-tap1* double mutant resulted in a synergistic delay in flowering (41 RL) compared to the *pft1* and *msi1-tap1* single mutants (14 and 17 RL respectively, **Table 1**), suggesting likewise independent effects of *MSI1* and *PFT1* in flowering promotion but likely on the same common targets. Together, these results propose a function of *MSI1* independent of the light quality pathway in floral induction.

MSI1 FUNCTIONS INDEPENDENTLY OF THE FLORAL REPRESSOR *FLC* ON FLOWERING

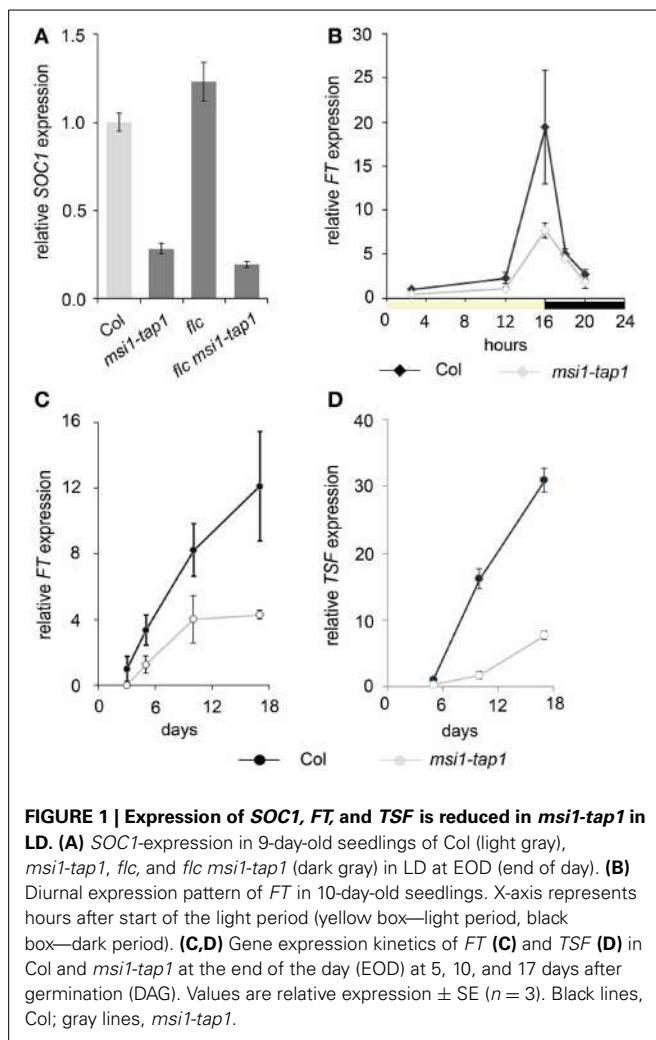
Next, we investigated the potential role of the flowering repressor *FLC* in *MSI1* effects on flowering time. *FLC* is one of the main regulators of flowering time in *Arabidopsis*, and altered flowering time is often caused by altered *FLC* expression. Consistent with earlier observations (Bouveret et al., 2006), analysis of *flc msi1-tap1* double mutants suggested that late flowering of *msi1-tap1* was independent of *FLC*, as evident from the largely unaffected late flowering of *msi1-tap1 flc* plants (**Table 1**). Previously, we observed reduced *SOC1* expression in *msi1-tap1* (Bouveret et al., 2006). Here, we tested whether the reduced expression of *SOC1* was independent of *FLC*. *SOC1* expression in the double mutant was as low as in *msi1-tap1*, suggesting that the *flc* mutation could not lift *SOC1* repression in *msi1-tap1* plants (**Figure 1A**). Because *MSI1* was recently shown to be involved in *FLC* control as part of plant PRC2 complexes (De Lucia et al., 2008; Derkacheva et al., 2013), we performed additional genetic tests of a potential role of *FLC* in *msi1-tap1* late flowering. The active *FRI*-allele of the late flowering *Arabidopsis* accession San Feliu (Sf2) crossed into Columbia (Col *FRI*), was introgressed into *msi1-tap1*. As previously reported (Lee et al., 1993; Clarke and Dean, 1994), Col *FRI* flowered very late, possibly due to high *FLC* expression (**Table 1**). Col *FRI msi1-tap1* plants flowered much later (85 RL) than either

Table 1 | Flowering time of double mutants of *msi1-tap1* and different flowering time mutants in LD.

| | | Rosette leaves | Days to bolting |
|---|--------------------------|-------------------------|---------------------------|
| 1 | Col | 12.1 ± 0.4 | 28.8 ± 0.6 |
| | <i>msi1-tap1</i> | 19.2 ± 0.7 | 36.5 ± 0.6 |
| | <i>phyB</i> | 4.3 ± 0.2 | 18.9 ± 0.3 |
| | <i>phyB msi1-tap1</i> | 15.4 ± 0.6 | 30.9 ± 0.7 |
| 2 | Col | 9.1 ± 0.2 | 26.5 ± 0.2 |
| | <i>msi1-tap1</i> | 17.4 ± 0.6 | 33.7 ± 0.5 |
| | <i>pft1</i> | 14.4 ± 0.3 | 32.7 ± 0.5 |
| | <i>pft1 msi1-tap1</i> | 41.3 ± 3.7 | 59.8 ± 3.4 |
| 3 | Col | 7.3 ± 0.4 | 20.8 ± 0.9 |
| | <i>msi1-tap1</i> | 19.6 ± 1.4 | 40.1 ± 1.3 |
| | Col <i>FRI</i> | 72.7 ± 3.1 | 79.4 ± 2.7 |
| | Col <i>FRI msi1-tap1</i> | 84.6 ± 3.1 ^a | 143.8 ± 12.2 ^a |
| | <i>flc</i> | 6.2 ± 0.1 | 21.0 ± 1.1 |
| | <i>flc msi1-tap1</i> | 16.9 ± 0.8 | 34.3 ± 0.5 |
| 4 | Col | 10.4 ± 0.5 | 27.8 ± 0.5 |
| | <i>msi1-tap1</i> | 19.9 ± 0.5 | 36.9 ± 0.5 |
| | <i>esd1</i> | 5.0 ± 0.1 | 24.0 ± 0.0 |
| | <i>esd1 msi1-tap1</i> | 11.9 ± 0.4 | 33.3 ± 0.3 |
| 5 | Col | 7.1 ± 0.4 | 23.9 ± 0.6 |
| | <i>msi1-tap1</i> | 16.5 ± 0.8 | 39.0 ± 0.6 |
| | <i>fca</i> | 68.6 ± 2.5 | 75.9 ± 4.0 |
| | <i>fca msi1-tap1</i> | 57.0 ± 1.1 ^a | 289.6 ± 41 ^a |

Shown are mean value ± SE ($n \geq 14$).

^aAnalysis of flowering time was stopped when ~1/3 of the plants had died before bolting. Number of rosette leaves and days until death or termination of the experiment are shown.



parent (73 and 20 RL for Col *FRI* and *msi1-tap1*, respectively). Some of the Col *FRI* *msi1-tap1* plants were not able to flower at all and died without completing their life cycle. This additive delay in flowering suggests an independent role of *MSI1* and *FRI* in flowering. Previously, we found a strongly synergistic interaction between *MSI1* and *FVE* (Bouveret et al., 2006). *FVE* is part of the autonomous pathway, which represses *FLC*, and genes in this pathway were grouped in two epistasis groups. While *FVE* represents one of the two groups, *FCA* is a gene from the second group. Here, we tested the genetic interaction between *MSI1* and *FCA*. The *fca msi1-tap1* double mutants were extremely delayed in flowering. They ceased to produce leaves without starting to bolt or flower leading to a smaller rosette leave number than for *fca*. After an extended period of developmental inactivity they eventually died (Table 1). The strongly synergistic interaction suggests that *MSI1* and *FCA* do not function in the same genetic pathway to control flowering time.

Another activator of *FLC* is *EARLY IN SHORT DAYS1* (*ESD1*, also known as *SUPPRESSOR OF FRIGIDA 3* and *ACTIN RELATED PROTEIN 6*). Mutations in *ESD1* hasten flowering through reduced *FLC* expression in LD and SD (Martin-Trillo

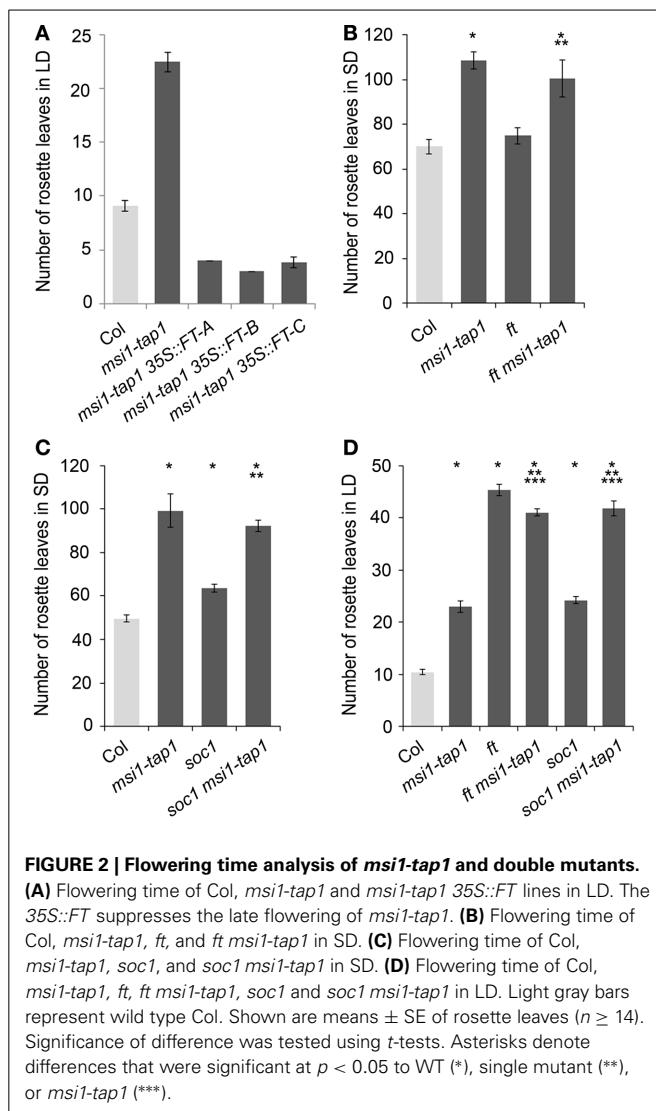
et al., 2006; Choi et al., 2007; Lazaro et al., 2008). An early flowering *esd1* mutant allele was crossed into *msi1-tap1*. In LD, the *esd1 msi1-tap1* double mutant flowered intermediate (12 RL) to both parents *esd1* and *msi1-tap1* (5 and 20 RL, respectively) disclosing an additive effect between *ESD1* and *MSI1* on flowering (Table 1). These data suggest that *ESD1* and *MSI1* function in separate genetic pathways. Together, these results firmly established that *MSI1* can function independently of *FLC* to affect flowering time in LD.

CHANGES IN *MSI1* LEAD TO REDUCED LEVELS OF *FT* AND *TSF*

FT and its homolog *TSF* are activators of *SOC1* (Yamaguchi et al., 2005; Yoo et al., 2005). Increased *FT* expression was found in *msi1-tap1* suppressor mutants, which rescued the *msi1-tap1* late flowering phenotype (Exner et al., 2009, 2010). In LD-grown wild-type Arabidopsis, CO activates *FT* at the end of the day (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). To test whether the diurnal rhythm of *FT* was affected in *msi1-tap1*, *FT* expression was profiled throughout the light-dark cycle in seedlings (Figure 1B). In wild type, *FT* had its expression peak toward the end of the light and beginning of the dark period as previously reported. The *FT* expression in *msi1-tap1* followed the same pattern as in wild type, but expression values were lower, especially at the end of the day (EOD), when expression of *FT* was reduced by up to 50%. Additionally, we tested whether *MSI1* affected the temporal activation of *FT* or its homolog *TSF* (Figures 1C,D). Under LD conditions, *FT* and *TSF* levels increased steadily in wild type between 3 and 17 days. In *msi1-tap1*, *FT* transcripts started to accumulate similarly to wild type but the increase was much slower leading to considerably reduced *FT* levels. The accumulation of *TSF* transcripts was even stronger reduced in *msi1-tap1* leading to 70% lower levels than in wild type at 17 days after germination. These results demonstrate that normal *MSI1*-function is needed for typical activation of *FT* and its homolog *TSF* in LD.

To test whether higher *FT* expression can be sufficient to suppress the late flowering phenotype of *msi1-tap1*, a 35S::*FT* transgene was introduced into *msi1-tap1*. The *FT* over-expression caused extremely early flowering (Figure 2A), which is consistent with the notion that reduced *FT* expression contributed to the late flowering of *msi1-tap1*.

To substantiate that delayed activation of *FT* and therefore of *SOC1* was responsible for the late flowering of *msi1-tap1*, a *ft* mutant allele was crossed into *msi1-tap1* for flower time measurements. The double mutant *soc1 msi1-tap1*, which was already described in LD before (Bouveret et al., 2006), was included into the analysis (Figures 2B–D; Supplementary Figure 2). Under SD conditions, *ft* flowered similar to wild type, and the *ft msi1-tap1* line flowered similar to *msi1-tap1*, confirming that *FT* does not play a major role under these conditions (Figure 2B) (Yanovsky and Kay, 2002; Corbesier et al., 2007). In contrast to *FT*, *SOC1* functions in induction of flowering in SD (Borner et al., 2000) and the *soc1* single mutant flowered later than wild type (Figure 2C). While the *soc1 msi1-tap1* line needed longer until flowering than either parent, it produced a similar number of RL as the *msi1-tap1* parent suggesting that delayed activation of *SOC1* contributes at least partially to the late flowering of *msi1-tap1* in SD. Thus,



during flowering induction in SD, MSI1 and SOC1 appear to function partially in the same genetic pathway.

Under LD conditions, both *ft* and *soc1* flowered later than wild type consistent with their roles in photoperiodic flowering (Borner et al., 2000). The double mutant *soc1 msi1-tap1* exhibited an additive late flowering phenotype (42 RL) compared to the *msi1-tap1* and *soc1* parents (23 and 24 RL, respectively, Figure 2D) confirming earlier results (Bouveret et al., 2006). The *ft msi1-tap1* line flowered with 42 RL similar to the *ft* parent (45 RL) supporting the notion that reduced *FT* expression is the main reason for late flowering of *msi1-tap1* in LD (Figure 2D). In summary, MSI1 affects full activation of *FT*, *TSF* and *SOC1* expression to promote timely flowering.

MSI1 FUNCTION IS CONNECTED TO THE PHOTOPERIOD PATHWAY

Because CO is a main activator of *FT*, *SOC1*, and *TSF* (Suarez-Lopez et al., 2001; Hepworth et al., 2002; Yamaguchi et al., 2005), we asked whether reduced expression of *FT*, *SOC1*, and *TSF* in *msi1-tap1* was caused by defects in CO regulation. CO is under

strong circadian and diurnal control (for review see Searle and Coupland, 2004), and CO expression in *msi1-tap1* was tested throughout an entire light-dark cycle. This experiment revealed that CO expression was considerably lower in *msi1-tap1* than in wild type (Figure 3A). The CO expression in wild type showed the previously reported peak toward the end of the day and beginning of the dark. Similarly, this expression pattern was observed for *msi1-tap1* suggesting that diurnal regulation was not grossly altered. This conclusion was supported by normal diurnal cycling of *CCA1* and *TOC1*, two components of the central circadian oscillator. However, under the tested conditions, *CCA1* and *TOC1* showed lower amplitudes of peak expression values in *msi1-tap1* (Figure 3B). Further, we analyzed the CO transcript levels at different developmental time points until 17 days after germination (Figure 3C). Under our conditions, CO increased steadily in wild type during 10 days after germination. In *msi1-tap1*, CO transcripts started to accumulate similarly to wild type but the increase was slower leading to considerably reduced CO levels. Together, the expression data suggest the hypothesis that MSI1 affects expression of *FT*, *TSF*, and *SOC1* and flowering time in LD via CO.

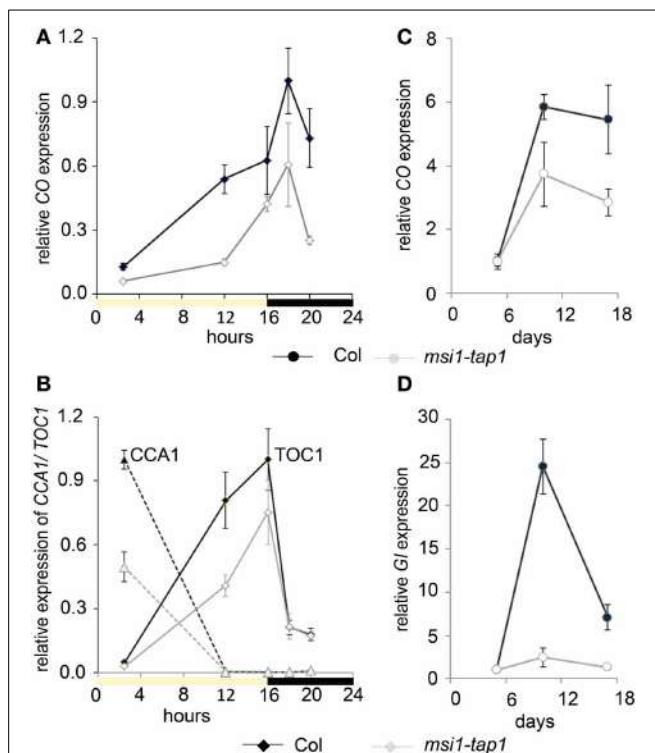
To test genetically whether reduced CO expression was responsible for delayed flowering of *msi1-tap1*, a *co* mutant allele was introduced into the *msi1-tap1* line. Consistent with earlier findings (Koornneef et al., 1991; Robson et al., 2001), the *co* mutant was late flowering in LD. While *msi1-tap1* delayed flowering substantially in the CO wild-type background, it only slightly delayed flowering of a *co* mutant (Figure 4A) suggesting that late flowering in *msi1-tap1* is caused mainly by effects on CO. The similar flowering time of *ft msi1-tap1* and the *ft co msi1-tap1* triple mutant (Figure 4A) further supported the notion of an epistatic genetic interaction between MSI1 and CO.

Because GIGANTEA (GI) is a major activator of CO expression (Imaizumi et al., 2005; Sawa et al., 2007), we tested whether GI expression was altered in *msi1-tap1*. At 5 d after germination, when CO levels did not differ between WT and *msi1-tap1*, GI was also not affected (Figure 3D). In contrast, at 10 d and 17 d, not only CO but also GI expression was substantially reduced in *msi1-tap1*.

Together, these data suggest that MSI1 acts on flowering in response to the photoperiod through GI and CO on *FT*.

MSI1 IS NEEDED FOR NORMAL SENSITIVITY OF THE PHOTOPERIOD PATHWAY

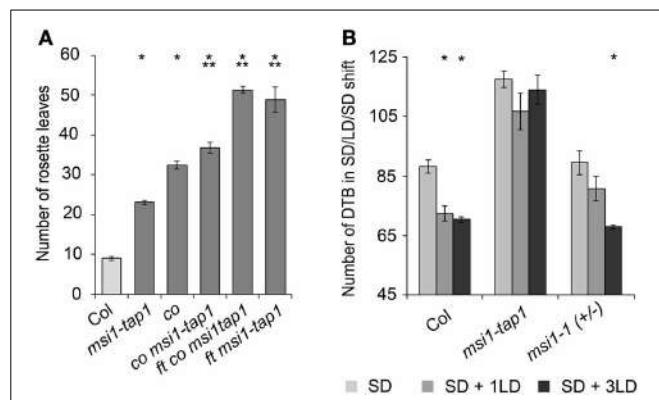
To further test the importance of MSI1 in the photoperiodic pathway, we performed a SD-LD-SD shift experiment. In SD, *FT* is only very weakly activated due to immediate destabilization of CO protein after synthesis, and flowering is very much delayed. A brief LD experience can be sufficient to activate *FT* and induce flowering if the photoperiodic pathway functions normally (Corbesier et al., 1996; King et al., 2008). We cultivated plants under SD conditions interrupted by 1 or 3 days of LD at 45 days after germination and measured flowering time (Figure 4B). Under these conditions, Col was highly sensitive to the additional LD exposures, and flowering was accelerated by about 3 weeks. In contrast, the effect on flowering time of *msi1-tap1* was minor and not statistically significant. Heterozygous *msi1-1* mutants reacted



like wild type to three additional LDs but showed a reduced response to a single additional LD (Figure 4B). Together, these results demonstrate that MSI1 is needed for normal sensitivity of the photoperiodic pathway.

DISCUSSION

In plants, flowering at the right time is determined by several endogenous and external signals. One of the genes affecting flowering is *MSI1*. Late flowering was observed in lines expressing either tagged (TAP-, GFP-, HA) or untagged MSI1 under a 2 kb *MSI1*-promoter fragment in a *msi1* mutant background (Bouveret et al., 2006; Alexandre et al., 2009; this work). In addition, *MSI1*-antisense lines and heterozygous *msi1* mutants were late flowering, together establishing that normal *MSI1* function is needed for normal timing of flowering. The *MSI1-TAP* construct did not affect flowering in WT plants nor did these plants in any other way differ from WT. Similarly, when a 35S::*MSI1* construct was introduced into *msi1-tap1*, the late flowering was suppressed (Bouveret et al., 2006). Therefore, we consider it unlikely that the late flowering of *msi1-tap1* plants was caused by a dominant negative effect of the fusion protein. Here, we used this line as a tool to dissect the function of the essential *MSI1* gene in flowering time control.



One signal affecting flowering is light quality, which gives information about competition by neighboring plants and is sensed mainly by phytochromes, in particular *PHYB* (for review see Thomas, 2006). In light-quality sensing, *PHYB* functions via *PFT1* both to activate *CO* and to activate *FT* in a *CO*-independent way (Cerdan and Chory, 2003; Iriarte et al., 2012a,b). In addition, *PHYB* has also functions in photoperiod sensing (for review see Thomas, 2006). Genetic interaction analysis between *PHYB* and *MSI1* showed an additive flowering time phenotype suggesting a function of *MSI1* independent from the light quality pathway to promote flowering. Similarly, *PFT1* and *MSI1* did not show an epistatic interaction, suggesting that both genes function in distinct genetic pathway. The finding that *PFT1* and *MSI1* showed a tendency for a synergistic genetic interaction with a greater than additive flowering delay, is consistent with the notion that both genes commonly affect *CO* and *FT* in flowering time control. Interestingly, *MSI1* and *PFT1* both affect not only flowering time but also drought stress responses (Alexandre et al., 2009; Elfving et al., 2011). Because *PFT1* is a subunit of the Mediator complex (Bäckström et al., 2007), future studies should aim to test whether *MSI1* and Mediator share direct targets.

In *Arabidopsis*, *FLC* is a major repressor of flowering and mutants deficient in *FLC* repression are often late flowering. *FLC* is repressed both by vernalization and also by the autonomous pathway to allow flowering even without vernalization (Baurle et al., 2007). Previously, it was shown that *MSI1* functions both in the major *FLC*-dependent vernalization pathway and in a *FLC*-independent vernalization pathway that regulates *AGL19* (Schönrock et al., 2006a; De Lucia et al., 2008; Derkacheva et al., 2013). Here we find that *MSI1* can affect flowering independent of vernalization and of *FLC*. This conclusion is based on genetic

interaction studies between (i) *MSI1* and *FLC*, (ii) *MSI1* and *FRI*, an *FLC*-activator (Michaels and Amasino, 2001), (iii) *MSI1* and *FCA* or *FVE*, two *FLC* repressors from the autonomous pathway, and (iv) *MSI1* and *ESD1/SUF3/ARP6*, a *FLC*-activator and putative subunit of the SWR1 complex (Martin-Trillo et al., 2006; Choi et al., 2007; Lazaro et al., 2008).

In summary, *MSI1* affects flowering time independent of the light quality pathway and of *FLC*.

The late flowering of *msi1-tap1* could be explained as a consequence of reduced expression of *FT*, *SOC1*, and *TSF*. Genetic interaction analysis showed epistatic effects of *FT* with *MSI1*, demonstrating that *MSI1* functions through the main flowering time integrators to promote flowering. *FT*, which is a major activator of *SOC1*, is in turn activated by *CO* in the photoperiod pathway to promote flowering in LD (Putterill et al., 1995). Strict diurnal regulation of *CO* protein levels is controlled by several complex pathways coupled to the core circadian oscillator and light conditions (for review see Andres and Coupland, 2012). *CO* is repressed in the morning by *PHYB* and activated in the evening by *GI*. The diurnal expression pattern of *CO* appeared not significantly altered in *msi1-tap1*, where *CO* still shows an expression peak late in the day. The level of detectable *CO* mRNA, however, was substantially reduced in *msi1-tap1*. The lower abundance of *CO* mRNA is associated with reduced *GI* expression. Together, reduced *GI* expression in *msi1-tap1* could cause the reduced *CO* expression that in turn delays activation of *FT* and eventually *SOC1* and could explain the delay in flowering.

Although *GI* is thought to function mainly by directly activating *CO*, *GI* can also directly activate *FT* and accelerate flowering in the absence of *CO* (Sawa and Kay, 2011). Notably, *msi1-tap1* did not cause any further delay of *ft* mutants but could slightly delay *co* mutants. These observations are consistent with a model in which reduced *GI* expression in *msi1-tap1* does not only affect flowering via reduced *CO* levels but also directly via compromising *FT* activation.

Here, we studied the role of *MSI1* in flowering under LD conditions and identified its function upstream of the photoperiodic *CO-FT* module. However, *MSI1* has also a function for flowering under SD conditions, and this is independent of *FT*. Flowering in SD depends on *SOC1* (Borner et al., 2000). We found not only that *SOC1* expression is reduced in *msi1-tap1* plants but also that *MSI1* and *SOC1* show a genetic interaction in SD suggesting that under these conditions *MSI1* affects flowering by contributing to normal *SOC1* expression. It remains to be tested which other flowering time genes are affected by *MSI1* and contribute to the late flowering phenotype of *msi1-tap1* in SD.

This work and earlier studies have established that *MSI1* affects flowering in multiple pathways (Figure 5). First, *MSI1* represses flowering via its functions in the EMF-PRC2-complex to represses *AGL19* prior to vernalization and *FT* prior to photoperiodic activation (Schönrock et al., 2006a; Jiang et al., 2008). Second, *MSI1* favors flowering via its function in the VRN-PRC2 complex to repress *FLC* after vernalization (De Lucia et al., 2008; Derkacheva et al., 2013). Here, we have shown that *MSI1* affects flowering in a third way—by contributing to *CO* expression *MSI1* allows to rapidly respond to photoperiod. The relative importance of these diverse functions will depend on conditions, such as LD vs. SD or

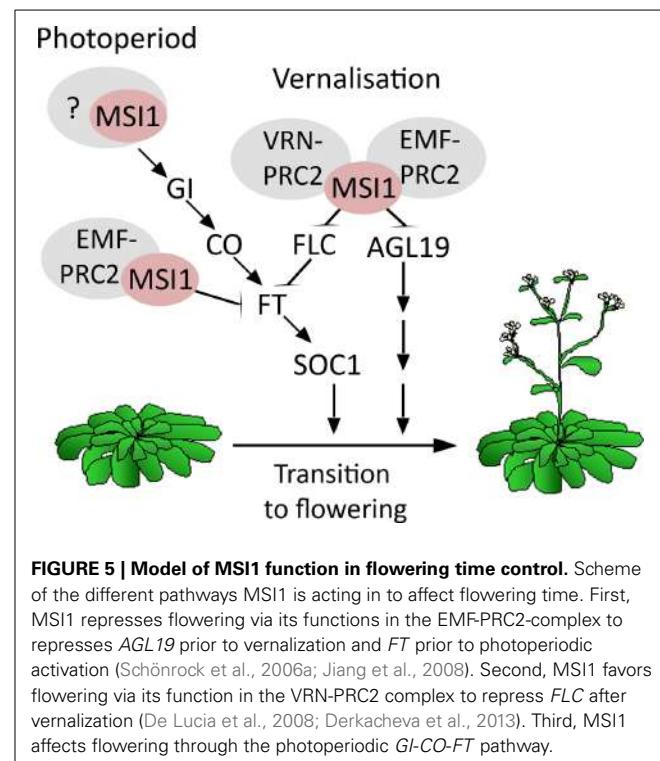


FIGURE 5 | Model of *MSI1* function in flowering time control. Scheme of the different pathways *MSI1* is acting in to affect flowering time. First, *MSI1* represses flowering via its functions in the EMF-PRC2-complex to represses *AGL19* prior to vernalization and *FT* prior to photoperiodic activation (Schönrock et al., 2006a; Jiang et al., 2008). Second, *MSI1* favors flowering via its function in the VRN-PRC2 complex to repress *FLC* after vernalization (De Lucia et al., 2008; Derkacheva et al., 2013). Third, *MSI1* affects flowering through the photoperiodic *GI-CO-FT* pathway.

with or without vernalization treatment. Given that the histone adaptor *MSI1* may be part of additional complexes, it is possible that *MSI1* affects flowering in even other ways.

AUTHOR CONTRIBUTIONS

Yvonne Steinbach conceived and carried out the experiments and analyzed the data. Yvonne Steinbach and Lars Hennig planned the study and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00077/abstract>

Supplementary Figure 1 | Characterization of a new *msi1* allele and the transgenic *msi1-HA* lines. (A) Insertion site of the WiscDsLox302B08 T-DNA in the second exon of *MSI1*-gene locus (AT5G58230). (B) Scheme of the *pMSI1::MSI1:HA* transgene containing the *MSI1* cDNA fused to a triple HA-tag at the C-terminus under control of 2 kb of the *MSI1*-promoter.

(C) Flowering time of heterozygous *msi1-1* and *msi1-5* mutants in LD (gray bars) and SD (dark bars) in RL ± SE ($n \geq 14$). **(D)** Flowering time of *msi1-HA* lines expressed in RL ± SE ($n \geq 14$). **(E)** Seed abortion rate in heterozygous *msi1-5* mutants and homozygous *msi1-HA* lines. Siliques of *msi1-5* contained about 50% normal and 50% aborted seeds, similar to the embryo lethal phenotype of the *msi1-1* mutant (Köhler et al., 2003). The *pMSI1::MSI1-HA* construct can rescue the seed abortion phenotype similar to *pMSI1::msi1-tap1* (Bouveret et al., 2006).

Supplementary Figure 2 | Flowering time of *msi1-tap1* and double mutants. **(A-C)** Flowering time in LD of **(A)** Col, *msi1-tap1*, *soc1*, *soc1 msi1-tap1*. **(B)** Col, *msi1-tap1*, *ft*, *ft msi1-tap1*. **(C)** Col, *msi1-tap1*, *co*, *co msi1-tap1*, *ft co msi1-tap1* and *ft msi1-tap1*. **(D,E)** Flowering time in SD of **(D)** Col, *msi1-tap1*, *soc1*, *soc1 msi1-tap1*, **(E)** Col, *msi1-tap1*, *ft*, *ft msi1-tap1*. Values are shown in mean DTB ± SE ($n \geq 14$). Significance of difference was tested using *t*-tests. Asterisks denote differences that were significant at $p < 0.05$ to WT (*), of the double mutants to the appropriate single mutant (***) or *msi1-tap1* (****).

Supplementary Table 1 | Primers used for genotyping and cloning.

Supplementary Table 2 | qRT-PCR primers used in this study. Shown are the forward and reverse primers with the appropriate Universal probe library (UPL) (Roche) probe number.

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Interplay between sugar and hormone signaling pathways modulate floral signal transduction

Ianis G. Matsoukas^{1,2*}

¹ Institute for Renewable Energy and Environmental Technologies, University of Bolton, Bolton, UK

² Systems and Synthetic Biology, Institute for Materials Research and Innovation, University of Bolton, Bolton, UK

Edited by:

Christian Jung, Christian Albrechts University of Kiel, Germany

Reviewed by:

Christina Kuehn, Humboldt University, Germany
Li Yang, University of North Carolina Chapel Hill, USA
Christian Jung, Christian Albrechts University of Kiel, Germany

***Correspondence:**

Ianis G. Matsoukas, Institute for Renewable Energy and Environmental Technologies, Institute for Materials Research and Innovation, University of Bolton, Deane Road, Bolton BL3 5AB, UK
e-mail: i.matsoukas@bolton.ac.uk

NOMENCLATURE

The following nomenclature will be used in this article:

- Names of genes are written in italicized upper-case letters, e.g., *ABI4*.
- Names of proteins are written in non-italicized upper-case letters, e.g., *ABI4*.
- Names of mutants are written in italicized lower-case letters, e.g., *abi4*.

The juvenile-to-adult and vegetative-to-reproductive phase transitions are major determinants of plant reproductive success and adaptation to the local environment. Understanding the intricate molecular genetic and physiological machinery by which environment regulates juvenility and floral signal transduction has significant scientific and economic implications. Sugars are recognized as important regulatory molecules that regulate cellular activity at multiple levels, from transcription and translation to protein stability and activity. Molecular genetic and physiological approaches have demonstrated different aspects of carbohydrate involvement and its interactions with other signal transduction pathways in regulation of the juvenile-to-adult and vegetative-to-reproductive phase transitions. Sugars regulate juvenility and floral signal transduction through their function as energy sources, osmotic regulators and signaling molecules. Interestingly, sugar signaling has been shown to involve extensive connections with phytohormone signaling. This includes interactions with phytohormones that are also important for the orchestration of developmental phase transitions, including gibberellins, abscisic acid, ethylene, and brassinosteroids. This article highlights the potential roles of sugar-hormone interactions in regulation of floral signal transduction, with particular emphasis on *Arabidopsis thaliana* mutant phenotypes, and suggests possible directions for future research.

Keywords: *Arabidopsis thaliana*, florigenic and antiflorigenic signaling, juvenile-to-adult phase transition, juvenility, signal transduction, sugar-hormone interactions, vegetative-to-reproductive phase transition

INTRODUCTION

The greatest advances in our understanding of the genetic regulation of developmental transitions have derived from studying the vegetative-to-reproductive phase transition in several dicot and monocot species. This has led to the elucidation of multiple environmental and endogenous pathways that promote, enable and repress floral induction (reviewed in Matsoukas et al., 2012). The photoperiodic (Kardailsky et al., 1999; Kobayashi et al., 1999) and vernalization (Schmitz et al., 2008) pathways regulate time to flowering in response to environmental signals such as daylength, light and temperature, whereas the autonomous (Jeong et al., 2009), aging (Yang et al., 2013; Yu et al., 2013) and gibberellin (GA)-dependent (Porri et al., 2012) pathways monitor endogenous indicators of the plant's age and physiological status. In addition, other factors and less characterized pathways also play a role in regulation of floral signal transduction. These include ethylene (Achard et al., 2006), brassinosteroids (BRs; Domagalska

et al., 2010), salicylic acid (Jin et al., 2008) and cytokinins (D'aloia et al., 2011).

The photoperiodic pathway is probably the most conserved of the floral induction pathways. It is known for its promotive effect by relaying light and photoperiodic timing signals to floral induction (reviewed in Matsoukas et al., 2012). This pathway involves genes such as *PHYTOCHROMES* (*PHY*s; Sharrock and Quail, 1989; Clack et al., 1994) and *CRYPTOCHROMES* (*CRY*s; Ahmad and Cashmore, 1993; Guo et al., 1998; Kleine et al., 2003), which are involved in the regulation of light signal inputs. Other genes such as *GIGANTEA* (*GI*; Fowler et al., 1999), *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*; Wang et al., 1997), and *LATE ELONGATED HYPOCOTYL* (*LHY*; Schaffer et al., 1998) are components of the circadian clock, whereas *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*; Kardailsky et al., 1999; Kobayashi et al., 1999), *TWIN SISTER OF FT* (*TSF*; Yamaguchi et al., 2005), and *FLOWERING LOCUS D* (*FD*;

Abe et al., 2005) encode proteins that specifically regulate floral induction. The actions of all pathways ultimately converge to control the expression of so-called floral pathway integrators (FPIs), which include *FT* (Corbesier et al., 2007), *TSF* (Yamaguchi et al., 2005), *SUPPRESSOR OF CONSTANS1* (*SOC1*; Yoo et al., 2005), and *AGAMOUS-LIKE24* (*AGL24*; Lee et al., 2008; Liu et al., 2008). These act on floral meristem identity (FMI) genes *LEAFY* (*LFY*; Lee et al., 2008), *FRUITFUL* (*FUL*; Melzer et al., 2008), and *APETALA1* (*API*; Wigge et al., 2005; Yamaguchi et al., 2005), which result in floral initiation. On the other hand, pathways that enable floral induction regulate the expression of floral repressors or translocatable florigen antagonists, known as antiflorogens (reviewed in Matsoukas et al., 2012). The pathways that regulate the floral repressor *FLOWERING LOCUS C* (*FLC*) are the best-characterized (reviewed in Michaels, 2009).

The vegetative-to-reproductive phase transition is preceded by the juvenile-to-adult phase transition within the vegetative phase (reviewed in Poethig, 1990, 2013; Matsoukas et al., 2013; Matsoukas, 2014). During the juvenile phase plants are incapable of initiating reproductive development and are insensitive to environmental stimuli such as photoperiod and vernalization, which induce flowering in adult plants (Matsoukas et al., 2013; Matsoukas, 2014; Sgamma et al., 2014). The juvenile-to-adult phase transition is accompanied by a decrease in microRNA156 (miR156A/miR156C) abundance and a concomitant increase in abundance of miR172, as well as the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL3/4/5*) transcription factors (TFs; Wang et al., 2009; Wu et al., 2009; Jung et al., 2011, 2012; Kim et al., 2012). Expression of miR172 activates *FT* transcription in leaves through repression of AP2-like transcripts *SCHLAFMÜTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*), and *TARGET OF EAT 1-3* (*TOE1-3*; Jung et al., 2007, 2011; Mathieu et al., 2009), whereas the increase in *SPLs* at the shoot apical meristem (SAM), leads to the transcription of FMI genes (Schwab et al., 2005; Schwarz et al., 2008; Wang et al., 2009; Yamaguchi et al., 2009). Therefore, from a molecular perspective juvenility can be defined as the period during which the abundance of antiflorogenic signals such as miR156/miR157 is sufficiently high to repress the transcription of *FT* and *SPL* genes (Matsoukas, 2014).

Carbohydrates serve diverse functions in plants ranging from energy sources, osmotic regulators, storage molecules, and structural components to intermediates for the synthesis of other organic molecules (reviewed in Rolland et al., 2006; Smeekens et al., 2010; Eveland and Jackson, 2012). Carbohydrates also act as signaling molecules (Moore et al., 2003) and by their interaction with mineral networks (Zakhleniuk et al., 2001; Lloyd and Zakhleniuk, 2004) affect the juvenile-to-adult and vegetative-to-reproductive phase transitions (Matsoukas et al., 2013). Interestingly, sugar signaling has been shown to involve extensive interaction with hormone signaling (Zhou et al., 1998; Arenas-Huertero et al., 2000; Moore et al., 2003). This includes interactions with hormones that are also important for the regulation of juvenile-to-adult and vegetative-to-reproductive phase transitions, including GAs (Yuan and Wysocka-Diller, 2006), abscisic acid (ABA; Arenas-Huertero et al., 2000; Laby et al., 2000), ethylene (Zhou et al., 1998), and BRs (Goetz et al., 2000;

Schluter et al., 2002). Several molecular mechanisms that mediate sugar responses have been identified in plants (reviewed in Rolland et al., 2006; Smeekens et al., 2010). The best examples involve hexokinase (HXK; Moore et al., 2003), trehalose-6-phosphate (Tre6P; Van Dijken et al., 2004) and the sucrose non-fermenting 1-related protein kinase1 (SnRK1; Baena-Gonzalez et al., 2007) complex. SnRK1 has a role when sugars are in extremely limited supply, whereas HXK and Tre6P play a role in the presence of excess sugar.

The panoptic themes of floral signal transduction, sugar sensing and signaling, and hormonal regulation of growth and development have attracted much attention, and many comprehensive review articles have been published (Rolland et al., 2006; Amasino, 2010; Smeekens et al., 2010; Depuydt and Hardtke, 2011; Huijser and Schmid, 2011; Andres and Coupland, 2012). This article, however, focuses specifically on sugar-hormone interactions and their involvement in regulation of floral signal transduction, with particular emphasis on *Arabidopsis thaliana* mutant phenotypes. The review is divided into two sections: the first provides several pieces of evidence on the interactions between sugars and different hormones in floral induction; whereas the second describes potential mechanisms that might be involved in regulation of floral signal transduction, in response to sugar-hormone interplay.

SUGAR/HORMONE INTERACTIONS AND FLORAL SIGNAL TRANSDUCTION

THE SUGAR AND GIBBERELLIN SIGNALING CROSSTALK

GAs are a group of molecules with a tetracyclic diterpenoid structure that function as plant growth regulators influencing a range of developmental processes. Several *Arabidopsis* mutants in the GA signal transduction and GA biosynthesis pathway have been isolated (Table 1; Peng and Harberd, 1993; Peng et al., 1997; Hедден and Phillips, 2000). Null mutations in the early steps of GA biosynthesis (e.g., *ga1-3*) do not flower in short days (SDs), whereas weak mutants (e.g., *ga1-6*; Koornneef and Van Der Veen, 1980), or GA signal transduction mutants [e.g., *gibberellin acid insensitive* (*gai*)], flower later than wild type (WT; Peng and Harberd, 1993). In contrast, mutants with increased GA signaling such as *rga like2* (*rgl2*; Cheng et al., 2004; Yu et al., 2004) and *spindly* (*spy*; Jacobsen and Olszewski, 1993) have an early flowering phenotype. Evidence has been provided that both *RGL2* and *SPY* might be involved in carbohydrate regulation of floral initiation, as mutation in both loci confers insensitivity to inhibiting glucose concentrations (Yuan and Wysocka-Diller, 2006). *SPY*, an O-linked B-N-acetylglucosamine transferase was shown to interact with the GI in yeast (Tseng et al., 2004). Mutants impaired in *GI* have a late flowering and starch-excess phenotype (Eimert et al., 1995). The interaction between *SPY* and *GI* suggests that functions of these proteins might be related, and that *SPY* might be a pleiotropic circadian clock regulator (Tseng et al., 2004; Penfield and Hall, 2009). In addition, the early flowering phenotype of the glucose insensitive *spy* may be via *FT*, as *spy4* suppresses the reduction of *CO* and *FT* mRNA in *gi2* genotypes (Tseng et al., 2004). This indicates that *SPY* functions in the photoperiod pathway upstream of *CO* and *FT*, involving glucose and GA metabolism-related events. Interestingly, it

Table 1 | List of genes in *Arabidopsis thaliana* that regulate floral signal transduction in response to sugar-hormone interplay.

| Gene name | Abbreviation | Allelic | Gene identifier | Description | Flowering mutant phenotype ^a | | References |
|--------------------------------------|--------------|------------------------------------|-----------------|-----------------------------------------------------------------------------------------------------------------|-----------------------------------------|--------------------------|------------------------------------------------------------------------------------|
| | | | | | SD | LD | |
| SUGAR-GA SIGNALING CROSSTALK | | | | | | | |
| GA REQUIRING 1-3 | GA1-3 | CPS, KSA | At4g02780 | GA biosynthesis; ent-copalyl diphosphate synthase/magnesium ion binding | No phenotype | No phenotype | Koornneef and Van Der Veen, 1980 |
| GA REQUIRING 1-6 | GA1-6 | CPS, KSA | At4g02780 | GA biosynthesis | Late | Late | Koornneef and Van Der Veen, 1980 |
| GIBBERELLIC ACID INSENSITIVE | GA1 | GRAS-3, RGA2 | At1g14920 | TF ^b ; repressor of GA responses; involved in GA mediated signaling | Late | Late | Peng and Harberd, 1993; Peng et al., 1997; Hedin and Phillips, 2000 |
| RGA LIKE 2 | RGL 2 | GRAS-15, SCL19, DELLA protein RGL2 | At3g03450 | TF; SCARECROW-like; GA signaling; encodes a DELLA protein | Early | Early | Cheng et al., 2004; Tyler et al., 2004; Yu et al., 2004 |
| SPINDLY | SPY | n/a | At3g11540 | Repressor of GA responses; positive regulator of cytokinin signaling; glucose insensitive mutant | Early | Early | Jacobsen and Olszewski, 1993; Swain et al., 2002; Greenbaum-Wainberg et al., 2005 |
| GIGANTEA | GI | n/a | At1g22770 | Starch excess mutant; component of the circadian oscillator | Late | Similar or later than WT | Eimert et al., 1995; Tseng et al., 2004; Penfield and Hall, 2009 |
| LEAFY | LFY | MAC9_13 | At5g61850 | TF; sugar and GA regulated | No phenotype | No phenotype | Blazquez et al., 1998; Eriksson et al., 2006 |
| SUGAR-ABA SIGNALING CROSSTALK | | | | | | | |
| ABA DEFICIENT 2 | AB2 | GIN1, IS14, SAN3, SDR1, SIS4, SRE1 | At1g52340 | Oxidoreductase; molecular link between sugar signaling and hormone biosynthesis | Early | Early | Laby et al., 2000; Rook et al., 2001; Cheng et al., 2002 |
| ABA DEFICIENT 3 | ABA3 | GIN5, SIS2, SIS3 | At1g16540 | Involved in the conversion of ABA-aldehyde to ABA; glucose insensitive mutant; molybdopterin cofactor sulfurase | Early | Early | Leon-Kloosterziel et al., 1996; Arenas-Huertero et al., 2000; Bittner et al., 2001 |
| ABA INSENSITIVE 3 | AB3 | SIS10 | At3g24650 | TF; molecular link between sugar signaling and hormone biosynthesis | Early | Early | Giraudat et al., 1992; Huang et al., 2008 |
| ABA INSENSITIVE 4 | AB4 | GIN6, SIS3, SIS5, SUN6 | At2g40220 | TF; molecular link between sugar signaling and hormone biosynthesis | Similar or slightly earlier than WT | Similar to WT | Finkelstein et al., 1998; Arenas-Huertero et al., 2000; Matsoukas et al., 2013 |
| CIRCADIAN CLOCK ASSOCIATED 1 | CCA1 | MYB-RELATED DNA BINDING PROTEIN | At2g46830 | TF; component of the circadian oscillator | Early | Similar to WT | Mizoguchi et al., 2002; Hanano et al., 2006 |

(Continued)

Table 1 | Continued

| Gene name | Abbreviation | Allelic | Gene identifier | Description | Flowering mutant phenotype ^a | | References |
|----------------------------------------------|--------------|---------------------------------|------------------|--------------------------------------------------------------------------------------------------------------------|-----------------------------------------|-----------------------------------|--------------------------------------------------------------------------------------------|
| | | | | | SD | LD | |
| TIMING OF CAB EXPRESSION 1 | TOC1 | Abi3/INTERACTIN PROTEIN 1, PRR1 | At5g61380 | TF; contributes to the plant fitness (carbon fixation, biomass) by influencing the circadian oscillator period | Early | Early | Kreps and Simon, 1997; Somers et al., 1998; Kurup et al., 2000; Pokhilko et al., 2013 |
| SUGAR-ETHYLENE SIGNALING CROSSTALK | | | | | | | |
| CONSTITUTIVE TRIPLE RESPONSE1 | CTR1 | GIn4, SIS1 | At5g03730 | Kinase; negative regulator of ethylene signaling; sugar signaling | Late | Late | Gibson et al., 2001; Cheng et al., 2002; Achard et al., 2007 |
| ETHYLENE INSENSITIVE 2 | EIN2 | CKR1, ERA3 | At5g03280 | Transporter; involved in ethylene signal transduction | Late | Late | Su and Howell, 1992; Fujita and Syono, 1996; Zhou et al., 1998; Alonso et al., 1999 |
| ETHYLENE OVERPRODUCER 1 | ETO1 | n/a | At3g1770 | Protein binding; promote ethylene biosynthesis | Early | Early | Bleecker et al., 1998; Guzman and Ecker, 1990; Roman et al., 1995; Chae et al., 2003 |
| ETHYLENE RESPONSE 1 | ETR1 | EIN1 | At1g66340 | Ethylene binding; ethylene receptor; protein histidine kinase | Late | Late | Bleecker et al., 1998; Guzman and Ecker, 1990; Chang et al., 1993; Chen and Bleecker, 1995 |
| ETHYLENE RESPONSE 2 | ETR2 | n/a | At3g23150 | Negative regulation of ethylene mediated signaling pathway; glycogen synthase kinase3; protein histidine kinase | Early | Similar or slightly later than WT | Sakai et al., 1998 |
| SUGAR-BR SIGNALING CROSSTALK | | | | | | | |
| BRASSINOSTEROID, LIGHT AND SUGAR 1 | BLS1 | n/a | n/a ^c | Component for BR and light responsiveness; involved in sugar signaling | Late | Late | Laxmi et al., 2004 |
| CONSTITUTIVE PHOTOMORPHOREGESIS AND DWARFISM | CPD | CBB3, CYP90, DWARF3 | At5g05690 | Electron carrier; heme binding; iron ion binding; monooxygenase; oxygen binding; under circadian and light control | Late | Late | Szekeres et al., 1996; Li and Chory, 1997; Choe et al., 1998; Domagalska et al., 2007 |
| DE-ETIOLATED 2 | DET2 | DWARF6 | At2g38050 | Similar to mammalian steroid-5-alpha-reductase; involved in the brassinolide biosynthetic pathway | Late | Late | Li et al., 1996; Noguchi et al., 1999; Tanaka et al., 2005 |

^aThe flowering mutant phenotype compared to WT under short (SD; 8 h light) and long day (LD; 16 h light) conditions.

^bTF transcription factor.

^cThe mutation has been mapped within a 1.4 Mb region of chromosome 5 (Laxmi et al., 2004).

has been suggested that *SPY4* may play a central role in the regulation of GA/cytokinin crosstalk during plant development (Greenboim-Wainberg et al., 2005).

Lines of evidence have demonstrated that there is a synergistic interaction between GAs and sucrose in the activation of *LFY* transcription (Blazquez et al., 1998; Eriksson et al., 2006). These pieces of evidence suggest a further link between GAs with sugar metabolism-related events and floral signal transduction. The effects of GA-sugar interplay on regulation of floral induction might be transduced by the *GIBBERELLIN INSENSITIVE DWARF1* (*GID1*), which act upstream of the DELLA (Feng et al., 2008; Harberd et al., 2009), and *PHYTOCHROME-INTERACTING FACTOR* (*PIF*; De Lucas et al., 2008; Nozue et al., 2011; Stewart et al., 2011) family of bHLH factors.

THE SUGAR-ABA SIGNALING CROSSTALK

ABA is regarded as a general inhibitor of floral induction. This is indicated in *Arabidopsis* where mutants deficient (e.g., *aba2*, *aba3*) in or insensitive [e.g., *aba insensitive4* (*abi4*)] to ABA are early flowering (Table 1; Martinez-Zapater et al., 1994). On the other hand, mutants with high ABA levels [e.g., *no hydrotropic response* (*nhr1*)] flower late or even later than WT under non-inductive SDs (Quiroz-Figueroa et al., 2010). However, many mutations affecting sugar signaling are allelic with components of the ABA synthesis or ABA transduction pathways. It has been shown that *aba2*, *aba3*, and *abi4* mutants are allelic to sugar-insensitive mutants *glucose insensitive1* (*gin1*)/*impaired sucrose induction4* (*isi4*)/*sugar insensitive1* (*sis1*; Laby et al., 2000; Rook et al., 2001), *gin5/isi2/isi3* (Arenas-Huertero et al., 2000) and *gin6/isi3/isi5/sun6* (Arenas-Huertero et al., 2000), respectively. In addition, ABA accumulation and transcript levels of several ABA biosynthetic genes are significantly increased by glucose (Cheng et al., 2002). These lines of evidence indicate that signaling pathways mediated by ABA and sugars may interact to regulate juvenility and floral signal transduction (Matsoukas et al., 2013).

The downstream effects of the sugar-ABA interaction might be mediated via the circadian clock. Photoperiodic induction requires the circadian clock to measure the duration of the day or night (reviewed in Harmer, 2009; Imaizumi, 2010). The clock modulates the expression of *CO*, the precursor of *FT* that accelerates flowering in response to several pathways (reviewed in Turck et al., 2008). It has been shown that glucose has a marked effect on the entrainment and maintenance of robust circadian rhythms (Dalchau et al., 2011; Haydon et al., 2013). In addition, circadian periodicity is also regulated by ABA via an unclear mechanism. This might be through *ABI3* (allelic to *sis10*; Huang et al., 2008) by binding to the clock component *TIMING OF CAB EXPRESSION1* (*TOC1*; also called *ABI3* Interacting Protein 1; Kurup et al., 2000; Pokhilko et al., 2013), and/or regulation of *CCA1* mRNA transcription levels by ABA (Hanano et al., 2006). Thus, gating of circadian clock sensitivity by the ABA and sugar crosstalk may constitute a regulatory module that coordinates the circadian clock with additional endogenous and environmental signals to regulate juvenility and floral signal transduction.

THE SUGAR-ETHYLENE SIGNALING CROSSTALK

Ethylene is another example of a phytohormone that regulates juvenility (Beyer and Morgan, 1971) and floral induction (Bleecker et al., 1988; Guzman and Ecker, 1990). *Arabidopsis* mutants impaired in ethylene signaling [e.g., *ethylene insensitive2* (*ein2*), *ein3-1*] or perception [e.g., *ethylene response1* (*etr1-1*)], flower late in inductive LDs (Table 1). This late flowering phenotype is significantly enhanced under non-inductive SDs. Mutants, which over-produce ethylene [e.g., *ethylene overproducer1* (*eto1*), *eto2-1*] flower at the same time or slightly earlier than WT under LDs, but dramatically later in SDs (Bleecker et al., 1988; Guzman and Ecker, 1990; Chen and Bleecker, 1995; Achard et al., 2007). Ample evidence has shown that ethylene can influence plant sensitivity to sugars. Ethylene-insensitive plants are more sensitive to endogenous glucose, whereas application of an ethylene precursor decreases glucose sensitivity (Zhou et al., 1998; Leon and Sheen, 2003). However, this interaction may also function in an antithetical way as several ethylene biosynthetic and signal transduction genes are repressed by glucose (Yanagisawa et al., 2003; Price et al., 2004).

Ethylene sensing and signaling pathways are also tightly interconnected with those for sugar and ABA (reviewed in Gazzarrini and McCourt, 2001; Leon and Sheen, 2003). Lines of evidence have shown that this crosstalk modulates the vegetative-to-reproductive phase transition. This is suggested by the glucose hypersensitive phenotype displayed by the late flowering mutants *ein2* [allelic to *enhanced response to aba3* (*era3*)], *ein3* and *etr1* (Chang et al., 1993; Zhou et al., 1998; Alonso et al., 1999; Cheng et al., 2002; Yanagisawa et al., 2003). Activation of the ethylene response [either in the presence of exogenous ethylene or by means of the *eto1* or *constitutive triple response1* (*ctr1*) mutations] attenuates the glucose effects (Zhou et al., 1998; Gibson et al., 2001). Further support for the sugar-ethylene crosstalk involvement on flowering time is derived by the epistatic analysis of the *etr1 gin1* (*aba2*) and *ein2 gin1* (*aba2*) double mutants in the elucidated role of *GIN1* (*ABA2*) in the ethylene signal transduction cascade. The *etr1 gin1* (*aba2*) and *ein2 gin1* (*aba2*) double mutants flower earlier than *etr1* and *ein2* single mutants, respectively (Cheng et al., 2002). The early flowering and glucose resistance phenotypes of the double mutants *etr1 gin1* (*aba2*) and *ein2 gin1* (*aba2*) under LDs, may suggest that ethylene affects glucose signaling, partially, through ABA to regulate floral induction (Zhou et al., 1998; Cheng et al., 2002; Ghassemian et al., 2006). Overexpression of *ETHYLENE RESPONSE2* (*ETR2*; Sakai et al., 1998) receptor in *Oryza sativa* reduced ethylene sensitivity and delayed floral induction (Wuriyanghan et al., 2009). Conversely, disruption of *ETR2* by T-DNA or with RNA interference (RNAi) conferred enhanced ethylene sensitivity and early flowering. Moreover, links of the ethylene signaling with starch accumulation responses and activation of sugar transporter genes have also been observed. *ETR2* promoted starch accumulation, whereas a monosaccharide transporter gene was suppressed in the *ETR2* over-expression lines (Wuriyanghan et al., 2009). Interestingly, when expression of *ETR2* was reduced in the *OSeTR2* T-DNA and RNAi lines, starch failed to accumulate, whereas sugar translocation was enhanced (Wuriyanghan et al., 2009).

Ethylene has dramatic effects on flowering time of mutants involved in activation of the ethylene response under SD conditions (Achard et al., 2007). *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*) is a major negative regulator of ethylene signaling that is allelic to *GIN4* (Cheng et al., 2002) and *SIS1* (Gibson et al., 2001). Loss-of-function *ctr1* mutations result in the constitutive activation of the ethylene response pathway, which indicates that the encoded protein acts as a negative regulator of ethylene signaling (Kieber et al., 1993). Under LDs *ctr1* has a flowering phenotype similar to WT. In antithesis with the other glucose insensitive genotypes, *ctr1* plants flower dramatically later than WT in SDs. This could be due to impaired involvement of GA pathway, which systematize floral initiation in SDs. Interestingly, evidence has been provided that ethylene dramatically prolongs time to flowering in *ctr1* under SDs by repressing the up-regulation of *LFY* and *SOC1* transcript levels via a DELLA-dependent mechanism, and decreasing the levels of the endogenous bioactive GAs (Achard et al., 2007).

THE SUGAR-BRASSINOSTEROIDS SIGNALING CROSSTALK

BRs are steroid hormones known to control various skotomorphogenic (Chory et al., 1991) and photomorphogenic (Li et al., 1996) aspects of development. Genetic and physiological analyses have revealed the critical role of BRs in floral induction (Table 1), establishing a new floral signal transduction pathway. The promotive role of BRs on floral induction is exerted by the late flowering phenotype of BR-deficient mutants *brassinosteroid-insensitive1* (*bsr1*; Clouse et al., 1996; Li and Chory, 1997), *brassinosteroid-insensitive2* (*bin2*; Li et al., 2001), *deetiolated2* (*det2*; Chory et al., 1991), *constitutive photomorphogenesis and dwarfism* (*cpd*; Szekeres et al., 1996; Domagalska et al., 2007) and *brassinosteroid, light and sugar1* (*bls1*; Laxmi et al., 2004). Conversely, mutations impaired in metabolizing BRs to their inactive forms, *phyB-activation-tagged suppressor1* (*bas1*; Neff et al., 1999) and *suppressor of phyB-4 7* (*sob7*; Turk et al., 2005) flower early (Turk et al., 2005). It has been reported that the response to exogenously applied BRs differs depending on the light quality and quantity (Neff et al., 1999), suggesting a potential interaction with sugars via light-mediated pathways (Goetz et al., 2000; Schluter et al., 2002). In addition, it has been demonstrated that BR responses are related to hormones such as GA (Gallego-Bartolome et al., 2012), ABA (Domagalska et al., 2010), and ethylene (Turk et al., 2005), which participate in sugar signaling. Furthermore, the sugar hypersensitive phenotype of the late flowering *bls1* can be repressed by exogenous BRs (Laxmi et al., 2004). Moreover, the late flowering mutant *det2*, as other constitutively photomorphogenic mutants have been found to have an altered response to applied sugars (reviewed in Chory et al., 1996; Laxmi et al., 2004, and references therein). Collectively, these data indicate interplay between BRs and sugars in regulation of floral signal transduction. The downstream effects of this crosstalk might be mediated through *BRASSINAZOLE RESISTANT1* (*BZR1*) and *BZR2*, as well as additional interacting TFs. Both *BZR1* and *BZR2* interact with PIF (Oh et al., 2012) and the GA signaling DELLA proteins (Oh et al., 2012). In addition, the BR-sugar interaction may also be indirectly involved in modulation of juvenility and floral signal transduction by influencing the photoperiodic pathway via

the circadian clock, as BR application shortens circadian rhythms (Hanano et al., 2006).

HOW DOES THE CROSSTALK BETWEEN SUGARS AND HORMONES REGULATE THE FLORAL SIGNAL TRANSDUCTION

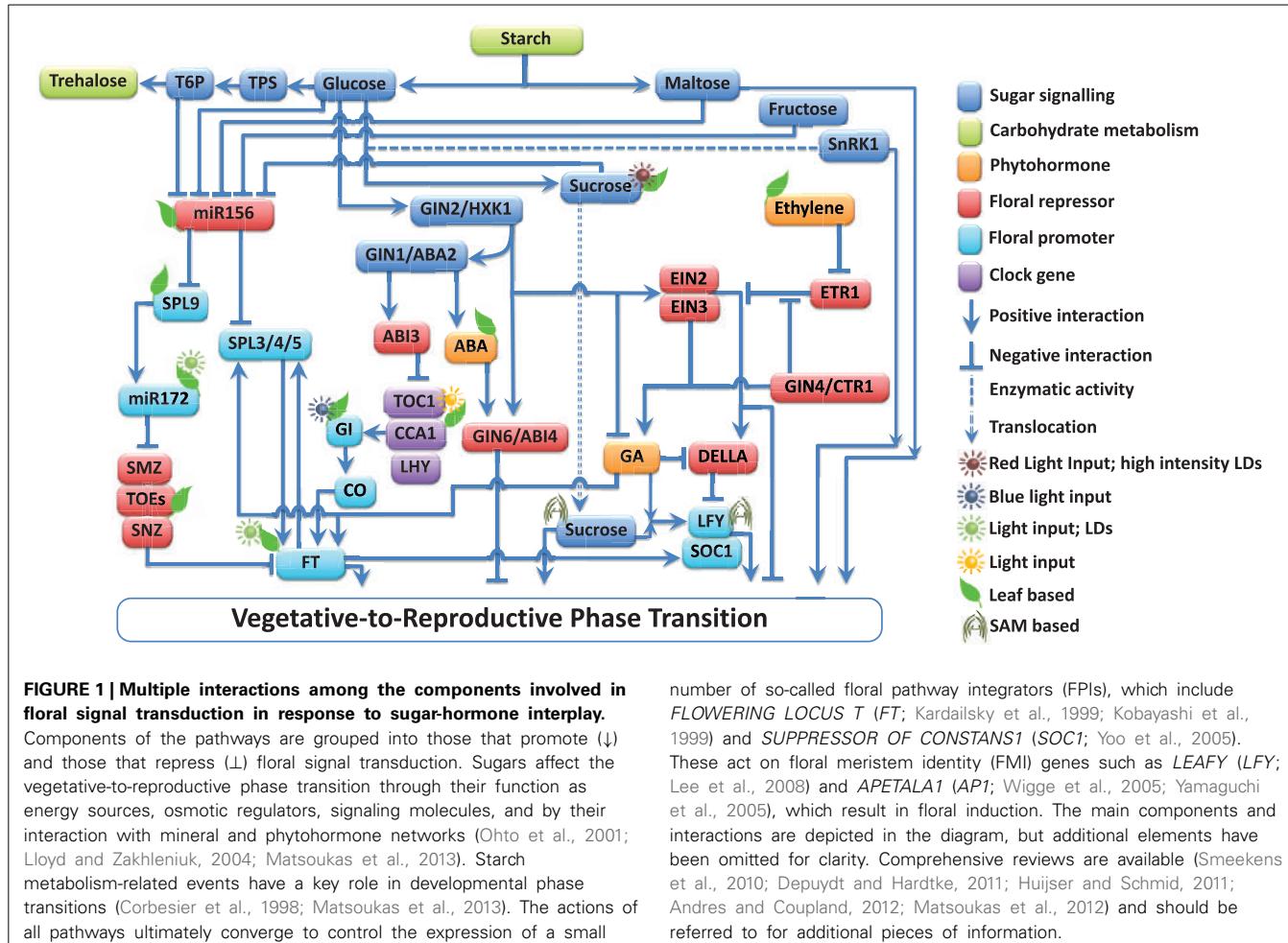
It is proposed that the effects of the sugar-hormone interplay might be mediated by hormones that enable tissues to respond to sugars, and/or hormone and sugar signaling, although essentially separate, could converge and crosstalk through specific regulatory complexes (Figure 1). One regulatory mechanism might be through metabolic enzymes, which also function as active members of transcriptional or posttranscriptional regulatory complexes (Cho et al., 2006). This cross-functionalization could be involved in mechanisms that modulate juvenility and floral signal transduction, by allowing interplay between different sugar and hormone response pathways or receptors.

THE HXK1-miR156 REGULATORY MODULE

Sugar signals can be generated either by carbohydrate concentration and relative ratios to other metabolites, such as hormones (Arenas-Huertero et al., 2000) and carbon-nitrogen ratio (Corbesier et al., 2002; Rolland et al., 2006), or by flux through sugar-specific transporters (Lalonde et al., 1999) and/or sensors (Moore et al., 2003). Sugar sensors perceive the presence of different sugars and initiate downstream signaling events. Glucose (Moore et al., 2003), fructose (Cho and Yoo, 2011; Li et al., 2011), sucrose (Seo et al., 2011), Tre6P (Van Dijken et al., 2004), and maltose (Niittyla et al., 2004; Stettler et al., 2009) function as cellular signaling molecules in specific regulatory pathways, which modulate juvenility and floral signal transduction. Of these signaling molecules, glucose has been studied the most comprehensively in plants.

Glucose-mediated floral signal transduction is largely dependent on HXK, HXK-independent, and SnRK1 signaling pathways. One possibility is that HXK1 controls juvenility and floral signal transduction by regulating the expression of miR156 (Yang et al., 2013). In this scenario, HXK1 that is largely dependent on ABA biosynthesis and signaling components (Zhou et al., 1998; Arenas-Huertero et al., 2000) promotes miR156 expression under low sugar levels. Above a threshold concentration, the circadian fluctuations of glucose, one of the final outputs of starch degradation (Stitt and Zeeman, 2012) that is regulated by starch and Tre6P (Martins et al., 2013) promotes GA biosynthesis (Cheng et al., 2002; Yu et al., 2012; Paparelli et al., 2013) and blocks HXK1 activity, resulting in downregulation of miR156 expression (Yang et al., 2013; Yu et al., 2013). Interestingly, defoliation experiments (Yang et al., 2011, 2013; Yu et al., 2013) show that removing the two oldest leaves results in increased miR156 levels at the SAM and a prolonged juvenile phase length. The fact that glucose, fructose, sucrose and maltose, partially, reverse this effect (Wang et al., 2013; Yu et al., 2013), indicates that photosynthetically derived sugars are potential components of the signal transduction pathway that repress miR156 expression in leaf primordia.

It seems highly probable that the differential regulation of SnRK1 by ABA and GAs (Bradford et al., 2003), and the



antagonism between ABA and GA, which function in an opposite manner, to activate specific *cis*-acting regulatory elements present in ABA- and GA-responsive promoters respectively (reviewed in Yamaguchi-Shinozaki and Shinozaki, 2005), may also be involved in this regulatory module (Achard et al., 2006; Yu et al., 2012; Wang et al., 2013).

THE TRE6P-miR156 REGULATORY MODULE

Tre6P is a metabolite of emerging significance in plant developmental biology, with hormone-like metabolic activities (reviewed in Smeekens et al., 2010; Ponnu et al., 2011). It has been proposed that Tre6P signals the availability of sucrose (Lunn et al., 2006), and then through the SnRK1 regulatory system orchestrates changes in gene expression that enable sucrose to regulate juvenility and floral signal transduction. In *Arabidopsis*, Tre6P is synthesized from glucose-6-phosphate by *TREHALOSE PHOSPHATE SYNTHASE 1* (*TPS1*; Van Dijken et al., 2004). Non-embryo-lethal weak alleles of *tps1* exhibit late flowering (Van Dijken et al., 2004) and ABA hypersensitive phenotypes (Gomez et al., 2010). Interestingly, the Tre6P pathway controls the expression of *SPL3*, *SPL4*, and *SPL5* at the SAM, partially via miR156, and partly independently of the miR156-dependent pathway via *FT* (Wahl et al., 2013). Several pieces of evidence suggest that

Tre6P inhibits SnRK1 when sucrose is above a threshold concentration (Polge and Thomas, 2007; Zhang et al., 2009). When the sucrose content decreases, with Tre6P decreasing as well, SnRK1 is released from repression, which leads to the induction of genes involved in photosynthesis-related events, so that more carbon is made available (Delatte et al., 2011). It has been shown that the Tre6P-SnRK1 module acts through a mechanism involving ABA (Gomez et al., 2010) and sugar metabolism (Van Dijken et al., 2004) to regulate several developmental events. The key link between sugars and ABA perception is exemplified by the *ABI* genes (Eveland and Jackson, 2012; Wang et al., 2013). Interestingly, *ABI4* encodes an AP2 domain TF that is required for normal sugar responses during the early stages of development (Arenas-Huerto et al., 2000; Laby et al., 2000; Rook et al., 2001; Niu et al., 2002). Taken together, these data could provide another mechanistic link, at the molecular level, on how the ABA-sugar interplay might be involved in regulation of juvenility and floral signal transduction.

PERSPECTIVES

Sugars serve diverse functions in plants ranging from energy sources, osmotic regulators, storage molecules, and structural components to intermediates for the synthesis of other organic

molecules. Sugars also act as signaling molecules and by their interaction with mineral and hormonal networks affect several aspects of growth and development.

There has been a long-standing interest in the role played by sugars and hormones in regulation of the juvenile-to-adult and vegetative-to-reproductive phase transitions. It has been proposed that the effects of sugar-hormone interactions might be mediated by key hormones that enable tissues to respond to sugars, and/or hormone and sugar signaling could converge and crosstalk through specific regulatory complexes and/or metabolic enzymes. However, how sugar and hormone signals are integrated into genetic pathways that regulate the juvenile-to-adult and vegetative-to-reproductive phase transitions is still incompletely understood. Recent studies have shown that metabolic enzymes, ABA, GA and Tre6P may integrate into the miR156/SPL-signaling pathway. However, despite this progress, mechanistic questions remain. Future challenges include the further clarification of the antagonistic and agonistic interactions between the sugar- and hormone-derived signals in a spatio-temporal manner at the molecular level, and their link to other known important transcriptional regulatory networks.

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Isolation and functional analysis of *CONSTANS-LIKE* genes suggests that a central role for *CONSTANS* in flowering time control is not evolutionarily conserved in *Medicago truncatula*

Albert C. S. Wong¹, Valérie F. G. Hecht¹, Kelsey Picard², Payal Diwadkar², Rebecca E. Laurie², Jiangqi Wen³, Kirankumar Mysore³, Richard C. Macknight² and James L. Weller^{1*}

¹ School of Biological Sciences, University of Tasmania, Hobart, TAS, Australia

² Department of Biochemistry, University of Otago, Dunedin, New Zealand

³ Plant Biology Division, Samuel Roberts Noble Foundation, Ardmore, OK, USA

Edited by:

Maria Von Korff Schmising, Max Planck Society, Germany

Reviewed by:

Steven B. Cannon, United States Department of Agriculture - Agricultural Research Service, USA

Chiara Campoli, Max Planck Institute for Plant Breeding Research, Germany

***Correspondence:**

James L. Weller, School of Biological Sciences, University of Tasmania, Private Bag 55, Hobart, TAS 7001, Australia
e-mail: jim.weller@utas.edu.au

The zinc finger transcription factor *CONSTANS* has a well-established central role in the mechanism for photoperiod sensing in *Arabidopsis*, integrating light and circadian clock signals to upregulate the florigen gene *FT* under long-day but not short-day conditions. Although *CONSTANS-LIKE* (*COL*) genes in other species have also been shown to regulate flowering time, it is not clear how widely this central role in photoperiod sensing is conserved. Legumes are a major plant group and various legume species show significant natural variation for photoperiod responsive flowering. Orthologs of several *Arabidopsis* genes have been shown to participate in photoperiodic flowering in legumes, but the possible function of *COL* genes as integrators of the photoperiod response has not yet been examined in detail. Here we characterize the *COL* family in the temperate long-day legume *Medicago truncatula*, using expression analyses, reverse genetics, transient activation assays and *Arabidopsis* transformation. Our results provide several lines of evidence suggesting that *COL* genes are unlikely to have a central role in the photoperiod response mechanism in this species.

Keywords: legume, flowering, photoperiod, *Medicago*, *CONSTANS*

INTRODUCTION

The length of the daily photoperiod is an important environmental variable that influences plant development. The most widely-recognized response to photoperiod is the induction of flowering, but photoperiod also controls other vegetative and reproductive characteristics, including formation of storage organs, axillary branching, and vegetative bud dormancy (Thomas and Vince-Prue, 1997). Within individual species, genetic variation for photoperiod responsiveness can be a major feature of adaptation to different latitudes and is therefore significant both in the natural environment and for agriculture.

As a result, there is widespread interest in the mechanism by which plants measure and respond to photoperiod, and this has been extensively examined in both *Arabidopsis* and rice. The study of induced mutants and natural variants affecting photoperiod responsiveness in both species has identified genes in the *FT* florigen family as the major target of photoperiod regulation, and have highlighted the general importance of light signaling pathways and the circadian clock for photoperiod measurement (Andres and Coupland, 2012; Brambilla and Fornara, 2013; Song et al., 2013; Tsuji et al., 2013).

In *Arabidopsis*, one gene in particular, *CONSTANS* (*CO*), has a central role in the mechanism of photoperiod measurement,

integrating clock and light signals to provide photoperiod-specific induction of *FT* expression (Andres and Coupland, 2012; Song et al., 2013). *CO* was originally defined on the basis of a long day (LD)-specific late-flowering mutant phenotype (Koornneef et al., 1991), and encodes a B-box zinc finger transcription factor (Putterill et al., 1995). Transgenic plants overexpressing *CO* are extremely early flowering, and epistatic and regulatory interactions position *CO* genetically between *GI* and *FT* (Onouchi et al., 2000; Suárez-López et al., 2001). It has subsequently been shown that *FT* is an early transcriptional target of *CO* (Samach et al., 2000), and that the *CO* protein binds to the *FT* promoter (Tiwari et al., 2010).

The LD-specificity for activation of *FT* by *CO* is achieved through regulation of *CO* protein abundance at both transcriptional and post-translational level. *CO* mRNA is rhythmically expressed under the control of the circadian clock, such that peak expression occurs at night under short days (SD) but in the afternoon under LD (Suárez-López et al., 2001). Afternoon *CO* expression in LD is reinforced by action of the FKF1 blue light photoreceptor, which interacts with *GI* to degrade CDF proteins, which are transcriptional repressors of *CO* (Fornara et al., 2009; Song et al., 2012). *CO* protein accumulation is prevented in darkness by the ubiquitin ligase COP1 (Jang et al., 2008) but permitted

in the afternoon under LD where phyA suppresses COP1 activity (Valverde et al., 2004) and FKF1 directly stabilizes CO (Song et al., 2012).

In rice, a warm-season crop with a short-day requirement for flowering, the CO-like gene *Hd1* also contributes to photoperiod measurement and photoperiod-specific regulation of *FT* family genes (Brambilla and Fornara, 2013). In contrast to Arabidopsis *CO*, *Hd1* appears to be a bifunctional regulator, acting to promote *FT* expression in SD and to repress it in LD (Izawa et al., 2002; Kojima et al., 2002). These observations have suggested that CO function may be widely conserved across the angiosperms. This conclusion has been tested in expression and functional analyses in a number of other species. In some species such as potato and sugar beet, CO-like genes do seem to be involved in photoperiod responses (Chia et al., 2008; Gonzalez-Schien et al., 2012), whereas evidence from other species such as barley, and poplar is less clear or inconclusive (Campoli et al., 2012; Hsu et al., 2012).

In the legume species pea (*Pisum sativum* L.), cloning of several flowering loci has demonstrated conserved roles for Arabidopsis circadian clock genes *GI*, *ELF4* and *ELF3* in the regulation of *FT* genes and the control of photoperiod-responsive flowering (Hecht et al., 2007; Liew et al., 2009; Weller et al., 2012). A similar role has also been demonstrated for *GI* in soybean (Watanabe et al., 2011). However, the endogenous function of CO-like (*COL*) genes in legumes has not been directly tested, and the possibility that they may participate in photoperiod measurement is still unresolved. In this study we have examined the potential involvement of *COL* genes in photoperiodic flowering of the temperate long-day legume *Medicago truncatula*, using expression analyses, Arabidopsis complementation, and loss-of-function mutants.

MATERIALS AND METHODS

PLANT MATERIAL

The experiments shown in **Figures 2, 4** used the *Medicago truncatula* line R108 and derived mutants obtained from reverse-screening the *Tnt1* insertion population described by Tadege et al. (2008). The *Medicago* sequences used for the experiments in **Figure 3** were obtained from cv Jester (*MtFTa1* promoter, *MtCOLa-d*) or R108 (*MtCOLe-h*).

GROWTH CONDITIONS

Arabidopsis plants were grown under long day photoperiod (16 h light/8 h dark) in growth cabinets maintained at 21°C with 30% to 40% humidity, and an irradiance of approximately 115 $\mu\text{mol m}^{-2} \text{s}^{-1}$. *Medicago* plants were grown in growth cabinets maintained at 22°C under either long (16-h) or short-day (8-h) photoperiods.

EXPRESSION ANALYSIS

Analysis of *MtCOL* expression followed procedures described by Hecht et al. (2011). Harvested material consisted of all expanded leaves from three-week-old plants, with each sample consisting of material pooled from two plants. Two technical replicates and three biological replicates were performed for each time-point. Transcript levels for experimental genes were evaluated as

previously described (Weller et al., 2009), relative to the reference gene *MtTEFIα*. Primer sequences are given in Supplemental Table 2.

ARABIDOPSIS TRANSFORMATION

DNA fragments containing full-length coding sequences of *MtCOLa-COLh* were amplified by PCR from cDNA and cloned into the pCR8/GW/TOPO TA vector (Invitrogen). The resulting entry vector was then recombined into plant transformation vector, pB2GW7 (Karimi et al., 2002) to generate the 35S:*MtCOLa-h* constructs. Transgenic plants were produced by applying *Agrobacterium tumefaciens* strain LBA4404 containing the pB2GW7 vectors to *Arabidopsis co-2* mutant flowers using the protocol described by Martinez-Trujillo et al. (2004). Seeds from these plants were collected and sown directly onto soil and selected using Basta herbicide. Putative transformants were confirmed by qRT-PCR analysis.

TRANSIENT ASSAYS

The transient expression assays were performed by infiltrating *Nicotiana benthamiana* leaves, as described by Hellens et al. (2005). Agrobacterium strains containing either the FT promoter-reporter construct or a 35S:*COL* construct were co-infiltrated into leaves using a mixture of the two strains at a ratio of 7:1, respectively. Firefly luciferase and Renilla luciferase were assayed 4 d after infiltration using the Dual-Luciferase Reporter Assay System (Promega) as described by Hellens et al. (2005).

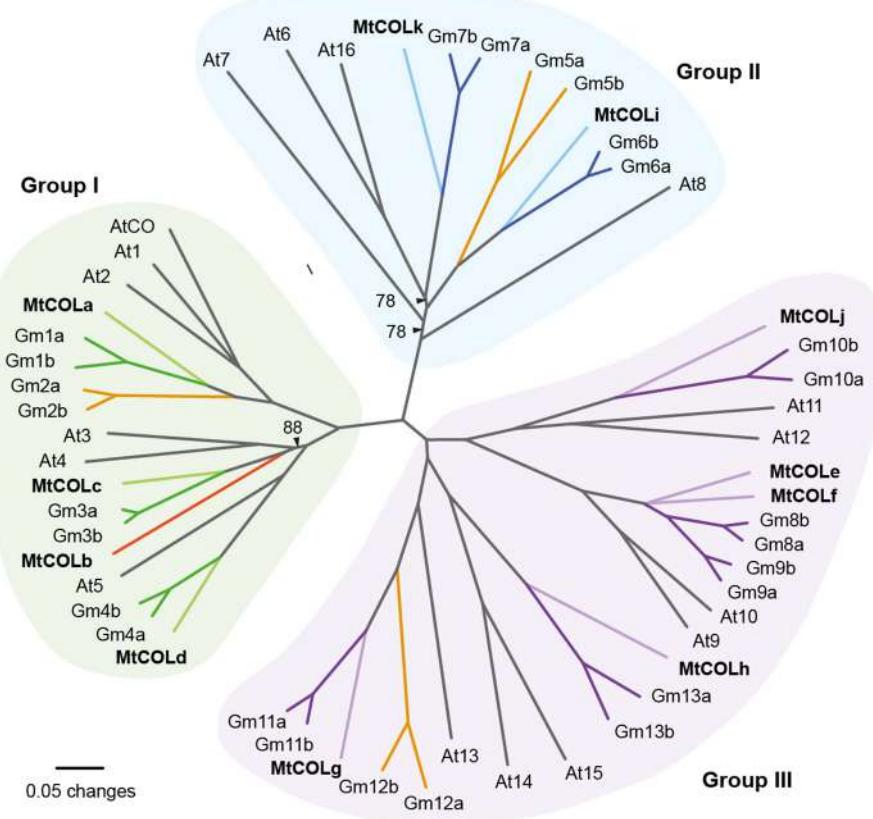
RESULTS

DEFINING THE CONSTANS-LIKE (*COL*) GENE FAMILY IN LEGUMES

We previously reported a partial characterization of the *COL* gene family in legumes (Hecht et al., 2005) focusing on the so-called Group I *COL* genes (Griffiths et al., 2003). This group of genes includes Arabidopsis *CO* and is characterized by two B-box domains within an N-terminal Zn finger region, and a conserved C-terminal (CCT) domain that is also found in the circadian clock-related pseudo-response regulator gene *TOC1* and related PRR (Strayer et al., 2000; Griffiths et al., 2003). To extend our understanding of legume *COL* genes, we used a combination of database searches and PCR-based approaches to isolate additional *COL* genes in *Medicago truncatula*. We identified a total of 11 expressed and apparently full-length *COL* coding sequences (**Figure 1**, Supplemental Figure 1) that included four Group I genes (*COLa-COLD*), two group II genes (*COLi*, *COLk*) and four Group III genes (*COLe-COLh*, *COLj*). It thus appears that all major groups within the *COL* family are represented in legumes, but some degree of independent expansion has occurred within Groups II and III.

Consistent with a previous report (Hecht et al., 2005) we identified only a single group Ia gene in *Medicago* (*MtCOLa*) and found that the three Arabidopsis group Ia genes *AtCO*, *AtCOL1* and *AtCOL2* were more similar to each other than to *MtCOLa*.

A recent report from soybean has identified 26 *COL* genes, representing 13 pairs of homeologs (Wu et al., 2014). For nine of these pairs, we identified a single *Medicago* ortholog (**Figure 1**), and the clade containing *GmCOL8a/b* and *COL9a/b* also included

**FIGURE 1 |** The CONSTANS-LIKE (COL) gene family in *Medicago*.

Phylogram of legume and *Arabidopsis* COL protein sequences. The analysis is based on the sequence alignment shown in Supplemental Figure 1 online. Sequence details are available in Supplemental Table 1 online. Groups I (green shading), II (blue shading) and III (purple shading) correspond to the classification of Griffiths et al. (2003). Branches representing legume proteins are shaded in color consistent with each group, with soybean proteins shown in

dark and *Medicago* genes in light shading. Branches shown in orange indicate soybean homeolog pairs for which no corresponding *Medicago* ortholog was found, and the single *Medicago* gene without a soybean counterpart is shown in red. Branches with bootstrap values <50% have been collapsed, and black arrowheads indicate branches with support >50% but <90%. All other branches have support >90%. At, *Arabidopsis thaliana*; Gm, *Glycine max*; Mt, *Medicago truncatula*.

two *Medicago* genes; *MtCOLe* and *COLf*. The *MtCOLb* gene had no corresponding pair of genes in soybean, and three soybean homeolog pairs were not represented by *Medicago* genes. This latter situation could imply the existence of additional *Medicago* COL genes not represented in the current genome build (Mt4.0), and we were particularly interested in a comparison of the Group Ia genes as this clade contains most of the genes known to have CO-like function in other species. In soybean, there are four Group Ia COL genes; *GmCOL1a/b* and *COL2a/b*. The single Group Ia gene *MtCOLa* is clearly orthologous to the *GmCOL1a/b* pair, implying that *Medicago* might possess a second Group Ia COL gene orthologous to *GmCOL2a/b*. To address the possibility, we examined the genomic regions containing *GmCOL2a* and *COL2b* for evidence of microsynteny with the *Medicago* genome. Supplemental Figure 2 shows that genes in the *GmCOL2a/COL2b* regions showed highest similarity to genes on *Medicago* chromosome 6, with clear evidence of microsynteny, but there was no *MtCOL* gene in this location, suggesting that this gene may have been lost from the *Medicago* lineage. Similarly, microsynteny between regions containing *GmCOL5a/b*

and another part of *Medicago* chromosome 6, and between regions containing *GmCOL12a/b* and *Medicago* chromosome 2 (Supplemental Figure 2) also suggests that orthologs of these genes are also absent from the *Medicago* genome. We therefore tentatively conclude that the 11 *Medicago* COL genes we have identified represent the entire gene family.

DIURNAL RHYTHMS OF COL GENE EXPRESSION

In *Arabidopsis*, the characteristic diurnal mRNA expression rhythm of CO is linked to its function in photoperiod measurement. Under SD, CO expression peaks in the night and is low throughout the day. Under LD, CO expression increases during the afternoon, and this increase is reinforced by an additional relief from repression through the action of the blue-light photoreceptor FKF1 (Imaizumi et al., 2003). We reasoned that if transcriptional regulation of COL genes was similarly important for photoperiod responses in temperate legumes, one or more COL genes might show distinctly different expression rhythms in long and short days. We therefore examined the diurnal expression rhythms for eight of the 11 *MtCOL* genes (*COLa-COLh*).

We previously reported that the single group Ia *COL* gene in pea, *PsCOLa*, shows a morning-phased expression rhythm in LD (Hecht et al., 2007) that is similar to the Arabidopsis Group Ia genes *COL1* and *COL2* (Ledger et al., 2001). **Figure 2** shows that *MtCOLa* expression also follows a similar LD rhythm with a peak at dawn. The level of expression under SD was not significantly different than under LD throughout the daily time-course, and under both conditions, *COLa* showed significant morning expression, which declined to basal level by ZT9. Under LD specifically, *COLa* expression remained very low during the afternoon, with no evidence of the afternoon “shoulder” to the LD rhythm that is characteristic of Arabidopsis *CO* (Imazumi et al., 2003). More generally, there was no evidence for any difference in *COLa* expression during the light phase in LD compared to SD.

Like *COLa*, the Group Ic genes *COLb* and *COLd* also showed a morning-phased rhythm. For both genes, the phase of the expression rhythm was earlier in SD than in LD, typical of the response of many rhythmically-regulated genes to photoperiod. However, as for *COLa*, there was no evidence of a qualitative difference in expression during the light phase between LD and SD conditions for either gene. *COLc* was only expressed at a very low level and showed minimal diurnal variation. In contrast to the Group I genes, the Group III genes generally showed an evening-phased rhythm under LD, which in most cases, was shifted earlier in SD. *COLe* showed the most strongly rhythmic expression with an afternoon peak in LD at around ZT12, and *COLf* expression was also clearly rhythmic, with peak expression under LD during the night. *COLg* and *COLh* were at most weakly rhythmic. The closest similarity to the Arabidopsis *CO* rhythm was seen for *COLf*,

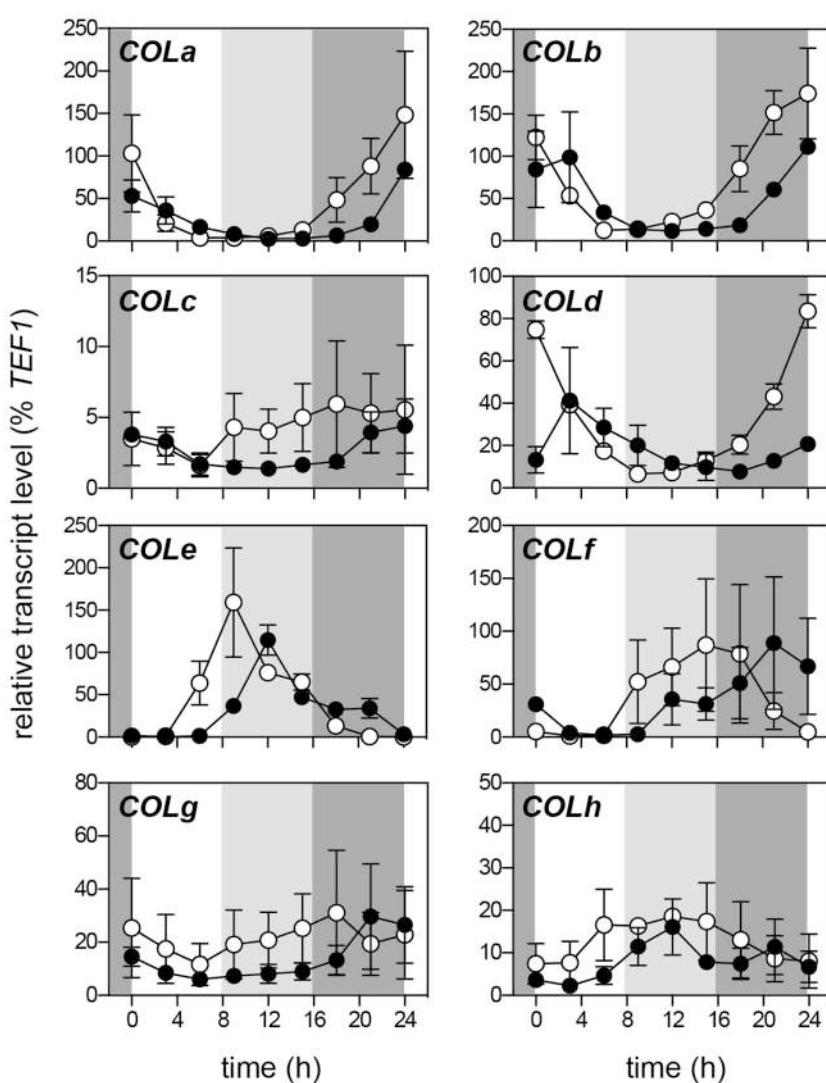


FIGURE 2 | Rhythmic regulation of *MtCOL* expression under SD and LD.

Transcript levels were determined in fully-expanded leaves taken from 3-week-old R108 seedlings grown under 8-h (short-day; open symbols) or 16-h long-day photoperiods (filled symbols) in growth cabinets at 22°C. The

night period common to both treatments is represented by dark gray shading, with the period in which plants are in the light in long days but not short days is represented by light gray shading. Data represent mean \pm SE for $n = 2$ biological replicates.

which was not expressed at dawn or at either of the two time-points during the light phase under SD, but showed significant expression at dawn and during the afternoon in LD.

ACTIVITY OF LEGUME COL GENES

Arabidopsis CO is a potent inducer of flowering, and Arabidopsis plants overexpressing CO flower very early under both LD and SD (Onouchi et al., 2000). To test whether any of the *MtCOL* genes might be similarly effective in flowering regulation, we assessed their ability to complement the late flowering phenotype of the Arabidopsis *co-2* mutant. **Figure 3A** shows that none of the eight *MtCOL* genes that we tested caused early flowering when over-expressed from the cauliflower mosaic virus 35S promoter in the late-flowering Arabidopsis *co-2* mutant plants.

Next, we examined the ability of the *MtCOL* genes to directly activate the *Arabidopsis FT* promoter using a transient assay system. In this system, the *Arabidopsis FT* promoter was fused to the luciferase reporter gene and infiltrated into *Nicotiana benthamiana* leaves together with different transcription factors. **Figure 3B** shows that expression of *AtCO* resulted in substantial upregulation of luciferase expression from the *Arabidopsis FT* promoter ($P < 0.0001$). In contrast, the majority of *MtCOL* genes had no clear statistically significant effect consistent with their inability to complement the *co-2* mutant. The one possible exception was *MtCOLf*, which showed a small increase in LUC signal with marginal statistical significance ($P = 0.045$).

The transient assay system was also used to investigate if any of the *MtCOL* genes are able to activate the *Medicago FTa1* promoter. The *Medicago FTa1* gene plays a key role in promoting flowering in response to both vernalization and LD (Laurie et al., 2011). When *Medicago* plants are shifted from SD to LD, *FTa1* is upregulated by exposure to a single long day (Laurie et al., 2011). An *MtFTa1* promoter sequence comprising 2017 bp upstream of the start codon was fused to the luciferase reporter gene and infiltrated into *Nicotiana benthamiana* leaves together with different *MtCOL* genes. Neither Arabidopsis *CO* nor any of the *MtCOL* genes was able to induce LUC expression from this promoter sequence ($P > 0.05$ in all cases) (**Figure 3C**).

Overall, these results provide further evidence that that none of the *MtCOL* genes are functionally equivalent to *AtCO*, with respect to their ability to induce expression of *AtFT*. In addition they also suggest that neither *AtCO* nor any of the tested *MtCOL* genes are able to induce *MtFTa1* expression. Although the specific reason for the inactivity of *MtCOL* genes on *AtFT*, and *AtCO* on *MtFTa1* is not yet clear, it could partially reflect divergence in FT promoter sequences and/or DNA binding characteristics of CO and COL proteins. An alignment of the *AtFT* proximal promoter with regions upstream of the transcriptional start site in the *Medicago* and chickpea *FTa1* genes (Supplemental Figure 3) shows that neither of the two CO-responsive (CORE) elements defined in the *AtFT* promoter are significantly conserved in the legume promoters, which may provide an explanation for the inactivity of *AtCO* on the *MtFTa1* promoter.

GENETIC ANALYSIS OF COL FUNCTION

Finally, in order to directly examine *COL* gene function, we made use of the *Medicago Tnt1* insertion platform (Tadege et al., 2008)

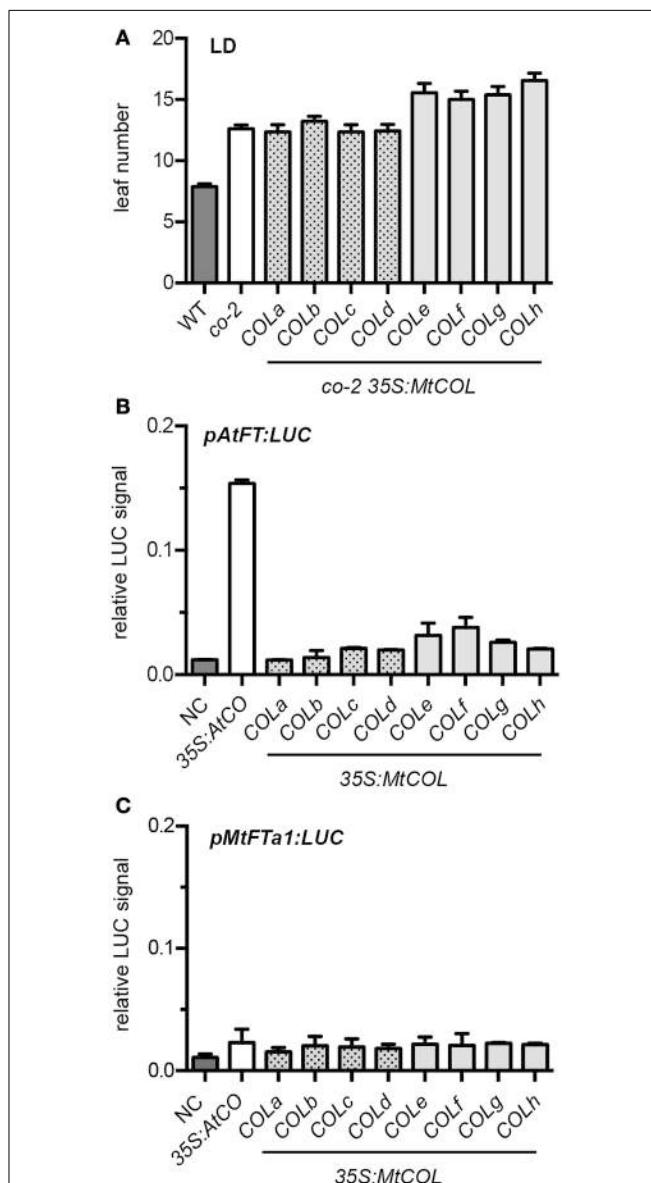


FIGURE 3 | Functional analyses of *MtCOL* genes. (A) Overexpression of *MtCOLa-COLh* genes does not promote flowering in the Arabidopsis *co-2* mutant. Flowering time is indicated by leaf number at flowering. Data represent a minimum of 10 plants for each line \pm SE. **(B)** *MtCOL* genes are unable to induce expression from the *Arabidopsis FT* promoter in transient expression assays. The 35S:*AtCO* construct and 35S:*MtCOL* constructs were co-infiltrated with *AtFT* promoter fused to the luciferase (LUC) reporter gene into *N. benthamiana* leaves. Only 35S:*AtCO* and 35S:*MtCOLf* resulted in statistically significant upregulation of the *AtCO* promoter compared with the NC (no construct) control, $P < 0.0001$ and $P = 0.045$, respectively **(C)**. *MtCOL* genes are unable to induce expression from the *Medicago FTa1* promoter in transient expression assays. The 35S:*AtCO* construct and 35S:*MtCOLa-h* constructs were co-infiltrated with *MtFTa1* promoter:LUC into *N. benthamiana* leaves. No statistically-significant difference in relative LUC signal between the NC control and 35S:*AtCO* or the 35S:*MtCOLs* was observed. NC (no construct) refers to leaves infiltrated with untransformed *Agrobacterium* along with the *AtFT* or *MtFTa1* promoter:LUC constructs. Relative LUC signal is a ratio of LUC activity versus Renilla luciferase activity to correct for variation

(Continued)

FIGURE 3 | Continued

in transformation efficiencies between infiltrated *N. benthamiana* leaves and data represent the mean \pm SE of three biological replicates. Statistical analysis was performed using Student's *t*-test. In all panels, Group I and Group III *MtCOL* genes are represented by dark gray and light gray shading, respectively.

to identify putative insertion mutants for three of the four Group I *MtCOL* genes. Insertions in *COLa*, *COLb* and *COLc* were verified by sequencing and mutant lines shown to specifically lack the corresponding transcript (**Figure 4A**). For phenotypic comparisons we vernalized seeds for 2 weeks at 4°C and grew seedlings under an 18 h photoperiod. In addition to a pure line of the progenitor line R108, we also included WT lines selected from individual segregating progenies for each mutant as controls. **Figure 4B** shows that neither *colb* nor *colc* mutants flowered significantly different from their corresponding control lines in terms of either flowering time ($P > 0.5$ and $P > 0.2$ for *colb* and *colc*, respectively) or for node of first flower ($P = 0.088$ and $P > 0.5$, respectively). The genetic background carrying the *cola* mutation was slightly later flowering than the R108 control line, in terms of days (18.9 vs. 16.2 days, $P < 0.001$), but slightly earlier in terms of nodes (5.2 vs. 5.9 nodes, $P < 0.01$). The *cola* mutant line was marginally later than its WT control line for both time (19.9 vs. 18.9 days, $P = 0.044$) but not for node number (5.7 vs. 5.2 nodes, $P = 0.088$). However, importantly, the variation in flowering time or node observed within and between these lines was negligible relative to the strong delay of flowering in vernalized R108 plants under SD, indicating that none of these three *COL* genes contributes significantly to the promotion of flowering by LD.

DISCUSSION

The CONSTANS protein has been a central feature of models explaining the molecular basis for plant responses to photoperiod, and the potential conservation of CO function across flowering plants has been a topic of considerable interest. In legumes, several studies have identified conserved elements of the photoperiod response pathway, including homologs of *GIGANTEA* (Hecht et al., 2007; Watanabe et al., 2011), *PHYA* (Weller et al., 2004; Liu et al., 2008a; Watanabe et al., 2009), *FT* (Kong et al., 2010; Hecht et al., 2011; Laurie et al., 2011; Sun et al., 2011) and circadian clock genes (Liew et al., 2009, 2014; Weller et al., 2012), but the potential role of CO-like genes has received less attention. In this study we have identified 11 *COL* genes in the model long-day legume *Medicago truncatula*, and investigated the regulation and function of eight of these. Collectively our results provide several strong lines of evidence that the three genes most similar to *Arabidopsis CO*, *MtCOLa*, *MtCOLb* and *MtCOLc*, genes do not participate in the induction of flowering by photoperiod. Our results also indicate that the five other genes we examined (*MtCOLd-COLh*) are also unlikely to function in a manner similar to *AtCO*.

The first line of evidence comes from regulation of *MtCOL* expression. Rhythmic expression of group I and group III *MtCOL* genes showed broad similarity to reported results from other

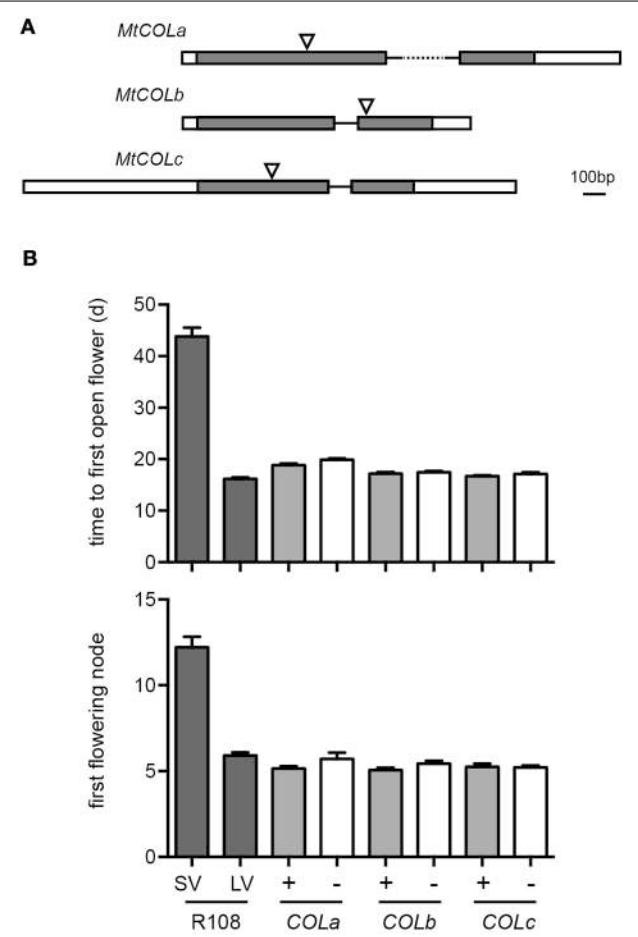


FIGURE 4 | Characterisation of mutants for *MtCOLa*, *COLb* and *COLc*.

(A) Diagram of *MtCOLa*, *COLb* and *COLc* genes showing gene structure and site of the *Tnt1* insertion. Exons are shown as boxes with the coding sequence in dark gray, and the 5' and 3' untranslated regions in white. **(B)** Flowering time and node of first flower for WT and *COL* mutant plants vernalized for 14 days at 4°C before transfer to LD at 22°C. Homozygous WT (+) and mutant (-) genotypes were selected from segregating progenies and are represented by shaded and empty bars, respectively. Vernalized R108 plants grown under LD (LV) or SD (SV) were included as a control. Values represent mean \pm SE for $n = 12\text{--}20$.

species, with group I genes showing peak expression around dawn and other genes generally more strongly expressed late in the day or in the early part of the night (**Figure 2**). However, with the possible exception of *MtCOLf*, we found no evidence for photoperiod-specific coincidence of *MtCOL* expression with the light phase in LD, or for the afternoon peak that is characteristic of the *AtCO* transcriptional rhythm under LD (Imaizumi et al., 2003) (**Figure 2**). Nevertheless, it should be noted that the absence of these regulatory features does not in itself exclude the possibility that these genes have CO-like function. First, because *Arabidopsis CO* is known to undergo significant post-transcriptional regulation, and it is conceivable that photoperiod-specific activity could be conferred on one or more of the *MtCOL* genes predominantly through regulation at the protein level. Second, because the link between

CO expression dynamics and photoperiod responsiveness has only been elaborated in detail for Arabidopsis and it is not yet clear how widely this may be conserved (Ballerini and Kramer, 2011).

We obtained more direct evidence on *MtCOL* functions from Arabidopsis complementation and experiments using a tobacco transient assay system. None of the *MtCOL* genes was able to promote flowering when overexpressed in the Arabidopsis *co-2* mutant (**Figure 3A**), in contrast to the strong flower-promoting activity of Arabidopsis *CO* (Onouchi et al., 2000). This contrast was also seen in transient assays, where none of the eight tested *MtCOL* genes was able to activate transcription from the Arabidopsis *FT* promoter, even though *AtCO* clearly possessed this ability (**Figure 3B**). Finally, transcript-null mutants for the Group Ia gene *MtCOLa* and two other Group I genes all flowered normally under LD after vernalization (**Figure 4**), clearly indicating that these genes are not needed for, and likely do not participate in, the promotion of flowering by LD.

Overall, the lack of any clear effect of *MtCOL* genes on flowering is somewhat surprising, particularly in the case of *COLa*, in view of the fact that Group Ia *COL* genes across a range of species have been shown to some degree of function in flowering regulation. Outside of Arabidopsis, the involvement of Group Ia *COL* genes in photoperiod responsiveness is most conclusive in rice, where the single group Ia *COL* gene *Hd1* underlies a major-effect QTL for flowering, and has a bidirectional role in regulation of *FT* homologs (Yano et al., 2000). The potato *CO* gene also has a clear endogenous role in photoperiod responsiveness, although this is less pronounced for flowering induction than for tuberization (Navarro et al., 2011). Group Ia *COL* genes in a number of other species have shown flower-promoting activity in Arabidopsis. Genes from potato, tomato, poplar and sugar beet are able to at least partially complement Arabidopsis *co* mutants (Ben-Naim et al., 2006; Chia et al., 2008; Gonzalez-Schain et al., 2012; Hsu et al., 2012). The barley *Hd1* ortholog *CO1* has no activity in transgenic Arabidopsis but does promote flowering when overexpressed in barley itself (Campoli et al., 2012).

In Arabidopsis, activation of the Arabidopsis *FT* promoter by *CO* requires several regulatory motifs located within 500 bp of the *FT* transcriptional start site (Adrian et al., 2010; Tiwari et al., 2010). Full *FT* activation in planta requires the additional association of *CO* with proteins bound to distal promoter regions and the formation of chromatin loops (Ben-Naim et al., 2006; Adrian et al., 2010; Cao et al., 2014). However, a 1 kb proximal fragment of the *AtFT* promoter is sufficient for maximal induction by *AtCO* in a transient assay system (Adrian et al., 2010) and our results show that *MtCOL* genes lack this activity, consistent with their lack of flower-promoting activity when overexpressed in Arabidopsis (**Figure 4A**). While the reason for this is not clear, the simplest explanation may be that *MtCOL* proteins either do not bind to the *CO*-responsive elements in this region, or simply do not function as transcriptional regulators. Our results also show that an equivalent region of the *MtFTa1* promoter is activated neither by *AtCO* nor *MtCOL* genes. The lack of *AtCO* activity may reflect the fact that none of the

functionally validated proximal elements in the Arabidopsis *FT* promoter are significantly conserved in the corresponding regions of the *Medicago* or chickpea *FTa1* genes (Supplemental Figure 3).

The argument may also be made that *CO* function may be preserved in the *MtCOL* family but is comprised of small individual contributions from multiple members. Clearly the present data also do not exclude this possibility, with some features of *MtCOLf* (**Figures 2, 3B**) consistent with a weak *CO*-like effect. However, it is worth noting that loss-of-function variants for Arabidopsis *CO* and rice *Hd1* both have large phenotypic effects and were first identified through relatively direct forward genetic analysis. This is not proof that deep redundancy within the *COL* family is not an explanation for our results, but does make it seem less likely. It also remains possible that *CO* function could be carried out one or more of the other *MtCOL* genes that we did not examine, which include both group II (*COLi*, *COLk*) and Group III (*COLj*) genes. Although no gene outside the group Ia *COL* clade has been implicated in photoperiodic flowering, a more general effect on flowering has been demonstrated for certain other *COL* genes. In Arabidopsis, the group Ic gene *COL3* (Datta et al., 2006) and the group III gene *COL9* (Cheng and Wang, 2005) both inhibit flowering, whereas a second group Ic gene, *COL5*, may have a promotive function (Hassidim et al., 2009). In rice, the Group Ic gene *COL4* gene inhibits flowering under SD and LD through repression of *FT* homologs *RFT* and *Hd3a* (Lee et al., 2010).

Recently, new information has emerged on *COL* genes in the short-day legume soybean. Soybean has multiple group Ia *COL* genes, which comprise two pairs of homeologs; *COL1a/b* and *COL2a/b*. Two recent studies show that one of these, *GmCOL2a*, is able to complement the Arabidopsis *co-2* mutant (Fan et al., 2014; Wu et al., 2014), and there is evidence that the remaining genes *COL1a/b* and *COL2b* may also have some activity in Arabidopsis (Wu et al., 2014). However, our phylogenetic comparisons indicate that *Medicago* has only a single Group Ia *COL* gene orthologous to Arabidopsis *CO/COL1/COL2*, and does not have an ortholog of the *GmCOL2* genes (Supplemental Figure 2). Sequence searches in other temperate legumes (including pea, and chickpea and *Lotus japonicus*) also identify only a single group Ia *COL* gene in these species, indicating that loss of *COL2* orthologs may have occurred relatively early in this temperate legume lineage.

Overall, it seems on balance likely that *COL* genes do not function as central integrators of photoperiod responsive flowering in *Medicago*. This may also be more generally true across the temperate legumes, at least for *COLa*, as *COLa* orthologs in pea and *Lotus japonicus* also do not show characteristic regulatory features of Arabidopsis *CO* (Hecht et al., 2007; Yamashino et al., 2013). Instead, *CO*-independent pathways may have a more prominent role in this plant group. In Arabidopsis, a number of other factors contribute to direct regulation of *FT* expression. These include the positive factors PIF4 and CIB1, bHLH proteins involved in light signaling, and SPL3, which is a target of the *miRNA156* pathway controlling juvenility (Liu et al., 2008b; Kim et al., 2012; Kumar et al., 2012). Factors repressing *FT* include the CDF family of Dof transcription factors, and a number of AP2 domain proteins that

are targets of *miR172* (Jung et al., 2007; Fornara et al., 2009). In particular, it is intriguing that despite the apparent absence of CO-like function in *Medicago*, evidence from the related legume pea shows a major role for the *GI* ortholog *LATE1* (Hecht et al., 2007, 2011). In *Arabidopsis* *GI* has been shown to promote *FT* transcription independently of *CO*, both by contributing to degradation of CDF proteins (Song et al., 2012), and also by positive effects on *miR172* biogenesis (Jung et al., 2007). In addition, the recently-identified B3-transcription factor-like gene *E1* in soybean is also important for photoperiod-dependent regulation of *FT* expression, and an ortholog is present in *Medicago* (Xia et al., 2012; Zhai et al., 2014). Whether one or more of these mechanisms contribute to the photoperiod response in temperate legumes will be an important question for future investigation.

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SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00486/abstract>

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Capturing sequence variation among flowering-time regulatory gene homologs in the allopolyploid crop species *Brassica napus*

Sarah Schiessl^{1*}, Birgit Samans¹, Bruno Hüttel², Richard Reinhard² and Rod J. Snowdon¹

¹ Department of Plant Breeding, IFZ Research Centre for Biosystems, Land Use and Nutrition, Justus Liebig University, Giessen, Giessen, Germany

² Max Planck Genome Centre Cologne, Max Planck Institute for Breeding Research, Cologne, Germany

Edited by:

Klaus Pillen,
Martin-Luther-University
Halle-Wittenberg, Germany

Reviewed by:

Iain Robert Searle, The University of
Adelaide, Australia
Rita Sharma, Jawaharlal Nehru
University, India

***Correspondence:**

Sarah Schiessl, Department of Plant
Breeding, Justus Liebig University,
Heinrich-Buff-Ring 26-32,
Giessen 35392, Germany
e-mail: sarah-veronica.schiessl@
agrar.uni-giessen.de

Flowering, the transition from the vegetative to the generative phase, is a decisive time point in the lifecycle of a plant. Flowering is controlled by a complex network of transcription factors, photoreceptors, enzymes and miRNAs. In recent years, several studies gave rise to the hypothesis that this network is also strongly involved in the regulation of other important lifecycle processes ranging from germination and seed development through to fundamental developmental and yield-related traits. In the allopolyploid crop species *Brassica napus*, (genome AACC), homoeologous copies of flowering time regulatory genes are implicated in major phenological variation within the species, however the extent and control of intraspecific and intergenomic variation among flowering-time regulators is still unclear. To investigate differences among *B. napus* morphotypes in relation to flowering-time gene variation, we performed targeted deep sequencing of 29 regulatory flowering-time genes in four genetically and phenologically diverse *B. napus* accessions. The genotype panel included a winter-type oilseed rape, a winter fodder rape, a spring-type oilseed rape (all *B. napus* ssp. *napus*) and a swede (*B. napus* ssp. *napobrassica*), which show extreme differences in winter-hardiness, vernalization requirement and flowering behavior. A broad range of genetic variation was detected in the targeted genes for the different morphotypes, including non-synonymous SNPs, copy number variation and presence-absence variation. The results suggest that this broad variation in vernalization, clock and signaling genes could be a key driver of morphological differentiation for flowering-related traits in this recent allopolyploid crop species.

Keywords: copy number variation, CNV, presence-absence variation, PAV, rapeseed, sequence capture

INTRODUCTION

As a recent allopolyploid species, *Brassica napus* L. (genome AACC, $2n = 38$) is also a very interesting model to investigate polyploidization and adaptation during crop evolution. Although oilseed rape/canola (*B. napus* ssp. *napus*) is today the second-most important oilseed crop worldwide, it is thought that the species originated only during the last few thousand years, after spontaneous interspecific hybridization events between Asian *Brassica rapa* (genome AA, $2n = 20$) and Mediterranean *Brassica oleracea* (genome CC, $2n = 18$) (Snowdon et al., 2006).

No wild forms of *B. napus* are known, and intensive selection and breeding following its anthropogenically-influenced polyploidization has led to cultivation of very different phenological types. This has caused the diversification of distinct gene pools adapted to highly different eco-geographic zones of Europe, Asia/Australia and North America. Very early-flowering morphotypes, without vernalization requirement, are today widely grown in Canada (as canola) and northern Europe (as spring oilseed rape), where harsh winters prohibit autumn-sown crops. Later-flowering “semi-winter” oilseed forms, requiring only

mild vernalization, are prevalent in China and Australia, while autumn-sown oilseed rape is today the most important oilseed crop in temperate regions of Europe (Friedt and Snowdon, 2010). The subspecies *napus* also includes leafy forms that sometimes need strong vernalization before flowering and are grown in parts of Europe and eastern Asia as fodder rape or kales. A second subspecies, *B. napus* ssp. *napobrassica*, comprises swede forms with an enlarged hypocotyl that is harvested as a vegetable or used as a grazing fodder. Swedes generally have a strong vernalization requirement but tend to lack the strong winter-hardiness of winter oilseed rape (Friedt and Snowdon, 2010).

Brassica napus is the most closely related major field crop species to the model crucifer *Arabidopsis thaliana*. This enables considerable insight into major biochemical and developmental pathways using information from the model species. For example, important *Brassica* orthologs of *A. thaliana* genes responsible for vernalization and floral transition are highly conserved between the model and the crop (Lagercrantz et al., 1996; Osborn et al., 1997; Wang et al., 2009; Zou et al., 2012). In *Arabidopsis*, the optimization of flowering in respect to environment is achieved

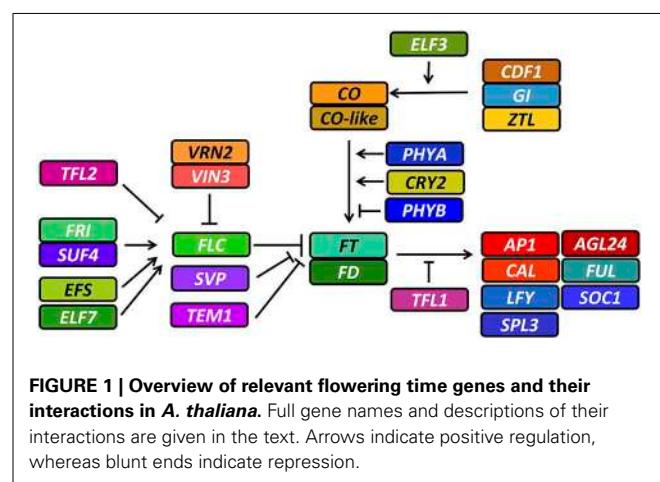
by a tightly regulated gene network determining the transition from the vegetative to the reproductive phase (Jaeger et al., 2006; Jung and Muller, 2009; Srikanth and Schmid, 2011; Andrés and Coupland, 2012). There is increasing evidence that this network not only regulates flowering time *per se*, but also plays a role throughout the whole plant life cycle (Deng et al., 2011). The pleiotropic or direct influence of flowering time regulators on multiple agronomic traits, like the number and size of seeds, seedling vigor, biomass gain and resistance to biotic or abiotic stress (Quijada et al., 2006; Chen et al., 2007; Ni et al., 2008; Chianga et al., 2009; Basunanda et al., 2010; Li et al., 2010), not only makes them a major driver of crop evolution and adaptation, but also subjects them to strong selection for useful diversity during crop breeding.

To meet the needs of their respective climate zone, plants developed several sensor systems to assess the correct time to flower. Of particular importance in this regard is an ability to sense temperature, day length, light quality and stress signals (Jaeger et al., 2006; Jung and Muller, 2009; Srikanth and Schmid, 2011; Wigge, 2013). In temperate climates zones where winter limits growth completely, the most important mechanism of plant flower regulation is vernalization, the induction of flowering after a period of prolonged cold (Preston and Sandve, 2013). The second condition for plants to flower after winter is day length (Song et al., 2013), whereas light quality and other forms of stresses can only modulate the flowering response. Moreover, the transition to flowering can also be influenced by endogenous factors like gibberellins and autonomous pathways like the circadian clock (Pak et al., 2009; de Montaigu et al., 2010). Understanding the role and interplay of these factors could assist in improving yield and adaption in *B. napus*.

Knowledge of flowering in *Brassica* species is largely based on *A. thaliana*. The most important *Arabidopsis* genes involved in flowering time have already been shown to have orthologs in *Brassica* crops (Wang et al., 2009; Zou et al., 2012), whereby comparisons of *A. thaliana* and *B. rapa* suggest that this congruence might be true for the whole flowering-time gene network (<http://brassicadb.org/brad/flowerGene.php#>). In *A. thaliana* the network features two major thresholds controlling the main flowering signal, *FLOWERING LOCUS T* (*FT*). The first threshold, the vernalization pathway, acts via removal of a factor repressing *FT* expression upon perception of the stimulus, while the second threshold, the photoperiod pathway, acts via *FT* activation. Repression of *FT* in the vernalization pathway is achieved by several factors, the most important being *FLOWERING LOCUS C* (*FLC*), assisted by other factors like *SHORT VEGETATIVE PHASE* (*SVP*) and *TEMPRANILLO 1* (*TEM1*). *FLC* is constitutively expressed before vernalization by activation of *FRIGIDA* (*FRI*), which acts in complex with other factors like *SUPPRESSOR OF FRIGIDA 4* (*SUF4*) as a transcriptional activator for *FLC*. Expression of *FLC* is also enhanced by other factors like *EARLY FLOWERING 7* (*ELF7*) and *EARLY FLOWERING IN SHORT DAYS* (*EFS*). The signal for *FLC* silencing is transmitted via upregulation of *VERNALIZATION INSENSITIVE 3* (*VIN3*) in response to prolonged cold. *VIN3* binds to a complex named PCR2, a major component of this complex being *VERNALIZATION 2* (*VRN2*). The PCR2 complex

is associated with the *FLC* gene segment and silences *FLC* transcription by heterochromatic changes upon binding of *VIN3*. During this process, *TERMINAL FLOWER 2* (*TFL2*) also binds to the *FLC* gene and may be responsible for conserving the vernalized state. *FLC* is then effectively silenced and not responsive to further activation by the *FRI* complex, making *FT* accessible for activation by the photoperiod pathway. *FT* is activated by the transcription factor *CONSTANS* (*CO*), which is only stably expressed at the end of a long day. This expression pattern is controlled by the circadian clock, transmitting its signal via *GIGANTEA* (*GI*) in complex with *ZEITLUPE* (*ZTL*), and *CYCLING DOF FACTOR 1* (*CDF1*). This transmission is also modulated by ambient temperature via *EARLY FLOWERING 3* (*ELF3*). Protein stability of *CO* is further controlled by photoreceptors. *PHYTOCHROME A* (*PHYA*) and *CRYPTOCHROME 2* (*CRY2*) stabilize *CO* protein, whereas *PHYTOCHROME B* (*PHYB*) destabilizes it. As soon as vernalization and photoperiod pathway allow for *FT* expression, *FT* is translocated to the shoot apex, triggering the vegetative-to-generative transition in a complex with *FLOWERING LOCUS D* (*FD*), via direct or indirect activation of several meristem identity genes like *APETALA 1* (*AP1*) and *CAULIFLOWER* (*CAL*). These are further modulated by an interwoven network of transcription factors including the miRNA-regulated *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* (*SPL3*), *AGAMOUS-LIKE 24* (*AGL24*), *LEAFY* (*LFY*), *FRUITFUL* (*FUL*) and *SUPPRESSOR OF CONSTANS 1* (*SOC1*). The function of *FT* is antagonized by *TERMINAL FLOWER 1* (*TFL1*), which contributes to the fine regulation of flowering time in response to ambient temperature, independently from vernalization (reviewed in depth in Jaeger et al., 2006; Jung and Muller, 2009; Pak et al., 2009; de Montaigu et al., 2010; Srikanth and Schmid, 2011; Wigge, 2013) (summarized in Figure 1).

Despite the common ancestry and functionality of most genes, a major difference between the genetic control particularly of complex traits in *B. napus* and *A. thaliana* is the influence of polyploidy. The progenitor genomes making up the *B. napus* genome are still basically intact (Parkin et al., 1995; Axelsson et al., 2000; Bancroft et al., 2011). This means that every gene may



have homologous alleles present in the A genome as well as in the C genome, which are hard to differentiate from alleles within each subgenome (Bancroft et al., 2011). Furthermore, each gene may have multiple paralogs within each subgenome as a consequence of whole-genome triplication and gene duplication in the diploid species (Town, 2006; Wang et al., 2011b). Gene expression studies revealed an average of 4.4 functional gene copies present in *B. napus* (Parkin et al., 2010). Furthermore, during allopolyploidization the two *B. napus* subgenomes frequently exchange gene material in a process called homoeologous recombination (Gaeta and Pires, 2010; Udall et al., 2006). This dynamic genome formation process has been shown to result in gene copy-number variation (CNV) and presence/absence variation (PAV), affecting traits with relevance for adaptation, selection and breeding (Harper et al., 2012). The extent of such variants within high-impact regulatory networks like the circadian clock, vernalization and floral transition pathways has yet to be investigated in the context of adaptive traits in *B. napus*.

Analysis of the *B. rapa* genome sequence has also revealed an expansion of transposons (Wang et al., 2011b). Transposons play a major role in creating genetic variation, the most important prerequisite of adaptation (reviewed in Lisch, 2012). Recently it was shown that a Tourist-like MITE insertion in the promoter region of a FLOWERING LOCUS C (*FLC*) homolog in the *B. napus* A-genome is associated with vernalization requirement in European winter rapeseed morphotypes (Hou et al., 2012). Transposon activity is highly accelerated in case of genomic shock caused by interspecific hybridization and chromosomal breakage (Lisch, 2012), both of huge relevance in *Brassica* species. As much as 8–15% of the *B. napus* genome is comprised of repetitive sequences, indicating a potentially high degree of transposon activity (Samans, unpublished data). As the C genome is larger than the A genome (Johnston, 2005), transposons might be expected to be more prominent in C-genome gene homologs.

Generally, polyploids are considered to be more stable and adapt easier to new environments (Chen, 2010). One reason is the number of gene copies, which can be a regulating factor. More copies offer the possibility of (1) simultaneous transcription, therefore accelerating or strengthening regulation responses, (2) separate regulation in order to reach a more elaborate fine-tuning, and (3) gene back-ups to reduce loss-of-function risks. In plants, copy number variation has been observed to be widespread (Zmienko et al., 2013). Due to the high degree of genome and gene duplication and genome rearrangements during polyploid formation, a relatively high degree of copy number variation is expected in *B. napus* (Edwards et al., 2013). All the same, classical cloning and mapping strategies suffer from this complexity. Due to the high specificity of cloning, it is often not possible to evaluate the number of copies present in a genome without exact knowledge from a high-quality reference genome.

As a proof of principle, this study aimed to detect genetic variation in all homologous and paralogous copies of 29 selected flowering time genes in *B. napus*, based on sequences derived from the diploid progenitors *B. rapa* and *B. oleracea*. Four genotypes representing the broad phenological variation for vernalization requirement, flowering transition and day-length

dependent flowering in *B. napus* were sequenced with an RNA-based sequence capture approach. The objectives were (1) to establish an effective RNA bait library for sequencing of flowering time regulatory genes in the allopolyploid *B. napus*, (2) to investigate gene losses and gains amongst flowering-related genes in different *B. napus* ecotypes, and (3) to determine the extent of genetic variation among flowering time and vernalization pathway genes in *B. napus*.

MATERIALS AND METHODS

PLANT MATERIAL

A large panel of genetically diverse *B. napus* inbred lines was previously tested for winter survival, date of flowering and duration of flowering under short and long day conditions. The plant material used to select the different morphotypes was the ERANET-ASSYST consortium diversity set, a panel of over 500 genetically diverse *B. napus* accessions described in (Bus et al., 2011; Körber et al., 2012). The panel was grown either in full or in part at a number of different locations in Germany from 2009 until 2013, in southwest China from 2011 to 2013 and in central Chile from 2012 until 2013. In Germany, where winters generally have prolonged periods with temperatures well below freezing, accessions requiring vernalization and known to have moderate or good winter survival (“winter-type” rapeseed) were grown in autumn-sown trials (sowing in late August or early September, with harvest the following July). A large panel of swede genotypes, which require vernalization before flowering but generally have considerably lower winter survival, were also grown in the autumn-sown trials. Spring-type accessions with poor winter survival and no vernalization requirement were grown in Germany in spring-sown trials (sowing in March or April, harvest generally around September). In Temuco, central Chile, where the winter is mild but has a sufficient cold period for vernalization of *B. napus*, the winter-type and spring-type accessions were grown together in a spring-sown trial to differentiate photoperiod sensitive flowering after short, mild vernalization. The winter-type and spring-type accessions were also grown in Chongqing, southwestern China, where the winter is mild and day-length variation is considerably less extreme than in northern Europe.

Based on the results of these field studies, an initial screening panel comprising four *B. napus* ideotypes with considerable phenological variation in terms of vernalization requirement, winter survival, flowering time and photoperiod sensitivity, was selected for the sequence capture experiment. The four selected genotypes were: (1) the winter-hardy, vernalization requiring but late-flowering winter oilseed rape “25629-3,” (2) the winter-hardy, vernalization requiring but early-flowering fodder rape “Silona,” (3) the winter-sensitive, spring-type canola “Campino,” which requires no vernalization and exhibits day-length dependent flowering (all *B. napus* ssp. *napus*), and (4) the swede “Magres Pajberg” (*B. napus* ssp. *napobrassica*), which has a low winter survival but requires vernalization and flowers very late.

Homozygous inbred lines of the four accessions were generated by self-pollination to at least the S5 generation over many years. Leaf material for genomic DNA extraction was harvested from each accession in spring 2012 from field trials performed in Giessen, Germany. Mixed leaf samples were taken from at least 5

different plants, immediately shock-frozen in liquid nitrogen and kept at -20°C until extraction.

DNA ISOLATION

Leaf material was ground with a mortar and pestle under liquid nitrogen. DNA was extracted using a common CTAB protocol modified from Doyle and Doyle (1990). Fifteen milliliter of hot (65°C) extraction buffer (1.4 M NaCl, 50 mM Cetyltrimethylammoniumbromid (CTAB), 50 mM $\text{Na}_2\text{S}_2\text{O}_5$, 0.1 M Tris/HCl pH 8.0, 20 mM EDTA, 30 mM mercaptoethanol) were added to 2 g of frozen ground leaf material, vortexed and incubated for 30 min at 65°C in a water bath. 15 ml of chloroform-isoamylalcohol (24:1, v/v) were added and mixed for 5 min at room temperature by inverting the tube. The mixture was centrifuged (Beckmann Coulter Allegra X-30R, [S/N 13D 1125], 3400 rpm, 4°C , 10 min) and the supernatant was transferred to a second tube. 12 ml of chloroform-isoamylalcohol (24:1, v/v) were added and again mixed for 5 min. The sample was centrifuged as before and the supernatant was transferred to a third tube. For precipitation of the amino acids 1 ml each of 3 M NaOAc and 10 M NH_4OAc were added together with cold (4°C) isopropanol in a volume of 2/3 of the supernatant. DNA was then separated by centrifugation (Beckmann Coulter Allegra X-30R, [S/N 13D 1125], 3000 rpm, 4°C , 10 min) and the pellet was washed in 500 μl washing ethanol (70% (v/v) ethanol, 10 mM NH_4OAc). The washed pellet was dried and diluted in TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA). 10 μl RNase A (1 mg/ml) per 100 μl TE were added and incubated for 16 h at room temperature. 3 M NaOAc and 10 M NH_4OAc were added to a volume of 10 μl each per 100 μl TE, followed by 80 μl isopropanol per 100 μl TE. The resulting pellet was separated again by centrifugation (sigma 2K15 (12148), 8000 rpm, 4°C , 10 min) and washed in 500 μl washing ethanol. The washed pellet was dried and diluted in the same amount of TE. DNA concentration was determined using a Qubit fluorometer and the Qubit dsDNA BR assay kit (Life Technologies, Darmstadt, Germany) according to the manufacturer's protocol. DNA quantity and purity was further checked on 0.5% agarose gel (3V/cm, 0.5xTBE, 120 min).

SELECTION OF TARGET GENES

A set of 29 flowering time genes was selected based on literature from *A. thaliana* and the *Brassicaceae*. The genes were selected to cover the entire genetic network controlling flowering time, including circadian clock regulators (*CDF1*, *ELF3*, *GI*, and *ZTL*), the input pathways for vernalization (*ELF7*, *EFS*, *FLC*, *FRI*, *SVP*, *SUF4*, *TFL2*, *VRN2*, *VIN3*), photoperiod sensitivity (*CO*, *CRY2*, *PHYA*, *PHYB*) and gibberellin (GA₃ox1), along with downstream signal transducers (*AGL24*, *AP1*, *CAL*, *FD*, *FT*, *FUL*, *LFY*, *SPL3*, *SOC1*, *TEM1*, *TFL1*).

RETRIEVAL OF GENE SEQUENCES FOR BAIT DEVELOPMENT

Full-length *A. thaliana* genomic sequences from all of the target genes were retrieved from NCBI. Because no reference genome for *B. napus* was available at the time of the bait construction, orthologous copies of the genes in the *Brassica* A genome were identified in the reference sequence of *B. rapa* using "synteny search" and "non-synteny search" at the database *BRAD* (<http://brad.ac.uk/>) accessed in June 2012).

brassicadb.org/brad/ accessed in June 2012). For homologs in the C genome, both *A. thaliana* and *B. rapa* sequences were blasted against the *B. oleracea* sequence database *bolbase* (<http://www.ocri-genomics.org/bolbase/> accessed in June 2012). The BLAST settings were: database: *B.oleracea.v1.0.DNA*, blastn (Default settings). Every hit with an *E*-value of e^{-50} or lower was taken into account. Full genomic sequences for the identified *B. oleracea* genes were kindly provided by Professor Shengyi Liu, Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan, China.

Full genomic sequences for 6 *B. napus* copies of *FT* were provided by Carlos Molina, Christian Albrechts University, Kiel, Germany. One copy (*Bna.FTA02*) included the promoter sequence. Full genomic sequences for two copies of *Bna.CO* were retrieved from NCBI (GenBank accession numbers AF016011.1 and AF016010.1).

BAIT DEVELOPMENT

120mer oligonucleotide sequences were developed using the Agilent Genomic Workbench program eArrayXD (Agilent Inc., Santa Clara, CA, USA; https://earray.chem.agilent.com/earray/helpPages/index.htm#earrayxd_and_the_earray_web_site.htm). For *B. rapa*, the reference sequence file from *BRAD* was loaded as custom genome. Alongside the full *B. rapa* reference genome sequence (v 1.1), each of the retrieved gene sequences was loaded into eArrayXD as a pseudo-chromosome to generate a custom reference for bait generation from the target genes.

Bait groups were created in eArrayXD using the "Bait Tiling" tool. The parameters were set as follows: Sequencing Technology: "Illumina," Sequencing Protocol: "Paired-End long Read (75 bp+)," "Use Optimized Parameters (Bait length 120, Tiling Frequency 1x)," Avoid Overlap: "20," "User defined genome," "Avoid Standard Repeat Masked Regions." The strand was selected manually depending on the location of the respective gene. Baits for genes on the minus-strand were developed in sense, while baits on the plus-strand were developed in antisense.

In total, 64 bait groups were created for *B. rapa* copies of the target genes, 68 bait groups for *B. oleracea* copies and 8 bait groups for *B. napus* copies.

SEQUENCE CAPTURE AND SEQUENCING

Custom bait production was carried out by Agilent Technologies using the output oligonucleotide sequences from eArrayXD. Sequence capture was performed using the SureSelectXT 1 kb-499 kb Custom Kit (Agilent Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. The resulting TruSeq DNA library (Illumina Inc., San Diego, CA, USA) was sequenced on an Illumina HiSeq 2500 sequencer at the Max Planck Institute for Breeding Research (Cologne, Germany) in 100 bp single read mode.

DATA ANALYSIS

Quality control of the raw sequencing data was performed using FASTQC. Reads were mapped onto a pre-publication draft (version 4) of the *B. napus* "Darmor-Bzh" reference genome sequence assembly, which was kindly made available prior to public release by INRA, France, Unité de Recherche en Génomique Végétale

(Boulos Chaloub, INRA-URGV, Évry, France, unpublished data). Mapping was performed using the SOAPaligner algorithm (<http://soap.genomics.org.cn/soapaligner.html>) with Default settings and the option $r=0$ to achieve uniquely aligned reads. Removal of duplicates, sorting and indexing was carried out with *samtools* version 0.1.19 (<http://samtools.sourceforge.net/>). Alignments were visualized using the IGV browser version 2.3.12 (<http://www.broadinstitute.org/igv/>). Enriched regions and coverage differences were calculated using the *bedtools* software genomeCoverageBed (<http://bedtools.readthedocs.org/en/latest/>) with the option $-bg$. Calling of single nucleotide polymorphisms (SNPs) was performed with the algorithm mpileup in the *samtools* toolkit. Calling of insertions/deletions (InDels) was performed with SOAPIndel and results of InDel mapping were compared using Bowtie2 (2.1.0, <http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>). Predicted sequences of the target genes in the *B. napus* Darmor-Bzh genome were annotated with BLAST2GO and used for comparisons with enriched positions. The target was defined using BLAST positions of respectively annotated genes and the bait pool (E -value cut-off e^{-100}) on the mapping reference, and used for fraction calculation.

Read coverage for each captured region was normalized as follows: $\text{coveragenorm} = (\text{number of reads of equally covered region} * \text{total length of genome}) / (\text{number of aligned reads} * \text{read length})$. Copy number variation (CNV) in a given target region was assumed if the ratio of normalized coverage(genotype)/normalized coverage(all genotypes) was smaller than 0.5 or higher than 1.5, respectively. Presence/absence variation (PAV) was assumed if the ratio was smaller than 0.05.

Gene coding sequences and translated peptide sequences were determined using GENSCAN (<http://genes.mit.edu/GENSCAN.html>), with settings for "Arabidopsis." The translated sequences were aligned to available protein sequences for *B. napus*, *B. rapa*, *B. oleracea*, and *A. thaliana* using the software CLC SequenceViewer (CLC Genomics, Aarhus, Denmark). Analysis of promoter regions was also done with CLC SequenceViewer. Sequences were aligned with gap open cost = 10, gap extension cost = 1 and settings of "very accurate," first in subgroups aligning to the closest public sequence and then as a total to allow alignment in different regions. From this alignment, a neighbor joining tree was constructed with bootstrapping, using Default settings.

RESULTS

SEQUENCE CAPTURE

Using the *aligner* algorithm of SOAP2, 83–88% of all sequence reads could be aligned successfully for the four accessions. Table S1 lists alignment results for the four genotypes. As expected, the reads from the winter oilseed 25629-3, which is the most closely related of the four accessions to the reference genotype Darmor-Bzh, showed the highest alignment rates. The lowest alignment rates were seen in the swede Magres Pajberg, which represents the divergent subspecies *B. napus* ssp. *napobrassica*. The alignment success was independent of the total number of reads.

The number of aligned reads per library varied from around 3 million (Campino) to over 13 million (Magres Pajberg), allowing us to test the effect of different levels of target coverage on the detection of additional homoeologous loci, CNV and PAV.

The normalized mean coverage of the total targeted sequence regions ranged from 879 times (879x) to 985x, with a target size of 614 kbp. Between 72 and 76% of the target was sequenced with a minimum coverage of 10 reads (equivalent to 0.2–0.5% of the genome). Between 19 and 22% of the intended target sequence was not captured, indicating a capture sensitivity (the fraction of target covered) of 78–81%. The ratio of absolute mean coverage in the target to total mean coverage suggests an enrichment factor of more than 760x. The two genotypes with over 10 million reads showed only a slightly higher fraction of covered target sequence than those sequenced with 3–5 mio reads. The specificity (fraction of reads covering the target) was also found to vary only slightly, from 50 to 52% (Table 1).

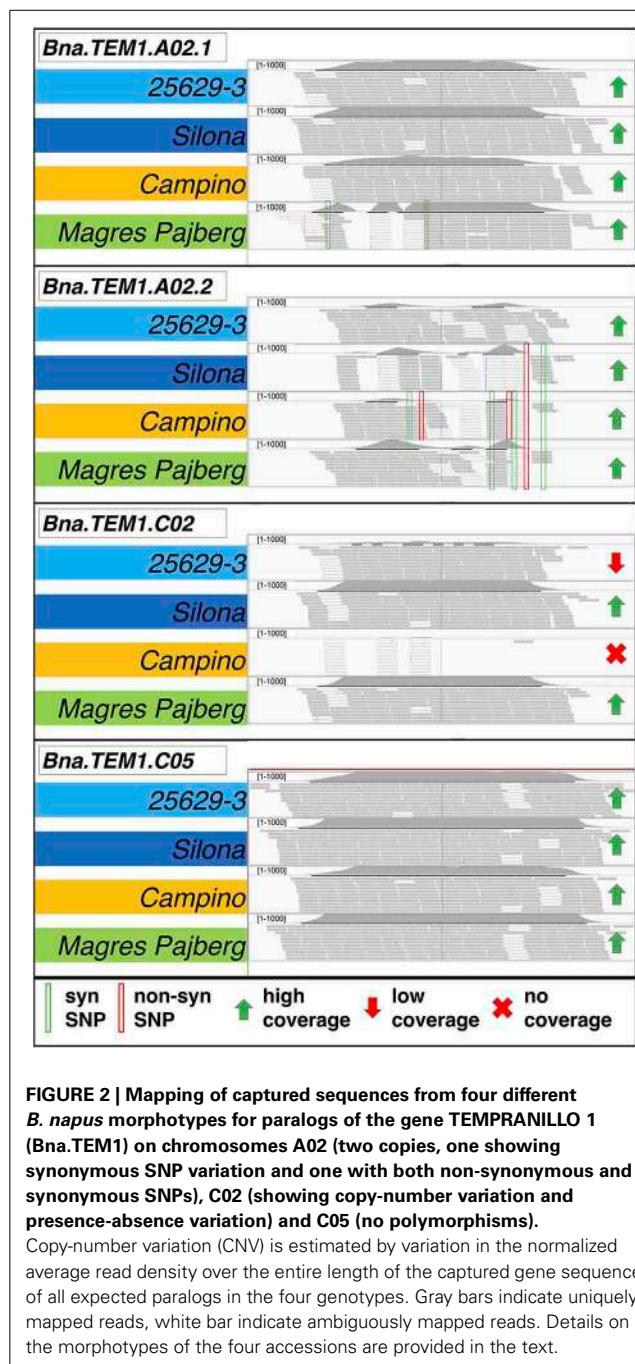
Figure 2 shows an example for read mapping, depth of coverage estimation and polymorphism detection in four *B. napus* homologs of the gene *TEMPRANILLO 1* (*Bna.TEM1*) on chromosomes A02 (two copies, one showing synonymous SNP variation and the other with both non-synonymous and synonymous SNPs), C02 (showing copy-number variation and presence-absence variation) and C05 (no polymorphisms). Despite the high sequence homology between homologs, use of the *B. napus* reference genome assembly enabled reads to be accurately mapped to their respective homologous locus, simplifying the detection of locus-specific sequence polymorphisms and allowing estimation of CNV from the average sequence coverage at each expected locus.

SNP CALLING

The results of the SNP calling are summarized in Table 2. After alignment with SOAP2, a total of 17,316 SNPs with a minimum read depth of 10 were called in the total dataset. The targeted region harbored 4269 SNPs, referred to here as target SNPs, resulting in average of 1 SNP per 144 nucleotides. Heterozygous hemi-SNPs representing multiple homologous loci made up 14–27% of the target SNPs, indicating mapping difficulties within duplicated or highly homologous gene regions. For subsequent analyses of potential functional mutations in the target sequences, only true homozygous SNPs in individual target gene loci were considered.

Table 1 | Coverage and genomic fractions of aligned reads in respect to target.

| Sequence coverage | 25629-3 | Silona | Campino | Magres Pajberg |
|------------------------------------------|---------|---------|---------|----------------|
| Mean genome-wide coverage | 0.47 | 1.35 | 0.38 | 1.48 |
| Mean target coverage | 362.19 | 1042.21 | 306.11 | 1150.26 |
| Enrichment factor | 767.20 | 773.41 | 802.27 | 779.80 |
| Normalized mean target coverage | 918.54 | 879.46 | 985.55 | 904.12 |
| Fraction of target covered (%) | 81.40 | 81.37 | 78.45 | 79.12 |
| Reads covering target (%) | 51.13 | 51.93 | 51.72 | 50.45 |
| Genome fraction covered by >10 reads (%) | 0.28 | 0.42 | 0.24 | 0.45 |
| Target fraction covered by >10 reads (%) | 75.90 | 76.61 | 71.90 | 73.30 |



DETECTED SEQUENCE VARIATION

Two or more copies of all targeted genes were recovered by the sequence capture, matching BLAST positions of all known homologs in the *B. napus* Darmor-bzh reference genome. In total we identified 160 individual homologs/paralogs for the 29 genes of the target panel. Of these, 23 sequences could not be translated *in silico* to proteins matching database records for *A. thaliana*, *B. rapa*, *B. oleracea*, or *B. napus*, and/or could not be uniquely mapped to a *B. oleracea* or *B. rapa* CDS database. Therein, we found 10 copies not translating to protein at all according to

Table 2 | High-quality SNPs called within the total enriched sequences (total SNPs) and the targeted gene sequences (target SNPs), respectively.

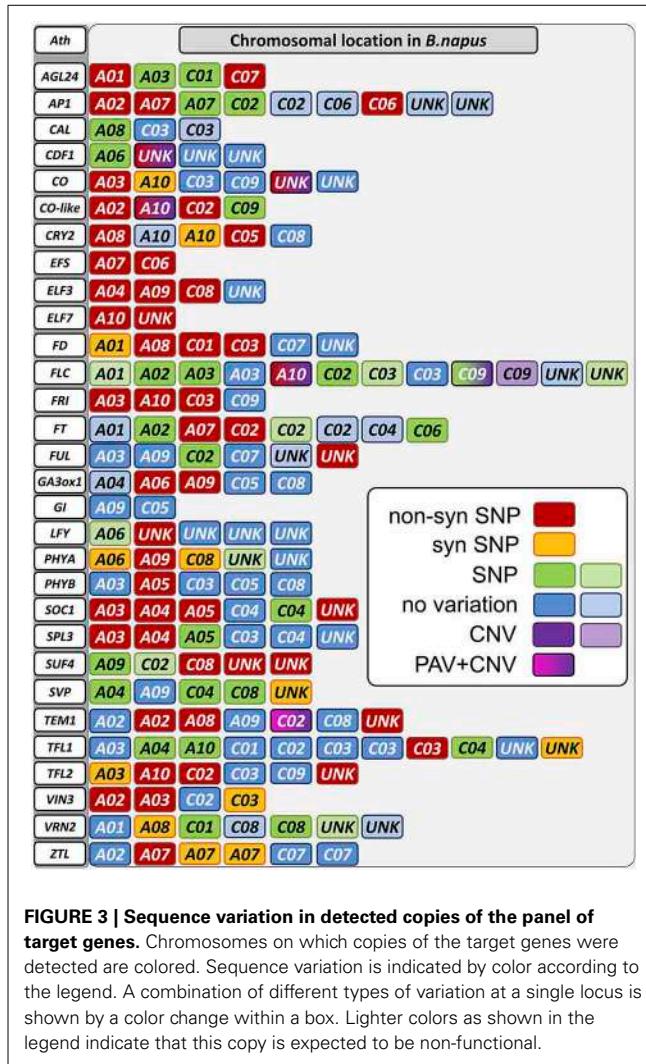
| Type of SNP | 25629-3 | Silona | Campino | Magres Pajberg |
|-------------------------------|---------|--------|---------|----------------|
| Total SNPs, homozygous | 2772 | 3974 | 4730 | 5849 |
| Total SNPs, heterozygous | 5259 | 4116 | 4283 | 4836 |
| Total SNPs, homozygous (%) | 16.01 | 22.95 | 27.32 | 33.78 |
| Total SNPs, heterozygous (%) | 30.37 | 23.77 | 24.73 | 27.93 |
| Target SNPs, homozygous | 546 | 990 | 1351 | 1538 |
| Target SNPs, heterozygous | 1145 | 599 | 813 | 771 |
| Target SNPs, homozygous (%) | 12.79 | 23.19 | 31.65 | 36.03 |
| Target SNPs, heterozygous (%) | 26.82 | 14.03 | 19.04 | 18.06 |

A SNP was called when one of the four test genotypes carried an alternative nucleotide to the reference genotype Darmor-bzh in all mapped reads covering a given target nucleotide position, with a minimum of 10 reads.

our prediction with GENSCAN, therefore they might be non-functional paralogs. Another 12 copies translated to fragmented or meaningless peptide *in silico*, having no (5 copies) or no unique hit to the respective CDS databases (7 copies). One copy was predicted to translate to meaningless peptide, but had a respective hit in the *B. rapa* CDS database. These copies were also considered non-functional. A further four copies had high homology but were partially missing in the reference genome assembly. 120 copies were captured over their full coding length, while 13 copies only translated to parts of the expected protein. Figure 6 and Figure S1 show the relative positions of all homologs between *A. thaliana*, *B. rapa*, *B. oleracea*, and *B. napus*. Considering the expected copy number based on the *B. rapa* and *B. oleracea* genomes, a total of 9 copies were lost, whereas 28 (including the 23 non-functional copies) were duplicated. This suggests that only 5 of the new gene duplications were functional, but also that relatively few duplicated paralogs of flowering time regulatory genes have been lost in *B. napus* after polyploidization. Considering all functional copies, this gives a ratio of 1.9:1 comparing the tetraploid with the diploid genomes, representing a 3% change to the expected 2:1 ratio (Figure 6, Figure S1).

Comparisons with gene expression data from the semi-winter *B. napus* variety “Ningyou 7” for different time points and treatments suggest that all of the loci we captured and considered functional are expressed in *B. napus* [Carlos Molina, Christian Albrechts University, Kiel, Germany, unpublished data]. Because of its homology to CO, the oligonucleotide baits also captured four *B. napus* homologs of the gene *CO-like 2*, although this gene was not included in the target panel. The four captured *Bna.CO-like 2* homologs were therefore included in the further analysis of variation.

DNA sequence variation was detected in 104 of the captured gene sequences. As expected, SNP variation was most predominant, with high-confidence SNPs being observed in 102 of the 104 variable genes. High-confidence CNV was observed at 7 gene loci, with one locus showing PAV. InDels were not detected by SOAPindel within our target regions, therefore no frameshifts are expected in this dataset. A comparative mapping with the



software Bowtie (using default settings) showed InDels only in regions of very low mapping quality, so we considered them to be mapping errors.

A total of 313 SNPs were located in exons of the captured genes. Out of these, 188 were synonymous, whereas 125 changed the amino acid sequence in one or more genotypes (Figures 3, 4). An amino acid change in at least one of the genotypes was predicted by 54 of the 141 functional target gene copies. The winter oilseed rape genotype 25629-3, belonging to the same eco-geographical flowering morphotype as the winter rapeseed Darmor-Bzh, differed from the Darmor-Bzh reference genome in only 10 gene copies with non-synonymous mutations. In contrast, the early-flowering fodder rapeseed Silona showed 21 gene loci with non-synonymous SNPs, while 31 loci with non-synonymous SNPs were detected in the cold-sensitive, day-length dependent spring rapeseed Campino and 35 loci in the swede Magres Pajberg, both of which have low winter-hardiness and flower under longer-day conditions.

A total of 54 paralogs of the target genes showed one or more non-synonymous mutations in the coding sequences

of the four sequenced genotypes compared to the winter oilseed rape reference genome sequence. Only four genes (*Bna.CAL*, *Bna.GI*, *Bna.SVP*, and *Bna.VRN2*) showed no nucleotide polymorphisms affecting the amino acid composition of the gene product of any paralog. In all other genes, including gene copies assigned to vernalization (*Bna.EFS*, *Bna.ELF7*, *Bna.FLC*, *Bna.FRI*, *Bna.SUF4*, *Bna.TFL2*, *Bna.VIN3*), photoperiod (*Bna.CO*, *Bna.CO-like*, *Bna.CRY2* and *Bna.PHYA*), gibberellin (*Bna.GA3ox1*), clock (*Bna.CDF1*, *Bna.ELF3*, *Bna.ZTL*) and signaling (*Bna.AGL24*, *Bna.API*, *Bna.FD*, *Bna.FT*, *Bna.FUL*, *Bna.LFY*, *Bna.SPL3*, *Bna.SOC1*, *Bna.TEM1*, and *Bna.TFL1*), we found potentially functional amino acid modifications in the gene products of at least one homolog/paralog within the four different *B. napus* morphotypes (Figure 4).

As expected, the degree of potentially functional sequence diversity in comparison to the *B. napus* reference genome sequence varied among the four sequenced genotypes in correspondence to their ecophysiological diversification from the winter oilseed rape reference genotype Darmor-Bzh. The winter oilseed rape 25629-3 and the winter-hardy fodder rape Silona showed the lowest degree of non-synonymous SNPs in comparison to Darmor-Bzh, while the spring-type canola genotype Campino and the swede Magres Pajberg showed considerable diversity in comparison to Darmor-Bzh. Campino, which flowers under long-day conditions, showed particularly high rates of non-synonymous mutations in photoperiod module genes, whereas Magres Pajberg was the most divergent from Darmor-Bzh in relation to vernalization, clock and signaling genes.

CNV AND PAV

In the winter rapeseed genotype 25629-3 we observed reductions in copy number for a copy of *Bna.CO* on chromosome C09 and a copy of *Bna.TEM1* on chromosome C02, respectively. One homolog of *Bna.CDF1*, which was unable to be assigned to a chromosome in the Darmor-Bzh reference genome, was reduced in copy number in the winter fodder rape Silona. On the other hand, the spring canola Campino was found to have a copy number increase in *Bna.CO-like* on chromosome A10, whereas no reads were captured corresponding to *Bna.TEM1* from chromosome C02; we therefore assume that this gene is deleted in Campino. The target coverage for a duplicated *Bna.FLC* locus on chromosome C09 indicated that this locus has been replaced in the swede Magres Pajberg by its homolog from a highly homoeologous chromosome segment on chromosome A10. Homoeologous non-reciprocal translocations are common in the allopolyploid *B. napus* genome (Samans, 2014). Figure 5 shows normalized coverage for the affected copies in each genotype, together with their flowering time. To avoid counting of homoeologous loci (Figure 6, Figure S1) in the CNV estimation, only gene loci for which no heterozygous SNPs were detected were included in the analysis. Figure 7 shows which of these copies carry the respective variation type. *Bna.GI* did not show variation in any of its copies, whereas other genes, (e.g. *Bna.FLC*), exhibited considerable sequence variation at most of their loci.

| Gene | AGL24 | API | CDF1 | CO | CO-like | CRY2 | EFS | ELF3 | ELF7 | FD | FLC | FRI | | | | | | | | | | | | | | | | | |
|----------------|-------|-----|------|-----|---------|------|-----|------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|---|
| copy on | A01 | C07 | A02 | A07 | C06 | unk | A03 | C09 | A02 | A10 | C02 | A08 | C05 | A07 | C06 | A04 | A09 | C08 | A10 | unk | A08 | C01 | C03 | A10 | A03 | A10 | C03 | | |
| 25629-3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Silona | 1 | | | | 1 | 2 | | | | | | | | | | | | | | | | | | | | | 1 | | |
| Campino | | | 1 | 1 | | | 3 | | | | | 1 | 1 | 2 | 2 | 7 | | 2 | 3 | 7 | | 1 | | | | 1 | 2 | 1 | |
| Magres Pajberg | | 1 | 1 | | | | 1 | 1 | | | 1 | | | | | 9 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

| Gene | FT | FUL | GA3ox1 | LFY | PHYA | PHYB | SOC1 | SPL3 | SUF4 | TEM1 | TFL1 | TFL2 | VIN3 | ZTL | | | | | | | | | | | | | | |
|----------------|-----|-----|--------|-----|------|------|------|------|------|------|------|------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|
| copy on | A07 | C02 | unk | A06 | A09 | unk | A09 | A05 | A03 | A04 | A05 | unk | A03 | A04 | C08 | unk | unk | A02 | A08 | unk | C03 | A10 | C02 | unk | A02 | A03 | A07 | |
| 25629-3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | 3 |
| Silona | | | 1 | | | 1 | | | 1 | 1 | 1 | 1 | 4 | | 1 | 1 | 1 | 1 | 2 | | | | | | 1 | 1 | 2 | |
| Campino | 1 | 1 | 3 | 2 | | | 2 | 2 | 1 | | | | | | 1 | 1 | 3 | 3 | 2 | | 1 | 2 | 1 | 1 | 1 | 3 | | |
| Magres Pajberg | 1 | 1 | 2 | | 1 | 1 | 1 | | | | | | | | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 4 | 9 | 3 | |

FIGURE 4 | Distribution of non-synonymous SNPs (gray) in copies of target genes from four diverse *Brassica napus* accessions with different morphophysiological flowering attributes (see text for details). Unk, unknown chromosome position.

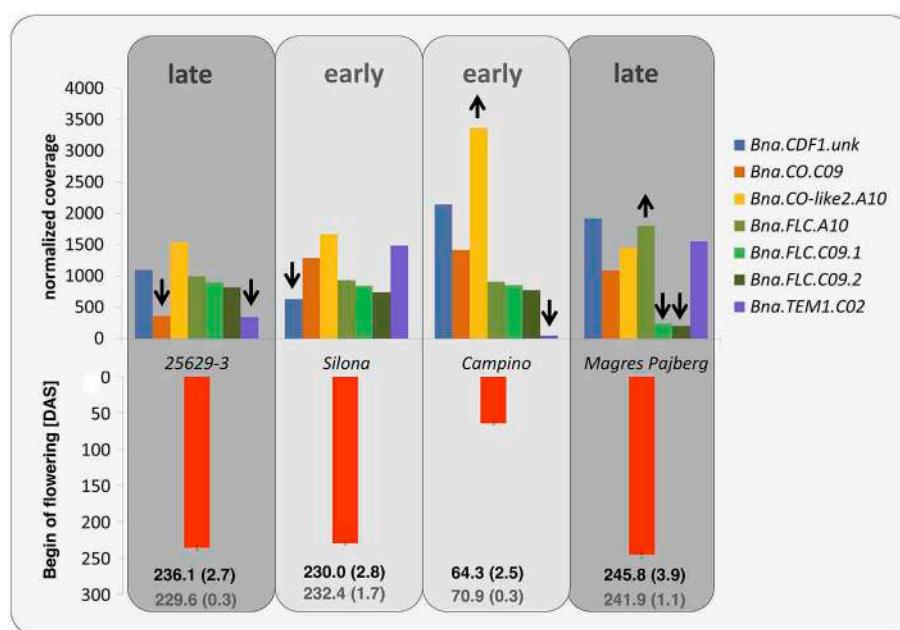


FIGURE 5 | Normalized coverage for gene copies with copy-number variation or presence-absence variation. A reduction in copy number compared to the Darmor-Bzh reference genome is indicated by the downwards arrows, an increased copy number by the upwards arrows. The red bars indicate flowering time in days after sowing (DAS), averaged over 3 years and 3 locations in Germany (with standard errors). In the field trials in Germany 25629-3, Silona and Magres Pajberg were tested in autumn-sown trials, whereas the winter-sensitive Campino was grown in a spring-sown trial. The black numbers indicate the genotype mean (with standard error), while the gray numbers indicate the population mean (with standard error).

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FT PROMOTER REGION

The bait library contained a full-length genomic sequence for *FT* on chromosome A02, including the promoter region. This successfully enriched for the targeted copy including promoter, but also for the promoter regions of *FT* copies on A01, A07, C02, and C06. Sequence alignments with previously known *B. napus* *FT* promoter sequences (Accession numbers JX193765.1, JX193766.1, and JX193767.1) revealed that the promoter of the newly detected *FT* locus on chromosome A07 is closely related to that of the locus on chromosome C06, whereas the other two newly detected *FT* promoters on chromosomes A01 and C02 diverge from all previously known *FT* promoter sequences (Figure 8). Interestingly, all detected *FT* promoter regions contained considerable SNP variation, with a total of 7 SNPs detected in the promoter region of *Bna.FT.A01*, 9 in *Bna.FT.A02*, 4 in

Bna.FT.A07, 14 each in *Bna.FTC02_1* and *Bna.FTC02_2* and 21 in *Bna.FTC06*. The promoter regions for two further *FT* copies on C02 and C04 were not detected. Since both are considered non-functional, this strengthens the hypothesis that both of these paralogs are pseudogenes.

DISCUSSION

Polyploidization was a major driver of crop evolution and many important crop plants are polyploids (e.g., wheat, cotton, sugar-cane, potato, rapeseed). This is a major restriction for knowledge transfer from well-studied model plants to crops. The high number of gene copies complicates model development for important traits, in particular for regulation of complex traits like timing of reproduction. The first step of elucidating the interplay between different gene copies of a regulation module is an assessment of

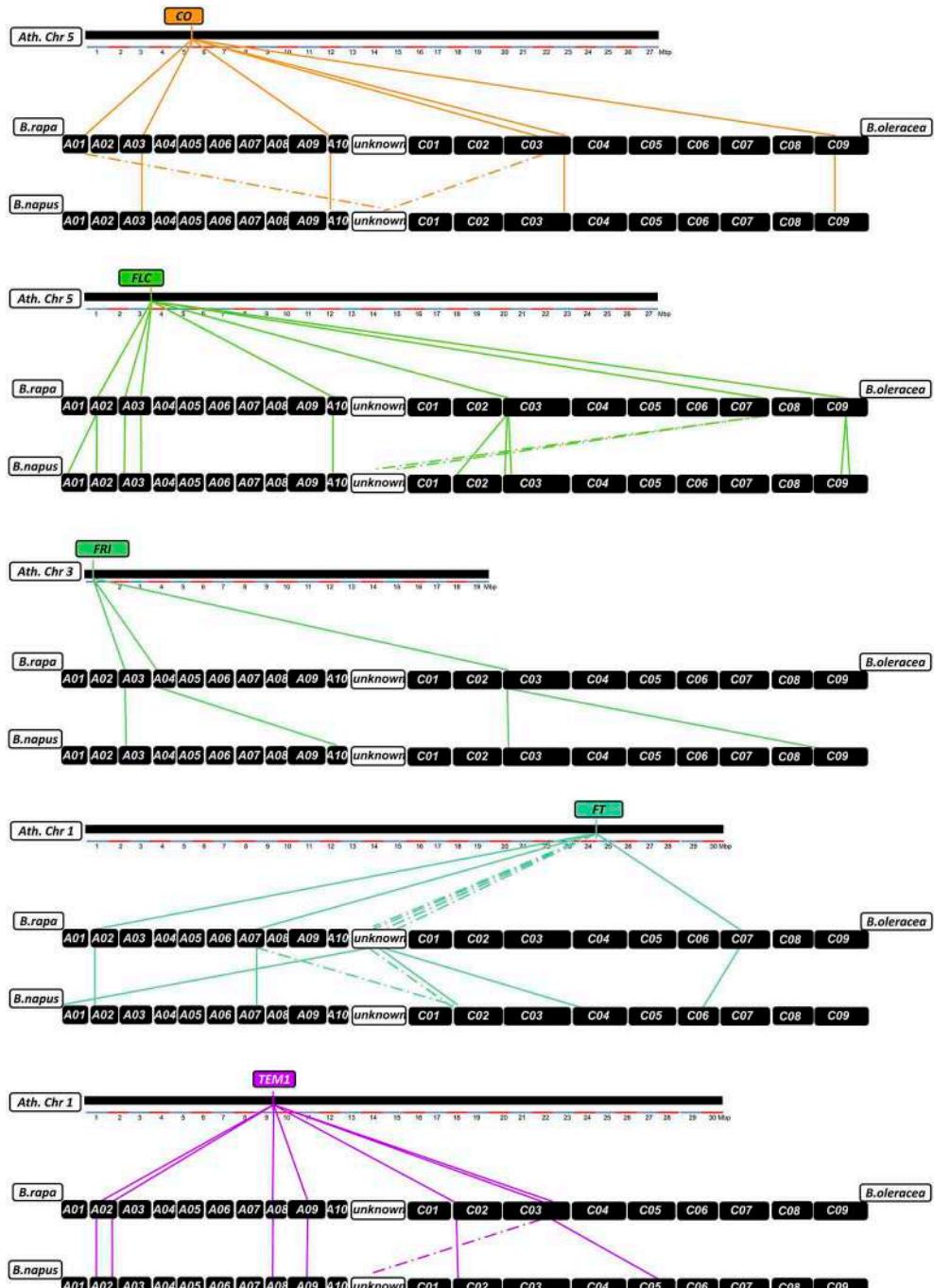


FIGURE 6 | Relationships between homologs from *Arabidopsis thaliana*, *Brassica rapa*, *Brassica oleracea*, and *Brassica napus* for the flowering time regulatory genes **CO, **FLC**, **FRI**, **FT**, and **TEM1**.** Chromosomes are shown as

black boxes. Colored lines connect relative chromosomal positions between *A. thaliana* and *B. rapa/B. oleracea* and between *B. rapa/B. oleracea* and *B. napus*. Dotted lines indicate positions that could not be verified by BLAST.

their number and sequence variation. The recent allopolyploid crop *B. napus* is an excellent model to study the influence of gene copy number and sequence variation on trait expression for two reasons: (1) the interspecific hybridization arose only a few thousand years ago and the ancestor genomes are still basically intact (Parkin et al., 1995), and (2) the close relationship to the

model plant *A. thaliana* and the recently sequenced *B. rapa* allows for comparisons between gene models and crop sequences. Gene cloning strategies and array technologies depend on very specific sequence information, whereas whole-genome sequencing cannot always provide the appropriate coverage for assessment of copy-number variation. Therefore we chose an in-solution

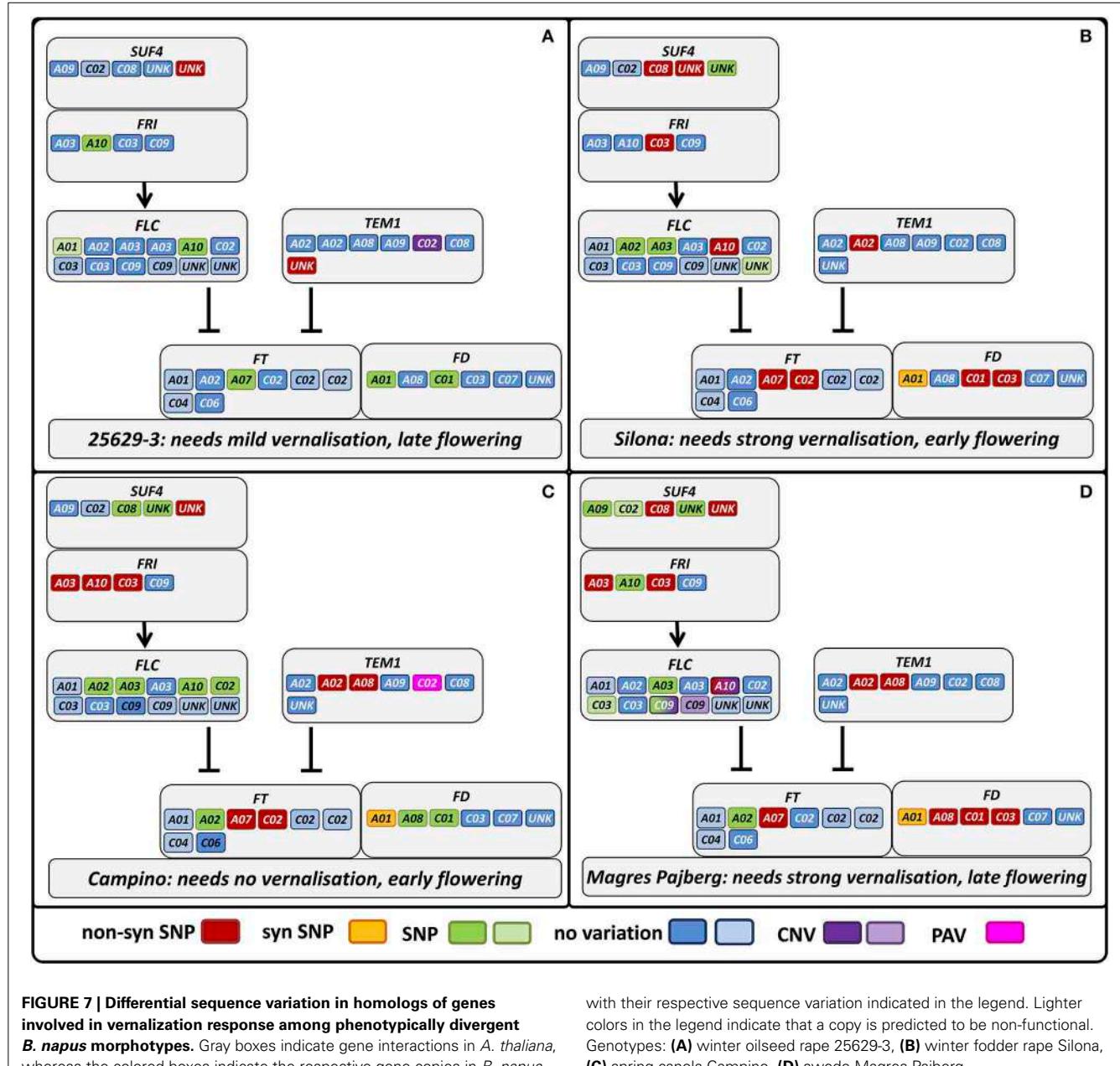


FIGURE 7 | Differential sequence variation in homologs of genes involved in vernalization response among phenotypically divergent *B. napus* morphotypes. Gray boxes indicate gene interactions in *A. thaliana*, whereas the colored boxes indicate the respective gene copies in *B. napus*

B. napus morphotypes. Gray boxes indicate gene interactions in *A. thaliana*, whereas the colored boxes indicate the respective gene copies in *B. napus*

hybridization sequence capture approach, aiming to detect all present copies of the targeted flowering time regulatory genes and mine for their variation in number and sequence.

We developed a RNA oligonucleotide pool designed to capture a core set of 29 flowering time genes in *B. napus*. This enabled us to enrich all 164 copies expected from the draft *B. napus* reference genome, proving the value of this technique to capture sequence variants across complex regulatory modules like the flowering time gene network.

All in all, 124 copies of the target genes were deep-sequenced over their full coding length; a further 17 copies were partially captured. This suggests an average copy number of 4.7, which is close to the average number of 4.4 copies per gene

with their respective sequence variation indicated in the legend. Lighter colors in the legend indicate that a copy is predicted to be non-functional. Genotypes: **(A)** winter oilseed rape 25629-3, **(B)** winter fodder rape Silona, **(C)** spring canola Campino, **(D)** swede Magres Pajberg.

expected over the entire *B. napus* genome (Parkin et al., 2010). Independent expression studies suggested that all of these captured flowering time gene copies are functional and expressed in *B. napus*. This represents a huge expansion of active flowering time regulatory genes in the allopolyploid *B. napus* genome in comparison to *A. thaliana*, where most of these genes are represented by only a single active copy. This expansion creates enormous potential for functional differentiation and regulatory plasticity across all pathways influenced the flowering time gene expression network. The selective potential inferred by this polyploidization-induced expansion in flowering-time genes can be speculated to have had a major impact on the natural and artificial selection of different ecophysiological morphotypes of

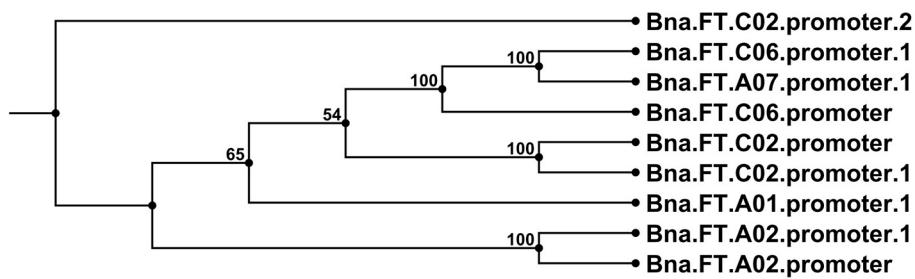


FIGURE 8 | Neighbor joining tree diagram of promoter sequences from all detected copies of the flowering gene *FT* in comparison with promoters from previously known *Bna.FT* loci retrieved from NCBI. Sequences

detected in the present study are labeled with the suffix 1 or 2, where one or two copies, respectively, were detected on the corresponding chromosome. Setting details can be found in the Materials and Methods Section.

B. napus, leading to their differential adaptation into the various cultivated forms.

Compared to whole genome sequencing data, alignment rates from our sequence capture data were high; only a low percentage of around 13% of the reads aligned non-uniquely. Enrichment was successful with an enrichment factor of more than 760x, indicating that baits developed from closely related species are able to efficiently enrich selected regions in *B. napus*; similar efficiency of sequence capture across close relatives was also shown in other species (Vallender, 2011; Bancock et al., 2012; Jupe et al., 2013; Mascher et al., 2013). Sensitivity and specificity were comparably low, with values of 78–81% and 50 to 52%, respectively (Mamanova et al., 2010). Lower values for sensitivity in case of multiplexed samples are reported (Mascher et al., 2013), so the lowered sensitivity may be attributed to multiplexing. Specificity in this case suffers from the artificial definition of the target (see Methods).

Comparing the detected copies to their ancestor genomes, we found only a 3% change in the expected ratio (considering only functional copies) of *B. napus* gene copies compared to the average copy number in the progenitor genomes. This is only a minor change in gene number compared to other polyploids. In wheat, the ratio of the hexaploid gene content compared to one of the diploid progenitors was 2.5–2.7:1, representing a 10–16% change (Brenchley et al., 2012). This illustrates that *B. napus* speciation is only a very recent event (Allender and King, 2010). The retention of functional copies provides more possibilities to introduce variation. More gene variants allow successful selection and adaptation in a wider range of environments, as non-functional copies can be replaced by functional homologs without loss of function. Moreover, environmental-specific beneficial alleles can exist at the same time, allowing for accumulation of a high adaptation potential. In *B. napus* the flexibility achieved by copy number expansion of flowering time regulatory genes is the basis for the great variation among different morphotypes in flowering time as well as in cold adaptation, winter hardiness and vernalization requirement.

We also report 14% non-functional copies, whereas some genes are more affected by non-functional copies than others. For example, we found 5 copies of *FLC* either fragmented or not expressed or both. *FLC* pseudogenes were reported for *Brassica oleracea* (*BoFLC4* and *BoFLC5*) (Razi et al., 2008) and a number of seven functional *BnFLC* copies was already estimated by others (Schranz et al., 2002; Pires et al., 2004).

Another important base for adaptation is copy number variation (CNV) (Żmieleńko et al., 2013). We observed CNV for the genes *Bna.CDF1.unk*, *Bna.CO.C09*, *Bna.CO-like2.A10*, *Bna.FLC.A10*, *Bna.FLC.C09*, and *Bna.TEM1.C02*, and presence-absence variation for *Bna.TEM1.C02*. This variation may have a strong influence on determination of flowering time in the respective morphotypes. For example, the winter oilseed rape 25629-3 is late flowering, winter hardy and needs mild vernalization. We would therefore expect a lower copy number of floral enhancers or a higher copy number of flowering repressors. Indeed, 25629-3 showed a copy-number reduction for *Bna.CO.C09*. The coverage differences in *Bna.TEM1.C02* further suggest that 25629-3 only possesses one copy of this locus, in contrast to the early flowering fodder rape Silona and the winter-sensitive Magres Pajberg.

CO is a central day length regulator necessary for flowering transition. Accumulation of the *CO* protein is crucial for flowering initiation (reviewed in Jaeger, 2008; Jung and Müller, 2009; Andrés and Coupland, 2012). In the case of 25629-3, the lower number of *Bna.CO* copies may relate to its late flowering behavior, as a lower number of transcripts can be synthesized at the same time.

TEM1 is known to bind to the 5'UTR region of *FT*, therefore repressing flowering (Castillejo and Pelaz, 2008). *TEM1* itself seems to be regulated by *FLC* (Deng et al., 2011) and *AP1* (Kaufmann et al., 2010). It has been assumed that the ratio of *CO/TEM* is decisive for *FT* expression in *A. thaliana* (Castillejo and Pelaz, 2008). In the present study, *Bna.TEM1.C02* was found to be absent in Campino, whereas two copies were observed in Silona and Magres Pajberg compared to the single copy found in Darmor-bzh and 25692-3, respectively (see also Figure 7). Considering that all paralogous loci are presumed to be expressed, this corresponds to a *Bna.CO/Bna.TEM1* ratio of 5:6 in 25629-3, 6:7 in Silona and Magres Pajberg, and 6:6 in Campino. Assuming gene dosage effects, these differences might be expected to accordingly influence the vernalization requirement via differential repression of *FT* expression. Correspondingly, Campino is a spring type without need for vernalization, making photoperiod signaling more important than pre-winter repression of

flowering. 25692-3 and Darmor-bzh are both winter types with mild vernalization requirement, presumably facilitated by the single copies of *Bna.CO.C09* and *Bna.TEM1.C02*, respectively. On the other hand, *Bna.TEM1.C02* seems to be duplicated in lines with stronger vernalization requirement (**Figure 7**). Therefore, the cold signal needs to be stronger to overcome the stronger repression.

In 25629-3, we further detected non-synonymous mutations in copies of *Bna.AP1*, *Bna.CO-like 2*, *Bna.ELF3*, *Bna.FLC*, *Bna.PHYB*, *Bna.SPL3*, *Bna.SUF4*, *Bna.TEM1*, *Bna.TFL2*, and *Bna.ZTL*. At present it is still unknown whether these mutations are beneficial or disadvantageous, however it is interesting to note that the non-synonymous mutations mainly affect genes involved in temperature perception (*Bna.ELF3*, *Bna.SPL3*) and vernalization modulation (*Bna.FLC*, *Bna.SUF4*, *Bna.TEM1*, *Bna.TFL2*). This could reflect differences in winter/spring perception among the different eco-physiological morphotypes; if so, the broad range of affected genes provides considerable potential for natural selection of adaptation traits to different environments, a potential advantage of paralog diversification following allopolyploidization.

Silona is a winter fodder rape with strong vernalization requirement. It flowers slightly later compared to winter oilseed types, but relatively early compared to other winter fodder or kale morphotypes. We found a copy number reduction in a *Bna.CDF1* paralog, on an unmapped scaffold (referred to here as *Bna.CDF1.unk*), which may relate to the early-flowering behavior of Silona. It has been shown that *CDF1* directly downregulates CO mRNA levels in Arabidopsis (Srikanth and Schmid, 2011), acting as link between the circadian clock and the photoperiod pathway (Niwa et al., 2007), and therefore can be regarded as flowering repressor. Reduction in *Bna.CDF1* transcript abundance due to a copy-number reduction could thus be expected to reduce floral repression and hence accelerate post-vernalization induction of flowering.

Silona was also found to carry non-synonymous mutations in one copy each of *Bna.AGL24*, *Bna.AP1*, *Bna.CDF1*, *Bna.CO-like 2*, *Bna.EFS*, *Bna.FLC*, *Bna.FRI*, *Bna.FUL*, *Bna.LFY*, *Bna.PHYB*, *Bna.SPL3*, *Bna.TEM1*, *Bna.TFL2*, *BnZTL*, in two copies of *Bna.FD*, *Bna.SUF4* and in four copies of *Bna.SOC1*. Mutations in *Bna.TEM1*, *Bna.EFS*, *Bna.FLC*, *Bna.FRI* and *Bna.TFL2* may relate to its stronger vernalization requirement than the winter oilseed Darmor-Bzh (as discussed before for the reduced *Bna.TEM1.C02* copy number; **Figure 7**). On the other hand, there also appears to be a stronger variation in downstream effectors, particularly in *Bna.SOC1*. *SOC1* is a signal integrator for the vernalization, photoperiod and GA signaling pathways and a direct regulator of LFY (Lee and Lee, 2010). As such *SOC1* can therefore be regarded as a floral activator. The results seen here support the assumption that the flowering time shift between earlier-flowering winter oilseed forms and later flowering, leafier winter fodder rape is more likely to be due to mutations in the effector pathways, with only slight modifications to be expected in the input pathways.

Campino is a vernalization-independent, early-flowering spring oilseed rape. We therefore would expect large differences in vernalization genes. As the vegetation period is shifted by 6–8 weeks in spring types compared to winter types, it is

necessary for the plants to adapt to warmer and longer days, so we also expect differences in photoperiod and temperature signaling pathway genes. The change from winter to spring behavior in *A. thaliana* is known to be caused by a mutation in either *FRI* or *FLC* or both (Choi et al., 2011). *FRI* is the main activator for *FLC*, while *FLC* is the major flowering repressor before vernalization (Choi et al., 2011). *Bna.FRI* has already been found to play a central role for variation in morphotype, not only for vernalization (Wang et al., 2011a). Correspondingly, we found 3 *Bna.FRI* paralogs carrying mutations in Campino in comparison to the winter rapeseed Darmor-Bzh, whereas only two *Bna.FRI* paralogs differed in Magres Pajberg and one in Silona (**Figure 7**). Campino is also the only genotype which does not show a mutation in the *Bna.FLC.A10*. This means that 25692-3, Silona and Magres Pajberg share an allele different from Darmor-Bzh and Campino in *Bn.FLC.A10*. It may be concluded that the Darmor-Bzh/Campino allele is less functional than the other, or, more generally, that the *Bna.FLC.A10* copy is not decisive for flowering time determination. As *Bna.FLC.A10* was found to be associated with vernalization behavior, this might be ruled out (Hou et al., 2012). As discussed before, presence-absence variation of *Bn.TEM1.C02* may also play a role in the change to the annual morphotype. As expected, these differences are also accompanied not only by further mutations in vernalization-related genes (*Bna.VIN3*, *Bna.TEM1*, *Bna.EFS*, *Bna.ELF7*, *Bna.SUF4*, *Bna.TEM1*, *Bna.TFL2*, *Bna.VIN3*), but also by numerous mutated sequences in genes from the photoperiod (*Bna.CO*, *Bna.CO-like 2*, *Bna.CRY2*, *Bna.PHYA*, *Bna.PHYB*, *Bna.ZTL*) and temperature signaling pathways (*Bna.SPL3*, three copies of *Bna.ELF3*) along with two copies of *Bna.GA3ox1*.

Magres Pajberg is a swede type belonging to the subspecies *napobrassica*. As such this genotype is typically strongly vernalization-dependent and flowers later than winter-type oilseed forms. Compared to the winter line Darmor-bzh, we detected wide sequence variation in Magres Pajberg affecting all pathways under study. Vernalization genes were particularly affected, along with flowering activators (*Bna.VIN3*, *Bna.TFL2*) and repressors (*Bna.EFS*, *Bna.ELF7*, *Bna.FRI*, *Bna.FLC*, *Bna.SUF4*, *Bna.TEM1*). We found further mutations in gene copies from the photoperiod pathway (*Bna.CO*, *Bna.CO-like 2*, *Bna.PHYA*, *Bna.PHYB*), gibberellin synthesis (*Bna.GA3ox1*), temperature signaling (*Bna.SPL3*, *Bna.ELF3*), the central signaling molecules (*Bna.FT*, *Bna.FD*) and downstream effectors (*Bna.AGL24*, *Bna.AP1*, *Bna.FUL*, *Bna.SOC1*, *Bna.TFL1*). We further observed a copy number reduction affecting two *Bna.FLC* paralogs on chromosome C09, which is mirrored by a corresponding copy number increase on A10. This suggests that one of the copies on C09 may have been replaced by a duplication of a locus originating from A10, a widespread effect of polyploidization in *B. napus* caused by homoeologous recombination (Gaeta et al., 2007). A comparison with genome-wide sequence data from different *B. napus* lines showed that these chromosome regions are indeed subject to homoeologous chromosome exchanges in resynthesized *B. napus* (Samans, unpublished data). This example underscores the potential of homeologous chromosome exchanges to generate functionally relevant copy-number variation among important adaptation

genes, illustrating the genomic plasticity of polyploid plants and the genetic potential they harbor for both drastic and more subtle modifications in flowering time and related adaptive phenotypes.

CONCLUSIONS

Different *B. napus* morphotypes show considerable sequence and copy number variation in paralogs of central flowering-time regulatory genes. We demonstrated that most flowering time gene copies arising from the ancestor genomes were retained after allopolyploidization, and many of the retained paralogs are still expressed. The consequence is a huge expansion in the number of flowering-related genes in *B. napus* compared to the related model plant, *A. thaliana*, and a correspondingly large increase in the complexity of the gene networks controlling flowering. Duplications during the recent polyploidization of *B. napus* also provide considerable scope for mutations leading to non-functional paralogs or also neofunctionalization. We demonstrate that sequence capture is a highly efficient method to analyse sequence variation for flowering time and other important pathways in polyploid crop species. Applying this technology to genetic mapping populations and breeding materials will allow us to link sequence variation in flowering time regulatory genes to phenotypic variation for flowering and other important agronomic traits.

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SUPPLEMENTARY MATERIAL

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Cloning of quantitative trait genes from rice reveals conservation and divergence of photoperiod flowering pathways in *Arabidopsis* and rice

Kazuki Matsubara¹, Kiyosumi Hori², Eri Ogiso-Tanaka² and Masahiro Yano^{1,2*}

¹ NARO Institute of Crop Science, Tsukuba, Japan

² Agrogenomics Research Center, National Institute of Agrobiological Sciences, Tsukuba, Japan

Edited by:

Christian Jung, Christian Albrechts University of Kiel, Germany

Reviewed by:

Peter Langridge, Australian Centre for Plant Functional Genomics, Australia

Christian Jung, Christian Albrechts University of Kiel, Germany

Rita Sharma, Jawaharlal Nehru University, India

***Correspondence:**

Masahiro Yano, Agrogenomics Research Center, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan
e-mail: myano@niaas.affrc.go.jp

†Present address:

Masahiro Yano, NARO Institute of Crop Science, Tsukuba, Japan

Flowering time in rice (*Oryza sativa* L.) is determined primarily by daylength (photoperiod), and natural variation in flowering time is due to quantitative trait loci involved in photoperiodic flowering. To date, genetic analysis of natural variants in rice flowering time has resulted in the positional cloning of at least 12 quantitative trait genes (QTGs), including our recently cloned QTGs, *Hd17*, and *Hd16*. The QTGs have been assigned to specific photoperiodic flowering pathways. Among them, 9 have homologs in the *Arabidopsis* genome, whereas it was evident that there are differences in the pathways between rice and *Arabidopsis*, such that the rice *Ghd7-Ehd1-Hd3a/RFT1* pathway modulated by *Hd16* is not present in *Arabidopsis*. In this review, we describe QTGs underlying natural variation in rice flowering time. Additionally, we discuss the implications of the variation in adaptive divergence and its importance in rice breeding.

Keywords: breeding, natural variation, photoperiodic flowering, quantitative trait gene, rice

INTRODUCTION

Photoperiodic flowering is one of the most important responses of plants to their environment (Thomas and Vince-Prue, 1997). In the last two decades, molecular genetics demonstrated that external light signals perceived by photoreceptors induce florigens (flowering signals). This process is regulated in part by the circadian clock, and promotes flowering in response to favorable daylength in both monocots and eudicots (reviewed by Andrés and Coupland, 2012).

Rice is a short-day (SD) plant, i.e., flowering is accelerated under SD conditions. Natural variation in rice flowering time is generated mainly by quantitative trait genes (QTGs) involved in photoperiod pathways, unlike in other cereals (such as wheat and barley) and *Arabidopsis thaliana*, which also need to be subjected to low temperature (vernalization requirement) (reviewed by Greenup et al., 2009; Tsuji et al., 2011; Bentley et al., 2013; Brambilla and Fornara, 2013; Itoh and Izawa, 2013).

To date, at least 12 QTGs, which belong to the two independent flowering pathways, have been mapped and cloned through quantitative trait locus (QTL) analysis of natural variation in flowering time (Figure 1 and Table 1). In this review, we describe the molecular basis of QTGs underlying natural variation in rice flowering time and discuss the implications on adaptive divergence and consequences for breeding.

MOLECULAR BASIS OF NATURAL VARIATION IN RICE FLOWERING

A genetic pathway resembling that the photoperiod pathway in *Arabidopsis* [a long-day (LD) plant] is conserved in rice. *Hd1* [a CONSTANS (CO) homolog in rice] was the first flowering time QTG cloned from natural rice variants. *Hd1* promotes flowering under SD conditions and represses it under LD conditions (Yano et al., 2000) (Figure 1). By contrast, the *Arabidopsis CO* gene promotes flowering under LD conditions (Putterill et al., 1995). The daylength-dependent conversion of *Hd1* activity between flowering activator and flowering repressor is caused by phytochrome-mediated signaling (e.g., Ishikawa et al., 2011). The repression of flowering by *Hd1* under LD conditions is enhanced by the kinase activity of *Hd6*, which encodes the α -subunit of casein kinase II (Takahashi et al., 2001; Ogiso et al., 2010). *Hd6* enhances the *Hd1* repressor function under LD conditions through the phosphorylation of an unknown protein (Ogiso et al., 2010).

Hd1 regulates *Hd3a*, a rice homolog of *Arabidopsis FLOWERING LOCUS T (FT)* (Kojima et al., 2002) (Figure 1). Tamaki et al. (2007) demonstrated that *Hd3a* functions as a florigen. Another florigen gene, *RFT1*, is a tandemly duplicated paralog of *Hd3a* (Kojima et al., 2002; Ogiso-Tanaka et al., 2013). Komiya et al. (2008, 2009) found that *RFT1* expression increases under LD conditions, indicating that *RFT1* is a LD-specific florigen. More recently, Ogiso-Tanaka et al. (2013) demonstrated

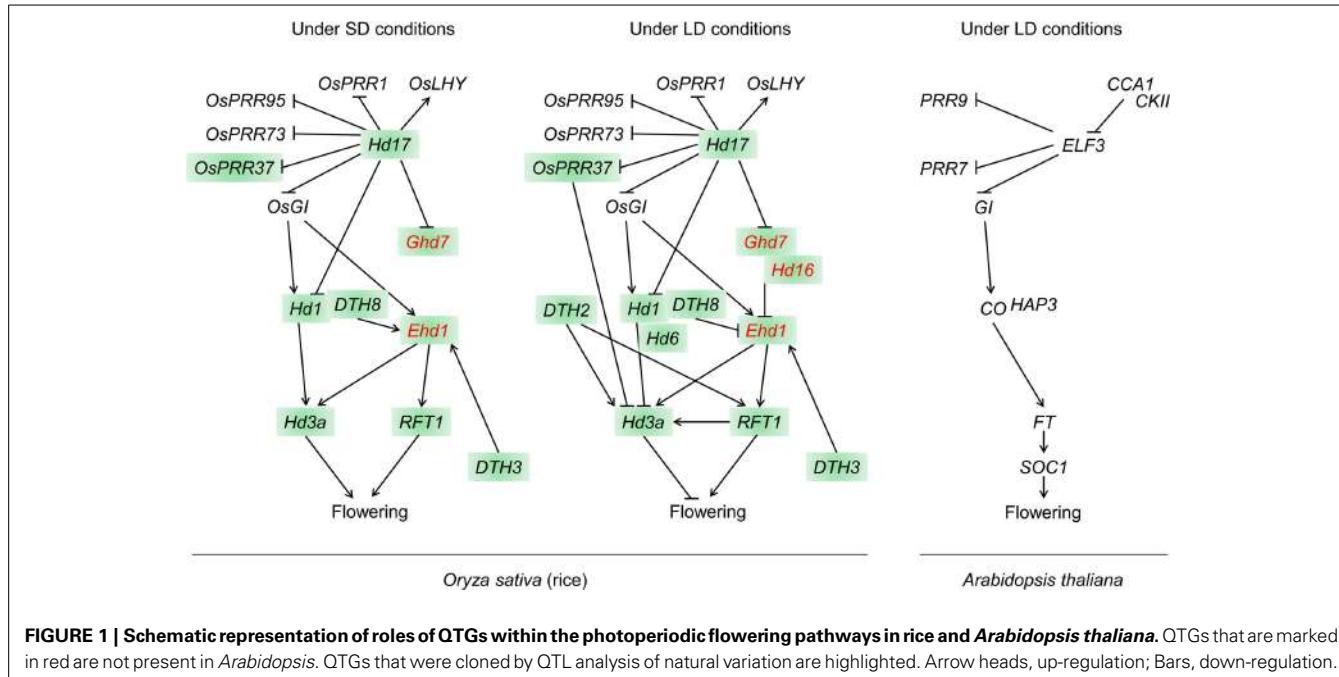


FIGURE 1 | Schematic representation of roles of QTGs within the photoperiodic flowering pathways in rice and *Arabidopsis thaliana*. QTGs that are marked in red are not present in *Arabidopsis*. QTGs that were cloned by QTL analysis of natural variation are highlighted. Arrow heads, up-regulation; Bars, down-regulation.

that functional defects, which were caused by sequence polymorphisms in the regulatory and coding regions of *RFT1*, contribute to late flowering under LD conditions in an *indica* cultivar.

In *Arabidopsis*, the *CO-FT* pathway is regulated by GIGANTEA (GI), which is a component of the circadian clock (Fowler et al., 1999; Park et al., 1999) (Figure 1). Similarly, regulation of the *Hd1-Hd3a* pathway is mediated by *OsGI*, a rice homolog of *GI* (Hayama et al., 2003). These findings reveal that a floral induction pathway from *GI* to *FT* in photoperiodic flowering is conserved between *Arabidopsis* (LD) and rice (SD), but that the photoperiod response differs between these plants.

A unique rice pathway with no obvious ortholog in *Arabidopsis* is also involved in photoperiodic flowering (Figure 1 and Table 1). *Ehd1* is a flowering promoter that encodes a B-type response regulator. *Ehd1* functions upstream of *Hd3a* and *RFT1* (Doi et al., 2004). *Ghd7*, which encodes a CCT (CO, CO-LIKE, and TIMING OF CAB1)-domain protein, was isolated by analysis of natural variations in flowering time. *Ghd7* affects the levels of *Ehd1* and *Hd3a* transcripts, but does not affect *Hd1* mRNA levels (Xue et al., 2008). *Ghd7* represses *Ehd1*, *Hd3a*, and *RFT1* under LD conditions, thereby delaying flowering.

Thus, two independent flowering pathways are present in rice, the conserved *Hd1-Hd3a* pathway and a unique *Ghd7-Ehd1-Hd3a/RFT1* pathway, which may integrate environmental photoperiod signals in the control of flowering. In the following sections, we describe additional QTGs that were more recently cloned.

Hd17, A RICE HOMOLOG OF ARABIDOPSIS ELF3

Subspecies *japonica* cultivars “Nipponbare” and “Koshihikari” differ in their flowering time and flowering responses to photoperiod. QTL analyses revealed that two QTLs on chromosomes 3 and 6 are involved in the difference in heading date between

the two cultivars (Matsubara et al., 2008). The QTL mapped on chromosome 3 was designated as *Hd16*, and the QTL mapped on chromosome 6 was designated as *Hd17*. Both *Hd16* and *Hd17* are involved in photoperiod response, as revealed by observation of heading date in near-isogenic lines (NILs) under SD and LD conditions.

Hd17 explained a small proportion of the variance in flowering time between “Nipponbare” and “Koshihikari.” Map-based cloning demonstrated that this difference may result in part from a single-nucleotide polymorphism (SNP) within a putative gene encoding a rice homolog of the *Arabidopsis* EARLY FLOWERING 3 (ELF3) protein (Matsubara et al., 2012). The SNP was observed among Asian rice cultivars, mainly in *japonica* cultivars. It seems that the wild-type allele has the “Koshihikari” SNP (i.e., the “Nipponbare” allele is a natural variant), because almost all *indica* cultivars and wild accessions surveyed in the study carry the “Koshihikari” SNP.

The amino acid change (serine to leucine) caused by this SNP in *Hd17* may reduce the mRNA level of the floral repressor *Ghd7* under LD conditions, causing ‘Nipponbare’ to flower earlier than NIL-*Hd17*, which carries a chromosomal segment including the “Koshihikari” allele in the “Nipponbare” background. On the other hand, a loss-of-function mutation *ef7* in the rice *ELF3*-like gene (= *Hd17*) seems to increase the *Ghd7* transcription level, and the mutants flower later than wild-type plants under both SD and LD conditions (Saito et al., 2012). This suggests that the *ELF3*-like gene acts as a floral promoter by attenuating the *Ghd7* transcription level (Figure 1).

Arabidopsis ELF3 regulates circadian rhythms by affecting the transcription of clock-associated genes such as *LATE ELONGATED HYPOCOTYL* (*LHY*), *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*), *PSEUDO-RESPONSE REGULATORs* (*PRRs*), and *GI*; the clock output gene *CHLOROPHYLL A/B*

Table 1 | QTGs underlying natural variation in flowering time of rice.

| Gene symbol | Full name | Locus ID | Synonym | Effect on flowering | <i>Arabidopsis</i> homolog | Note | References |
|----------------|-----------------------------------------------|--------------|--------------------------------------|----------------------------|----------------------------|-----------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| <i>Hd1</i> | Heading date 1 | Os06g0275000 | | SD promotion/LD repression | <i>CO</i> | B-box zinc-finger protein with CCT domain | Yano et al., 2000 |
| <i>Hd6</i> | Heading date 6 | Os03g0762000 | | LD repression | <i>CKII</i> | Casein kinase II α subunit | Takahashi et al., 2001; Ogiso et al., 2010 |
| <i>Hd3a</i> | Heading date 3a | Os06g0157700 | | SD promotion | <i>FT</i> | Similar to phosphatidylethanolamine-binding protein | Kojima et al., 2002 |
| <i>RFT1</i> | Rice <i>FT</i> -like 1 | Os06g0157500 | | LD promotion | <i>FT</i> | Similar to phosphatidylethanolamine-binding protein | Kojima et al., 2002; Ogiso-Tanaka et al., 2013 |
| <i>Ehd1</i> | Early heading date 1 | Os10g0463400 | <i>Ef1</i> | SD/LD promotion | No obvious ortholog | B-type response regulator | Doi et al., 2004; Saito et al., 2009 |
| <i>Ghd7</i> | Grain number, plant height and heading date 7 | Os07g0261200 | | LD repression | No obvious ortholog | CCT domain protein | Xue et al., 2008 |
| <i>DTH8</i> | Days to heading on chromosome 8 | Os08g0174500 | <i>Ghd8/LHD1/Hd5/LH8</i> | SD promotion/LD repression | <i>HAP3/NFYB/CBF-A</i> | A putative HAP3 subunit of the CCAAT-box-binding transcription factor | Wei et al., 2010; Dai et al., 2012; Fujino et al., 2013; Chen et al., 2014 |
| <i>DTH3</i> | Days to heading on chromosome 3 | Os03g0122600 | <i>OsMADS50</i> | SD/LD promotion | <i>SOC1/AGL20</i> | MLK3-type MADS-box protein | Lee et al., 2004; Bian et al., 2011 |
| <i>Hd17</i> | Heading date 17 | Os06g0142600 | <i>Hd3b/Ef7/OsELF3-1/OsELF3/Hd-q</i> | SD/LD promotion | <i>ELF3</i> | Similar to ELF3 protein | Matsubara et al., 2012; Saito et al., 2012; Yang et al., 2012; Zhao et al., 2012, 2013 |
| <i>DTH2</i> | Days to heading on chromosome 2 | Os02g0724000 | | LD promotion | <i>CO</i> | CONSTANS-like protein | Wu et al., 2013 |
| <i>Hd16</i> | Heading date 16 | Os03g0793500 | <i>EL1</i> | LD repression | No obvious ortholog | Casein kinase I | Dai and Xue, 2010; Hori et al., 2013; Kwon et al., 2014 |
| <i>OsPRR37</i> | Oryza sativa Pseudo-Response Regulator37 | Os07g0695100 | <i>Hd2</i> | LD repression | <i>PRR7</i> | Pseudo-Response Regulator | Koo et al., 2013 |

Locus ID was based on The Rice Annotation Project Database (<http://rapdb.dna.affrc.go.jp/>).

BINDING 2 (CAB2); and the floral promoter *CO* (reviewed by McClung, 2011) (**Figure 1**). The circadian clock function is conserved also in rice (Murakami et al., 2007). In the *ef7* mutant, the expression of the luciferase gene driven by the *CAB PROTEIN* (a rice homolog of *Arabidopsis CAB2*) promoter under constant darkness was not affected, but the period of free-running rhythms under constant light was slightly shortened, suggesting that *Ef7* mediates light input to the circadian clock but is not required for the clock function in the dark (Saito et al., 2012). By using an *ELF3*-like gene knockout and down-regulation of the expression of *ELF3*-like gene by RNAi, other groups revealed that the *ELF3*-like gene affects the mRNA expression of clock-associated genes and *Hd1* (Yang et al., 2012; Zhao et al., 2012) (**Figure 1**). These observations suggest that the rice *ELF3*-like gene is also involved in the function of the circadian clock.

***Hd16*, A GENE ENCODING CASEIN KINASE I**

The “Koshihikari” allele of *Hd16* decreased photoperiod response in comparison with the “Nipponbare” allele. Map-based cloning revealed an SNP in the gene encoding casein kinase I (CKI), which has no obvious ortholog in *Arabidopsis* (Hori et al., 2013). The SNP resulted in a non-synonymous substitution of an alanine (“Nipponbare”) with threonine (“Koshihikari”). CKI is a protein serine/threonine kinase that is highly conserved among plant and animal species (reviewed by Tuazon and Traugh, 1991; Gross and Anderson, 1998). CKI has various functions in both the cytoplasm and the nucleus, such as DNA repair, and regulation of the cell cycle and circadian rhythm (Liu et al., 2003; Rumpf et al., 2010). Phosphorylation of the clock components by CKI is the key step that initiates and regulates the circadian rhythm. The *tau* gene encodes CKIε in golden hamster (*Mesocricetus auratus*), and a missense mutation in this gene drastically reduces the period of the circadian rhythm (Ralph and Menaker, 1988). The non-synonymous substitution in the “Koshihikari” *Hd16* allele is located at a site close to the *tau* mutation site, and is within the activation loop of the catalytic domain of CKI (Hori et al., 2013). However, the expression patterns of clock-associated genes are similar in the presence of the “Nipponbare” and “Koshihikari” *Hd16* alleles. Therefore, *Hd16* regulates flowering time mediated by the photoperiodic flowering pathway without affecting the regulation of the circadian rhythm.

To reveal the role of *Hd16* in the photoperiodic flowering pathway, we investigated the genetic interactions between *Hd16* and other flowering time QTLs, and the expression levels of the latter (Hori et al., 2013). In rice NILs with functional or deficient alleles of flowering-time genes, significant pairwise interactions were observed between *Hd16* and four other QTLs: *Ghd7*, *Hd1*, *DTH8*, and *Hd2* (= *OsPRR37*). The transcription levels of *Ehd1*, *Hd3a*, and *RFT1* differed between NILs carrying the “Nipponbare” and “Koshihikari” *Hd16* alleles. Biochemical characterization indicated that the *Hd16* recombinant protein encoded by the “Nipponbare” allele specifically phosphorylated *Ghd7* (but not *Hd1*) *in vitro*. The kinase activity of “Koshihikari” *Hd16* was strongly decreased relative to that of “Nipponbare” (Hori et al., 2013). Thus, *Hd16* acts as a *Ghd7* inhibitor in the rice flowering-time pathway by enhancing the photoperiod response as a result of *Ghd7* phosphorylation (**Figure 1**).

Another missense mutation was found in the kinase domain of *Hd16* in the early-heading Korean cultivar “H143” (Kwon et al., 2014). *In vitro* kinase assays revealed that the “H143” *Hd16* allele is also defective. Thus, there are two defective natural variants of *Hd16*. The “Koshihikari” and “H143” alleles were found only among *japonica* cultivars in temperate areas (Hori et al., 2013; Kwon et al., 2014).

Hd16 was previously identified as *Early flowering 1 (EL1)* (Dai and Xue, 2010), which controls rice flowering time by down-regulating the gibberellin (GA) signaling pathway mediated by phosphorylation of *SLR1*, encoded by *Slender rice 1*. *SLR1* phosphorylation suppresses the GA response, whereas *Ghd7* phosphorylation enhances the photoperiod response. In both cases, phosphorylation leads to delayed flowering under LD conditions. Thus, *Hd16/EL1* appears to be associated with both photoperiod and GA responses in rice flowering.

OTHER QTGs UNDERLYING NATURAL FLOWERING TIME VARIATION IN RICE

In addition to the QTGs described above, four rice homologs of the *Arabidopsis* flowering-related genes have been cloned by using genotypes that show natural variation in flowering time (**Table 1**). *DTH8* encodes a rice homolog of the *Arabidopsis* HEME ACTIVATOR PROTEIN (YEAST) HOMOLOG 3 sub-unit of the CCAAT-box-binding transcription factor. Under LD conditions, *DTH8* down-regulates *Ehd1* and its downstream target *Hd3a* and therefore acts as a flowering suppressor (Wei et al., 2010) (**Figure 1**). A *DTH8* variant also promotes flowering under SD conditions (Yan et al., 2011). Most recently, interaction between *DTH8* and *Hd1* was demonstrated by yeast-two-hybrid assay (Chen et al., 2014).

DTH3 encodes a rice homolog of *Arabidopsis* SUPPRESSOR OF OVEREXPRESSION OF CO1, a MIKC-type MADS-box protein. *DTH3* up-regulates *Ehd1* and *RFT1* under both SD and LD conditions and thereby promotes flowering (Bian et al., 2011) (**Figure 1**). At this QTL, there is functional allelic variation between *O. sativa* and *O. glaberrima*, but probably not among *O. sativa* cultivars.

DTH2 encodes a CONSTANS-like protein. *DTH2* up-regulates *Hd3a* and *RFT1* under LD conditions and thus promotes flowering (Wu et al., 2013) (**Figure 1**). *OsPRR37* encodes a rice homolog of *Arabidopsis PRR7*, and down-regulates *Hd3a* expression to suppress flowering under LD conditions (Murakami et al., 2007; Koo et al., 2013) (**Figure 1**).

CONSERVATION AND DIVERSIFICATION OF FLOWERING TIME REGULATION BETWEEN RICE AND ARABIDOPSIS

Much progress has been made in understanding the natural genetic variation in rice flowering. 12 QTGs have been isolated and assigned to specific photoperiod flowering pathways. Among them, 9 have homologs in the *Arabidopsis* genome, suggesting that the genetic basis of photoperiodic flowering has an ancient origin in flowering plants (**Table 1**).

On the other hand, it is evident that there are differences in the photoperiodic flowering pathways between the two species (**Figure 1**). The *Ghd7–Ehd1–Hd3a/RFT1* pathway, which is modulated by *Hd16*, is not present in *Arabidopsis*. However, in many

cases even orthologous genes have divergent functions, as exemplified by *CO* (flowering promotion in LD) and *Hd1* (flowering promotion in SD), and by *ELF3* (flowering repression in SD) and *Hd17* (flowering promotion in both SD and LD). The differences between these orthologs may be associated with neofunctionalization, which is the evolution of new function of a duplicated gene, as suggested about the divergent function of orthologs of *FLOWERING LOCUS VE* and *FT* in sugar beet (*Beta vulgaris*, Pin et al., 2010; Abou-Elwafa et al., 2011). Actually, both *Hd1* and *ELF3* have putative paralog(s) in the rice genome.

Such differences may have resulted from evolution of these lineages in different geographic regions: rice in equatorial regions characterized by stable temperature and daylength all year round, *Arabidopsis* in temperate regions with fluctuating temperatures and changing daylength. As a result, rice has developed photoperiodic flowering, while *Arabidopsis* acquired an additional vernalization requirement as adaptation to the cold season (reviewed by Ballerini and Kramer, 2011).

Probably, this hypothesis should be examined among cereals, such as maize (*Zea mays*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) as well as rice, because they are more closely related to each other as rice and *Arabidopsis* (Magallón and Sanderson, 2005). Additionally, a similar difference in flowering phenology has been observed between tropical-origin (rice and maize) and temperate-origin (wheat and barley) cereals as between rice and *Arabidopsis* (e.g., Greenup et al., 2009; Jung and Müller, 2009).

We suggest that emphasizing divergence among plant species (particularly cereals) rather than conservation would help to better explain the genetic basis of flowering and adaptive divergence in rice.

APPLICATION OF NATURAL VARIATION IN FLOWERING TIME GENES IN BREEDING PROGRAMS

Adjustment of photoperiod response changes flowering time and enhances adaptability to local environmental conditions in many plant species, including rice (e.g., Jung and Müller, 2009; Hori et al., 2012). To introduce rice at high latitudes, breeders have selected lines with a weaker response to photoperiod to produce early-flowering cultivars and to ensure maturation during the optimal period (e.g., Izawa, 2007; Ebana et al., 2011). The weak allele of *Hd16* may have permitted the extension of the rice cultivation area into northern regions (Hori et al., 2013; Kwon et al., 2014). Deficient or weak alleles of *Hd1*, *Ghd7*, *DTH8*, *DTH2*, and *OsPRR37* are also distributed in northern rice cultivation areas at high latitudes (Xue et al., 2008; Takahashi et al., 2009; Wei et al., 2010; Koo et al., 2013; Wu et al., 2013), strongly suggesting that such alleles are involved in the expansion of rice cultivation areas.

In addition to several major flowering time QTGs isolated in previous studies, we have identified QTLs with minor effects: *Hd4* (likely allelic to *Ghd7*), *Hd7*, *Hd9* (Lin et al., 2002, 2003), and *Hd17* (Matsubara et al., 2012). These QTLs are necessary for breeders in fine tuning of the flowering time in rice cultivars. Breeders sometimes want to slightly change the flowering time of rice cultivars. Natural variations are observed at many loci (at least more than 20, according to a publicly available database, Q-TARO: <http://qtaro.abr.affrc.go.jp/>) involved in rice

photoperiodic flowering pathways. These variations are available for producing a number of allelic combinations of flowering time QTLs and for developing rice cultivars adjusted to diverse cultivation areas.

CONCLUSIONS AND PERSPECTIVES

Molecular cloning of QTGs underlying natural variation in flowering time of rice has improved our understanding of the genetic basis and provides insights into adaptive evolution and breeding in rice. However, we still know little about the distribution of QTG associated with flowering time in rice cultivars. We are still unable to predict exactly the relative effects of individual QTG on flowering time of rice cultivars in diverse natural field conditions, owing to limited knowledge of QTG × QTG and QTG × environmental interaction. This leads to the gap between genotype and phenotype (discussed by Benfey and Mitchell-Olds, 2008; Olsen and Wendel, 2013). To resolve this problem, we will need to assess the effect of each allele on flowering time in different allele combinations and in various environments by the combination of experimental populations, such as recombinant inbred lines (RILs), chromosome segment substitution lines (CSSLs), nested association mapping (NAM) population and multi-parent advanced generation inter-cross (MAGIC) population, and sequencing the alleles from each line. Furthermore, the transcriptome analysis of flowering-time genes under natural field conditions may also pave the way for the prediction of flowering time (Nagano et al., 2012).

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The *FLC*-like gene *BvFL1* is not a major regulator of vernalization response in biennial beets

Sebastian H. Vogt¹, Guy Weyens², Marc Lefèvre², Bettina Bork³, Axel Schechert³ and Andreas E. Müller^{1*}

¹ Plant Breeding Institute, Christian-Albrechts-University of Kiel, Kiel, Germany

² SESVanderHave N.V., Tienen, Belgium

³ Strube Research GmbH & Co. KG, Söllingen, Germany

Edited by:

Dorothee Staiger, Bielefeld University, Germany

Reviewed by:

Maria C. Albani, University of Cologne, Germany

Elizabeth Dennis, Commonwealth Scientific and Industrial Research Organisation, Australia

Adrian Simon Turner, John Innes Centre, UK

***Correspondence:**

Andreas E. Müller, Strube Research GmbH & Co. KG, Hauptstr. 1, 38387 Söllingen, Germany
e-mail: a.mueller@strube-research.net

†Present address:

Andreas E. Müller, Strube Research GmbH & Co. KG, Söllingen, Germany

Many plant species in temperate climate regions require vernalization over winter to initiate flowering. *Flowering Locus C (FLC)* and *FLC*-like genes are key regulators of vernalization requirement and growth habit in winter-annual and perennial *Brassicaceae*. In the biennial crop species *Beta vulgaris* ssp. *vulgaris* in the evolutionarily distant Caryophyllales clade of core eudicots growth habit and bolting time are controlled by the vernalization and photoperiod response gene *BTC1* and the downstream *BvFT1-BvFT2* module. *B. vulgaris* also contains a vernalization-responsive *FLC* homolog (*BvFL1*). Here, to further elucidate the regulation of vernalization response and growth habit in beet, we functionally characterized *BvFL1* by RNAi and over-expression in transgenic plants. *BvFL1* RNAi neither eliminated the requirement for vernalization of biennial beets nor had a major effect on bolting time after vernalization. Over-expression of *BvFL1* resulted in a moderate late-bolting phenotype, with bolting after vernalization being delayed by approximately 1 week. By contrast, RNAi-induced down-regulation of the *BvFT1-BvFT2* module led to a strong delay in bolting after vernalization by several weeks. The data demonstrate for the first time that an *FLC* homolog does not play a major role in the control of vernalization response in a dicot species outside the *Brassicaceae*.

Keywords: *Beta vulgaris*, bolting, *Flowering Locus C (FLC)*, photoperiod, vernalization

INTRODUCTION

Vernalization is the process by which the exposure of a plant to a prolonged period of cold temperatures over winter promotes the initiation of flowering. In temperate climate regions vernalization is an integral part of life cycle strategies as an evolutionary adaptation to changing seasons. In the annual dicotyledonous species *Arabidopsis thaliana* and its perennial relative *Arabis alpina*, the vernalization response is regulated by the MADS-box gene *Flowering Locus C (FLC)* and its ortholog *Perpetual Flowering 1 (PEP1)*, respectively (Michaels and Amasino, 1999; Sheldon et al., 1999; Wang et al., 2009; Zografas and Sung, 2012). By contrast, the vernalization response in monocotyledonous species like barley or wheat requires the *Vernalization 1-3 (VRN1-3)* genes, with *VRN1* being the only MADS-box gene of these three (Yan et al., 2003, 2004, 2006). Recent studies in *Beta vulgaris*, which on an evolutionary scale is similarly distantly related to *Arabidopsis* (~120 million years of evolution) and the monocots (~140 million years; Chaw et al., 2004; Davies et al., 2004), revealed a new mode of life cycle control in dicotyledonous species. In *B. vulgaris*, the pseudo-response regulator (PRR) gene *Bolting Time Control 1 (BTC1)* determines whether floral transition occurs in the first year of growth, as in annual accessions, or in the second year, as in biennials (Pin et al., 2012). *BTC1* mediates bolting and flowering by regulation of an antagonistic pair of *Flowering Locus T (FT)* homologs first described by Pin et al. (2010). Bienniality in beet derives from a recessive *BTC1* allele

(*btc1*) with a reduced responsiveness to the floral inductive stimulus of long days and/or reduced activity of the *BTC1* protein compared to annual beets. The perception of prolonged cold over winter after the first growing season restores the competence to bolt and flower in biennial beets. This process was suggested to involve up-regulation of *BTC1*, leading to suppression of the flowering repressor *BvFT1* and expression of the flowering activator *BvFT2*. Life cycle control by *BTC1* thus involves the integration of both photoperiod and vernalization signals. By contrast, the PRR genes in monocots such as *PPD1* in *Hordeum vulgare* or *SbPRR37* in *Sorghum bicolor* are only known to mediate photoperiod response, while a role in vernalization response or life cycle control has not been described (Turner et al., 2005; Murphy et al., 2011).

In *Arabidopsis*, *FLC* represses flowering by binding to *cis*-regulatory sequences in the floral integrator genes *FT* and *Suppressor of Overexpression of Constans 1 (SOC1)* (Helliwell et al., 2006; Searle et al., 2006). During vernalization, *FLC* is down-regulated and the repressed state is epigenetically maintained after vernalization. The repression of *FLC* allows activation of *FT* under long-day conditions through the photoperiod pathway and its central regulator *Constans (CO)*. *FT* protein expressed in the phloem companion cells of the leaves moves to the shoot apical meristem as part of the “florigen” signal and initiates flowering (Andrés and Coupland, 2012). Besides their highly conserved function as day length-induced floral activators, *FT* and

FT-like genes also control other processes like stomatal opening in *Arabidopsis* or tuberization in potato (Pin and Nilsson, 2012).

A dose-dependent positive correlation between *FLC* expression and circadian period length was shown by using genotypes with different functional and non-functional allele compositions at *FLC* and the *FLC*-regulatory locus *FRIGIDA* (*FRI*) as well as a 35S::*FLC* over-expressor line (Salathia et al., 2006). Furthermore, El-Assal et al. (2003) showed that *FLC* negatively and dose-dependently regulates expression of the photoreceptor gene *Cryptochrome 2* (*CRY2*). *CRY2* co-regulates circadian period length together with *CRY1* and tends to act as a negative regulator of period length (Devlin and Kay, 2000; Gould et al., 2013), suggesting that the *FLC*-induced increase in circadian period length may be mediated through cryptochromes. Vernalization resulted in a significant decrease in circadian period length, which was suggested to reduce the day length threshold required for photoperiodic induction of flowering and thus to accelerate flowering in spring (Yanovsky and Kay, 2002; Salathia et al., 2006). Finally, mutations in photoperiod pathway genes affected expression of *FLC*, providing further indication for the crosstalk between vernalization and photoperiod pathways (Rouse et al., 2002).

FLC and *FLC*-like genes belong to a major MADS-box gene clade that was recently shown to also include monocot genes (Ruelens et al., 2013). In dicots, *FLC*-like genes have been identified in two species outside the *Brassicaceae*, i.e., *B. vulgaris* in the Caryophyllales clade of core eudicots (Reeves et al., 2007) and the asterid species *Cichorium intybus* (Périlleux et al., 2013), which includes the biennial crop root chicory. Complementation analyses of *B. vulgaris FLC-LIKE 1* (*BvFL1*) and *C. intybus FLC-LIKE* (*CiFL1*) in *Arabidopsis* and down-regulation of *BvFL1* and *CiFL1* by vernalization in beet or chicory, respectively, suggested a conserved floral repressor function of these genes. However, instead of being epigenetically maintained in a transcriptionally silent state after vernalization, the expression of *BvFL1* and *CiFL1* after vernalization reverted to pre-vernalization levels (Reeves et al., 2007; Périlleux et al., 2013). Interestingly, the *FLC* ortholog *PEP1* in *A. alpina* also reverts to pre-vernalization expression levels after return to warm temperatures, which correlates with unstable histone modifications at the *PEP1* locus (Wang et al., 2009). Unstable repression of *PEP1* after vernalization was suggested to correlate with perennial life cycle strategies (Wang et al., 2009).

A more complex pattern of *BvFL1* regulation in beet emerged from a study of *BvFL1* expression in the shoot apical meristem (Trap-Gentil et al., 2011). According to this study, “bolting sensitive” biennial beet genotypes, which only require relatively short periods of vernalization for bolting to occur, are first down-regulated during vernalization, but up-regulated during a later stage of vernalization. The authors suggested that the early transient decrease in *BvFL1* expression during vernalization may account for the relatively high susceptibility to bolting in these genotypes. By contrast, “bolting resistant” biennial genotypes that require relatively long periods of vernalization exhibited a gradual increase in expression during vernalization. Furthermore, RNA methylation of *BvFL1* mRNA was detected in the shoot apical meristem of a bolting-resistant genotype after vernalization and was proposed to contribute to the control of vernalization response in sugar beet (Hébrard et al., 2013). However, a

clear picture of the functional role of *BvFL1* in beet has not yet emerged, and a characterization of this gene’s function through transgenic or mutational analyses in beet is still lacking.

Here, we further dissect the vernalization response in beet by over-expression and RNAi-mediated down-regulation of *BvFL1* and down-regulation of the *FT* homologs *BvFT1* and *BvFT2* in transgenic plants. Phenotypic analysis revealed a delay in bolting after vernalization by 1 week in transformants over-expressing *BvFL1*, while *BvFL1* RNAi neither had a major effect on bolting time after vernalization nor did it lead to bolting without vernalization. RNAi-induced concomitant down-regulation of the floral repressor *BvFT1* and the floral activator *BvFT2* resulted in a bolting delay by up to 7 weeks and a high percentage of non-bolting plants in a subset of transformation events. Taken together, our data support a dominant role of the *BvFT1-BvFT2* module in the control of vernalization response and show that, by contrast, *BvFL1* is not a major regulator of vernalization response in beet.

MATERIALS AND METHODS

VECTOR CONSTRUCTION AND PLANT TRANSFORMATION

For the *BvFL1* over-expression construct a 616 bp cDNA fragment covering the whole coding sequence of the splice variant *BvFL1_v3* (Reeves et al., 2007) was inserted downstream of a Cauliflower Mosaic Virus (CaMV) 35S promoter and the Tobacco Mosaic Virus (TMV) 5'UTR and upstream of an *Agrobacterium tumefaciens* nos 3' terminator. In *Arabidopsis*, over-expression of *BvFL1_v3* caused the strongest delay in flowering among *BvFL1* splice variants (Reeves et al., 2007). RNAi vectors were constructed by insertion of a 332 bp fragment of the *BvFL1* 3'UTR or a 361 bp cDNA fragment spanning most of the phosphatidylethanolamine-binding protein (PEBP) domain of *BvFT1* (Pin et al., 2010), respectively, as inverted repeats between the regulatory elements described above. A 91 bp sugar beet intron sequence was used as spacer between the sense and antisense repeat units. The *phosphinothricin acetyl transferase* (*PAT*) gene was inserted downstream of the RNAi cassettes for selection of transgenic plants with glufosinate. The constructs were introduced into the biennial sugar beet genotype SES01 (SESVanderHave, Tienen, Belgium) by polyethylene glycol-mediated DNA transfer as described previously (Hall et al., 1996; Pin et al., 2012). Transgenic protoplasts, calli and regenerating plantlets were selected using glufosinate and transgene integration was confirmed by PCR. Low copy number (1–3 transgene copies) transformants were selected by quantitative PCR using TaqMan® assays (Life Technologies, Carlsbad, California, USA) and DNA gel blot analysis for the effector transgene and the *PAT* gene (Table 1). For DNA gel blot analysis, genomic DNA was digested with two different restriction enzymes, *EcoRI* and *NcoI*, separated by gel electrophoresis and transferred to Hybond™-N membranes (GE Healthcare, Little Chalfont, UK). Construct-specific probes were amplified from the corresponding plasmid DNA using primers 5'-CTATTTACAATTACACC ATGGCAGGCG and 5'-TGAACGATCGGGAAATTCGAGC TCGG for analysis of *BvFL1* over-expression transformants, 5'-GGTTTTATGTACTACTGTTGTAGCTG and 5'-TGAA CGATCGGGAAATTCGAGCTCGG for *BvFL1* RNAi transformants, and 5'-GGTTTTATGTACTACTGTTGTAGCTG and

Table 1 |Bolting time after vernalization in independent sugar beet transformants carrying *BvFL1* or *BvFT1-BvFT2* RNAi or over-expression constructs.

| Effector construct type | Transgenic event number | Total number of vernal. plants ^a | Number of bolting plants | DTB ^b (mean ± SD ^c) | Unpaired t-test value for DTB (probability ^d) | Total number of non-bolting plants | PAT gene copy number ^e | Effector transgene copy number ^f | Target gene expression level |
|-------------------------------|-------------------------|---------------------------------------------|--------------------------|--------------------------------------------|-----------------------------------------------------------|------------------------------------|-----------------------------------|---------------------------------------------|------------------------------|
| <i>BvFL1</i> | | | | | | | | | |
| RNAi | 019-07G | 15 | 15 | 35.60 ± 3.20 | 6.85 ($p < 0.0001$) | 0 | n.a. | 2 | 2 unchanged |
| RNAi | 021-11G | 15 | 15 | 37.00 ± 2.71 | 5.45 ($p < 0.0001$) | 0 | 9 | 2 | 1 down |
| RNAi | 021-12A | 15 | 15 | 39.87 ± 4.84 | 1.16 ($p = 0.2500$) | 0 | n.a. | 2 | 2 down |
| RNAi | 021-12H | 15 | 15 | 37.67 ± 4.13 | 3.72 ($p = 0.0005$) | 0 | n.a. | 2 | 2 down |
| RNAi | 022-10F | 12 | 12 | 38.75 ± 3.24 | 2.68 ($p = 0.0100$) | 0 | n.a. | 2 | 3 unchanged |
| RNAi | 024-11E | 20 | 20 | 36.55 ± 2.22 | 7.15 ($p < 0.0001$) | 0 | n.a. | 1 | 1 down |
| RNAi | 024-12E | 20 | 20 | 44.15 ± 3.47 | -4.06 ($p = 0.0002$) | 0 | 6 | 1 | 1 down |
| Over-expression | 016-05C | 20 | 19 | 49.21 ± 6.31 | -6.96 ($p < 0.0001$) | 1 | 5 | 1 | 1 up |
| Over-expression | 016-10A | 15 | 14 | 41.07 ± 3.81 | -0.05 ($p = 0.9600$) | 1 | 8 | 2 | 3 up |
| Over-expression | 017-06C | 20 | 20 | 48.55 ± 5.62 | -7.05 ($p < 0.0001$) | 0 | n.a. | 2-3 | 2-3 up |
| Over-expression | 017-07C | 15 | 15 | 39.93 ± 4.19 | 1.20 ($p = 0.2400$) | 0 | n.a. | 1 | 2 up |
| <i>BvFT1-BvFT2</i> | | | | | | | | | |
| RNAi | 014-02G | 20 | 18 | 48.83 ± 9.21 | -4.79 ($p < 0.0001$) | 2 | n.a. | 1 | 1 down |
| RNAi | 014-07F | 15 | 13 | 73.46 ± 18.37 | -10.3 ($p < 0.0001$) | 2 | 14 | 1-2 | 1 down |
| RNAi | 014-08B | 20 | 20 | 43.80 ± 5.78 | -2.54 ($p = 0.0140$) | 0 | n.a. | 3 | 2 unchanged |
| RNAi | 018-06E | 15 | 4 | 68.75 ± 5.80 | -18.5 ($p < 0.0001$) | 11 | n.a. | 1 | 2 down ^g |
| RNAi | 018-09A | 15 | 15 | 52.40 ± 4.99 | -11.2 ($p < 0.0001$) | 0 | 10 | 2 | 1 down |
| RNAi | 019-01E | 16 | 9 | 93.67 ± 36.73 | -8.46 ($p < 0.0001$) | 7 | n.a. | 3-4 | 3 down ^g |
| RNAi | 020-01E | 15 | 13 | 63.00 ± 8.49 | -14.1 ($p < 0.0001$) | 2 | 6 | 2 | 2 down |
| RNAi | 018-12H | 15 | 15 | 57.73 ± 7.64 | -11.9 ($p < 0.0001$) | 0 | 9 | 2 | 1 down |
| RNAi | 020-01C | 15 | 14 | 63.93 ± 25.27 | -5.35 ($p < 0.0001$) | 1 | 8 | 2 | 1 down |
| RNAi | 020-05G | 15 | 12 | 60.00 ± 15.04 | -7.28 ($p < 0.0001$) | 3 | n.a. | 2-3 | 2 down |
| Non-transgenic control | | | | | | | | | |
| n.a. | n.a. | 37 | 37 | 41.03 ± 2.21 | n.a. | 0 | n.a. | n.a. | n.a. |

^aPlants were generated by in-vitro multiplication (cloning) of primary transformants.^bDays to bolting after vernalization.^cStandard deviation of the mean.^dProbability that the DTB value is significantly different from that obtained using the non-transgenic control.^eFor a subset of transformants, the number of plants indicated in this column was grown without vernalization in the greenhouse over spring and summer under optimal conditions for more than 6 months, but all of these plants failed to bolt.^fCopy numbers for the PAT gene and effector transgenes were determined by DNA gel blot analysis.^gHigh transgene expression.

n.a., not applicable.

5'-TGAACGATCGGGAAATTGAGCTCGG for *BvFT1-BvFT2 RNAi* transformants. A *PAT* gene-specific probe was amplified using primers 5'-AGATTAGGCCAGCTACAGCAGCTGATA and 5'-GCCTTGAGGAGCTGGCAACTCAAAT. Probes were radioactively labeled by random primer labeling (Feinberg and Vogelstein, 1983) using α -³²P-dCTP and the large (Klenow) fragment of DNA polymerase I (Life Technologies, Carlsbad, California, USA). Copy number was determined as the number of discrete bands after hybridization. In cases where the number of detectable bands for a given transformant differed between the two enzymes, the detected range of copy numbers is given in **Table 1**. Transgenic and non-transgenic control plants were clonally multiplied *in vitro* and transferred to soil according to standard procedures (Hall et al., 1996).

GROWTH CONDITIONS AND PHENOTYPING

Transgenic plants and non-transgenic SES01 control plants were grown side-by-side in the greenhouse in early spring with supplementary lighting under 16 h light/8 h dark cycles. Vernalization and phenotyping was as described previously for *Bvbtc1 RNAi* transformants (Pin et al., 2012). In brief, vernalization was carried out in a climate chamber at 4°C and 16 h light for 3 months. To avoid devernalization several acclimation steps were performed over a period of 6 weeks during which the temperature was raised from 4 to 25°C during the light cycle and from 4 to 15°C during the dark cycle. Plants were phenotyped for the occurrence and time of bolting three times per week until 6 months after vernalization. Bolting time was defined as the number of days after the end of vernalization for a plant to reach a stem height of 5 cm. For each independent transgenic event 12–20 plants were phenotyped for bolting time. The non-transgenic control comprised 37 clones of the host genotype that was used for transformation. The Student's *t*-test was used for statistical analysis of phenotypic data. A subset of plants was not vernalized but instead continued to be grown in the greenhouse over spring and summer for more than 6 months under natural daylight conditions with supplementary lighting (16 h).

GENE EXPRESSION ANALYSIS

For each transgenic event and the non-transgenic control genotype, leaf samples of three clones each were harvested before vernalization 2 months after transfer to soil and again at the end of a 12 week vernalization period at Zeitgeber time (ZT) 6–8. For diurnal expression analysis, leaf samples of three individual clones of the *BvFL1* over-expressing transformant 016-05C were taken every 2 h over a period of 24 h 4 weeks after the end of vernalization. RNA extraction and cDNA synthesis were done for each of the three biological replicates (clones) separately and exactly as described for *Bvbtc1 RNAi* transformants (Pin et al., 2012). Primer annealing temperatures and elongation times are given in Table S1. Three technical replicates were performed for each RT-qPCR reaction. RT-qPCR was performed on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, California, USA) as described in Pin et al. (2012). To determine RT-qPCR efficiencies and serve as positive controls, the endogenous target transcript regions analyzed by RT-qPCR were cloned into the pGEM-T vector (Promega Corporation, Madison,

Wisconsin, USA). All plant samples were assayed for expression of the respective RNAi or over-expression target gene and the housekeeping gene *BvGAPDH*, which was used as a reference gene for normalization. The comparative $C_T(2^{-\Delta\Delta CT})$ method was applied for analysis (Schmittgen and Livak, 2008).

RESULTS

RNAI AND OVER-EXPRESSION OF VERNALIZATION RESPONSE GENES IN BIENNIAL BEETS

BvFL1 and the *FT*-like gene pair *BvFT1-BvFT2* were analyzed by RNAi or over-expression in a biennial genetic background. Four to ten independent transformants were pre-selected for the presence of transgene inserts and low transgene copy numbers by PCR, TaqMan® assays and DNA gel blot analysis as described in Materials and Methods. Transformants with low copy numbers (1–3) were multiplied by clonal propagation and analyzed for changes in target gene expression and bolting time (**Table 1**; Table S1).

BvFL1

Down-regulation of *BvFL1* by RNAi to less than half of the expression level in the non-transgenic control plants was successful in four out of seven transgenic events (**Figure 1A**). Two of these events (021-11G, 024-12E) showed a reduction to less than 20% of the expression level in the control plants. Following vernalization, all *BvFL1 RNAi* transformants bolted (**Figure 1A; Table 1**). The mean days to bolting after the end of vernalization varied from 35.60 to 44.15 days, whereas bolting occurred on average 41.03 days after vernalization in the non-transgenic control plants. In one *BvFL1 RNAi* event, the mean days to bolting did not deviate significantly from the control plants. Five events bolted 2.28–5.43 days earlier and one event bolted 3.12 days later than the control plants. Of the four *BvFL1 RNAi* events in which *BvFL1* was down-regulated most, two (021-11G, 021-12H) bolted 3–4 days earlier than the control, one (024-12E) bolted 3 days later, and one (021-12A) did not deviate significantly from the control. Together, the data suggest a certain level of experimental noise but did not reveal a clear and consistent phenotypic effect of reduced *BvFL1* expression.

Of the four events derived from transformation with a *BvFL1* over-expression construct, all showed strong up-regulation of *BvFL1* expression (**Figure 1B**). Bolting time after vernalization varied from 39.93 to 49.21 days. In two events (016-05C, 017-06C), bolting was delayed by approximately 8 days, whereas in the two other events (016-10A, 017-07C) bolting time did not deviate significantly from the control (**Table 1**). Two events (016-05C, 016-10A) included one plant each which failed to bolt until the end of the experiment 6 months after the end of vernalization.

Of the two events with down-regulation of *BvFL1* to less than 20% of the control (021-11G, 024-12E) and two *BvFL1* over-expression events (016-05C, 016-10A), an additional 5–9 plants each were grown in parallel for more than 6 months over spring and summer under long-day conditions and without vernalization in the greenhouse, but none of these plants initiated bolting (**Table 1**).

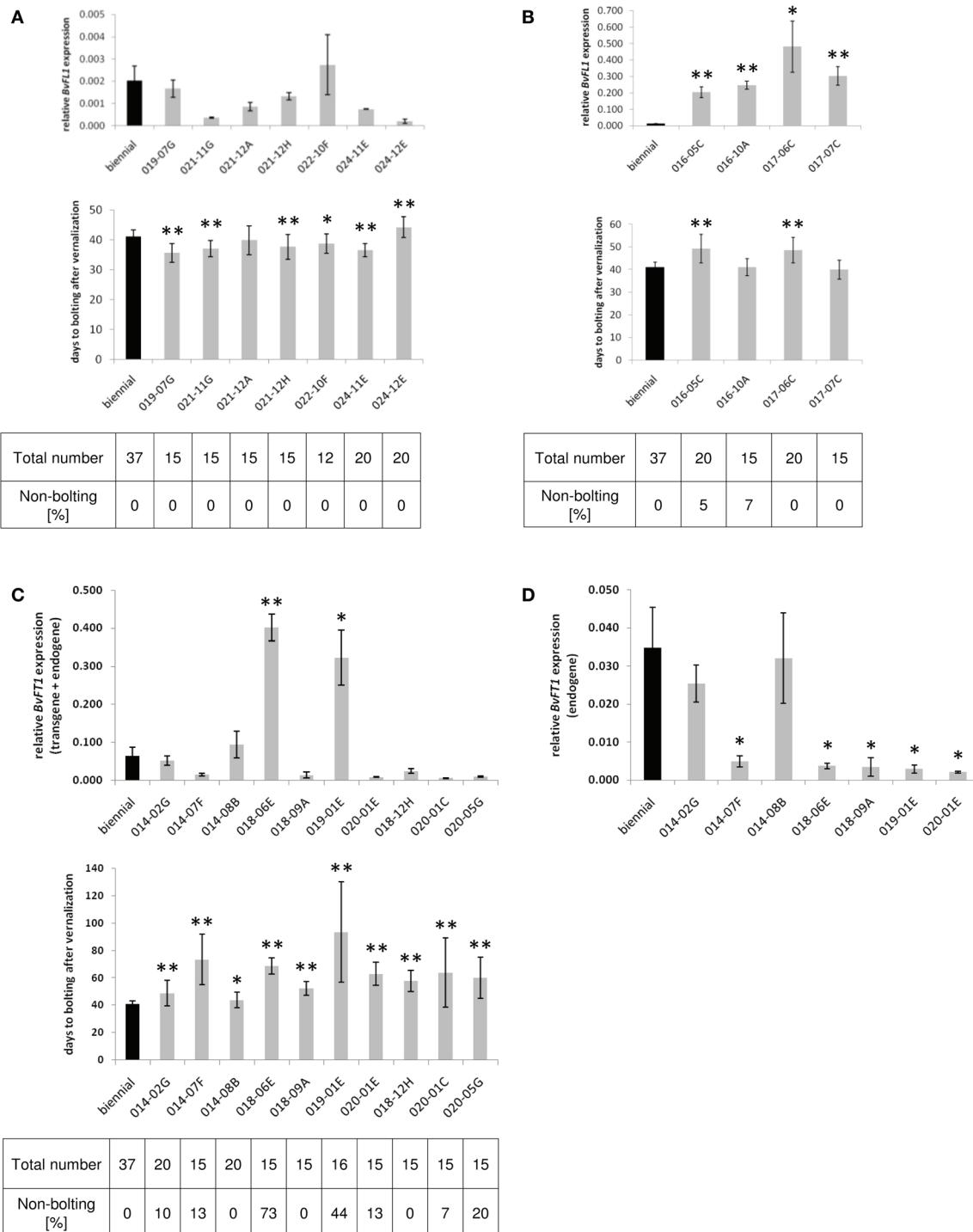


FIGURE 1 | Gene expression and bolting time phenotypes in *BvFL1* RNAi (A), *BvFL1* over-expression (B), and *BvFT1*-*BvFT2* RNAi transformants (C,D). Leaf samples of non-vernalized plants derived from independent sugar beet transformation events and the non-transgenic biennial control genotype were taken under long-day conditions at Zeitgeber time (ZT) 6-8. For each transgenic event, three clones were analyzed as biological replicates, and each RT-qPCR reaction was run in triplicate. Gene expression was normalized using the house-keeping gene *BvGAPDH* and the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Error bars represent mean \pm SE of the mean. Expression of *BvFT1* in *BvFT1*-*BvFT2* RNAi plants was determined with

primers which co-amplify endogenous and transgenic *BvFT1* transcripts (C) and with primers which specifically amplify the endogenous *BvFT1* transcript (D). Bolting time was measured in days to bolting and the SE of the mean are shown for plants which bolted within 6 months after the end of vernalization. Significant differences between expression levels in the transformants and the control plants and between bolting time are indicated by asterisks (* $\alpha = 0.05$, ** $\alpha = 0.01$ according to Student's *t*-test). The total number of plants per transgenic event and the percentage of plants which failed to bolt within this period are given in the tables below the bar graphs.

BvFT1-BvFT2

Out of ten transgenic events derived from transformation with an RNAi construct carrying part of the *BvFT1* cDNA, seven exhibited down-regulation of the *BvFT1* endogene (**Figure 1C**). A further analysis of several *BvFT1* RNAi transformants revealed that not only *BvFT1* but also *BvFT2* was down-regulated in these plants, presumably due to RNAi off-target effects (see further below). Therefore, the RNAi transformants expose the effects of co-silencing of both constituents of the *BvFT1*-*BvFT2* module and will be referred to as *BvFT1*-*BvFT2* RNAi events.

Bolting was delayed in all seven events and occurred approximately 8–32 days later than in the control plants (**Figure 1C; Table 1**). Besides a delay in bolting time, five of the seven *BvFT1*-*BvFT2* RNAi events also included one to three non-bolting plants each among the 15–20 plants that were phenotyped for each of the *BvFT1*-*BvFT2* RNAi events. An additional 47 plants (6–14 plants each of events 014-07F, 018-09A, 020-01E, 018-12H, 020-01C) were grown for more than 6 months without vernalization in the greenhouse and side-by-side with the *BvFL1* events mentioned above, but like these did not initiate bolting (**Table 1**).

Two of the transgenic events (018-06E and 019-01E) stood out in that they appeared to show over-expression of *BvFT1* rather than down-regulation (**Figure 1C**). The primer binding sites of the RT-qPCR assay for *BvFT1* expression were located within the segment of the cDNA that was used for construction of the inverted repeat cassette in the RNAi construct. Thus, both the endogenous *BvFT1* transcript and the transgene-derived transcript can be co-amplified, suggesting that transcription from the transgene may contribute to the observed high levels of transcript accumulation. To test this possibility, *BvFT1* expression was re-analyzed by RT-qPCR using endogene-specific primers (with binding sites outside the cDNA fragment used for RNAi transgene construction) in the two events in question as well as five of the events in which *BvFT1* expression was either down-regulated or largely unchanged. For the latter five events, this analysis confirmed the previous expression data. However, for the events in question the endogene-specific RT-qPCR now revealed clear down-regulation of the endogene (**Figure 1D**). Transcript accumulation in these two events was similarly low as in other transformants in which *BvFT1* was down-regulated (<20% of transcript accumulation in the control). Interestingly, the same two events also contained exceptionally high percentages of non-bolting plants (73% in 018-06E and 44% in 019-01E; **Figure 1C; Table 1**).

PRE- AND POST-VERNALIZATION EXPRESSION OF FLORAL REGULATORS

Two independent transgenic events each which showed either clear down- or up-regulation of the gene of interest were analyzed further. These events were assayed for target gene expression before vernalization and at the end of a 12-week vernalization period. Expression of *BvFL1* in the non-transgenic control plants was lower at the end of vernalization than before vernalization (**Figure 2A**). The strong down-regulation of *BvFL1* by RNAi in the transgenic events 021-11G and 024-12E when compared to the control plants was evident both before and at the end of vernalization. To test for possible regulatory effects on the three

central flowering time control genes thus far identified in beet, the *BvFL1* RNAi plants were analyzed for expression of *BTC1*, *BvFT1*, and *BvFT2* (**Figures 2B–D**). In the non-transgenic control plants, expression differences between samples harvested before and at the end of vernalization were largely consistent with previous reports (Pin et al., 2010, 2012), i.e., *BTC1* and *BvFT2* expression levels were higher at the end of vernalization than before vernalization, whereas *BvFT1* expression was strongly reduced at the end of vernalization. Down-regulation of *BvFL1* by RNAi did not result in consistent changes in expression of any of the central floral regulators.

In the two 35S::*BvFL1* events which were further analyzed (016-05C and 017-06C) *BvFL1* was stably over-expressed both before and at the end of vernalization (**Figure 2E**). The difference in expression levels between the two events was in approximate accordance with the respective transgene copy numbers (1 in 016-05C and 2–3 in 017-06C; **Table 1**). *BTC1* expression did not appear to be majorly affected by *BvFL1* over-expression (**Figure 2F**). *BvFT1* expression before vernalization was slightly higher in the 35S::*BvFL1* transformants than in the control plants but *BvFL1* over-expression did not prevent down-regulation of *BvFT1* by vernalization (**Figure 2G**). *BvFT2* expression was not detectable before vernalization in either the control or over-expression plants. *BvFT2* was expressed at the end of vernalization and was lower in the *BvFL1* transformants than in the controls (**Figure 2H**). None of the expression levels in the transformants deviated significantly from the control plants.

Expression analysis of *BvFT1*-*BvFT2* RNAi plants before and at the end of vernalization showed down-regulation of both of the *FT* genes (**Figure 3**). Because *BvFT2* is only expressed after vernalization, down-regulation of this gene was only detectable in the post-vernalization samples (**Figure 3C**). As described above, *BvFT1*-*BvFT2* RNAi transformants showed low accumulation of both endogene- and transgene-derived transcripts (including 014-07F and 020-01E; **Figure 3**) except for two events (018-06E, 019-01E) in which the transgene-derived transcripts accumulated to higher levels. The distinction between these two types of events was evident both before and at the end of vernalization.

DIURNAL EXPRESSION PROFILES OF FLORAL REGULATORS IN *BvFL1* OVER-EXPRESSION PLANTS

Previous reports for Arabidopsis indicated a regulation of the circadian clock by *FLC* (Swarup et al., 1999; El-Assal et al., 2003; Salathia et al., 2006). Therefore, the late-bolting *BvFL1* over-expression event 016-05C, which carries a single copy of the transgene, was assayed for changes in the diurnal expression profiles of the beet homolog of the circadian clock gene *GIGANTEA* (*GI*) (Pin et al., 2012) and the photoperiod response gene *BTC1*. *BvFT1*, *BvFT2* and *BvLHP1*, a homolog of the vernalization pathway gene *LIKE HETEROCHROMATIN 1* in Arabidopsis (GenBank accession number KJ636469), were also included in the analysis. Diurnal expression was analyzed under long-day conditions (16 h light, 8 h darkness) 4 weeks after the end of vernalization.

In the non-transgenic control plants, *BvFL1* had two broad peaks of expression at mid-day to mid-afternoon and in the second half of the night until early morning (**Figure 4A**), indicating

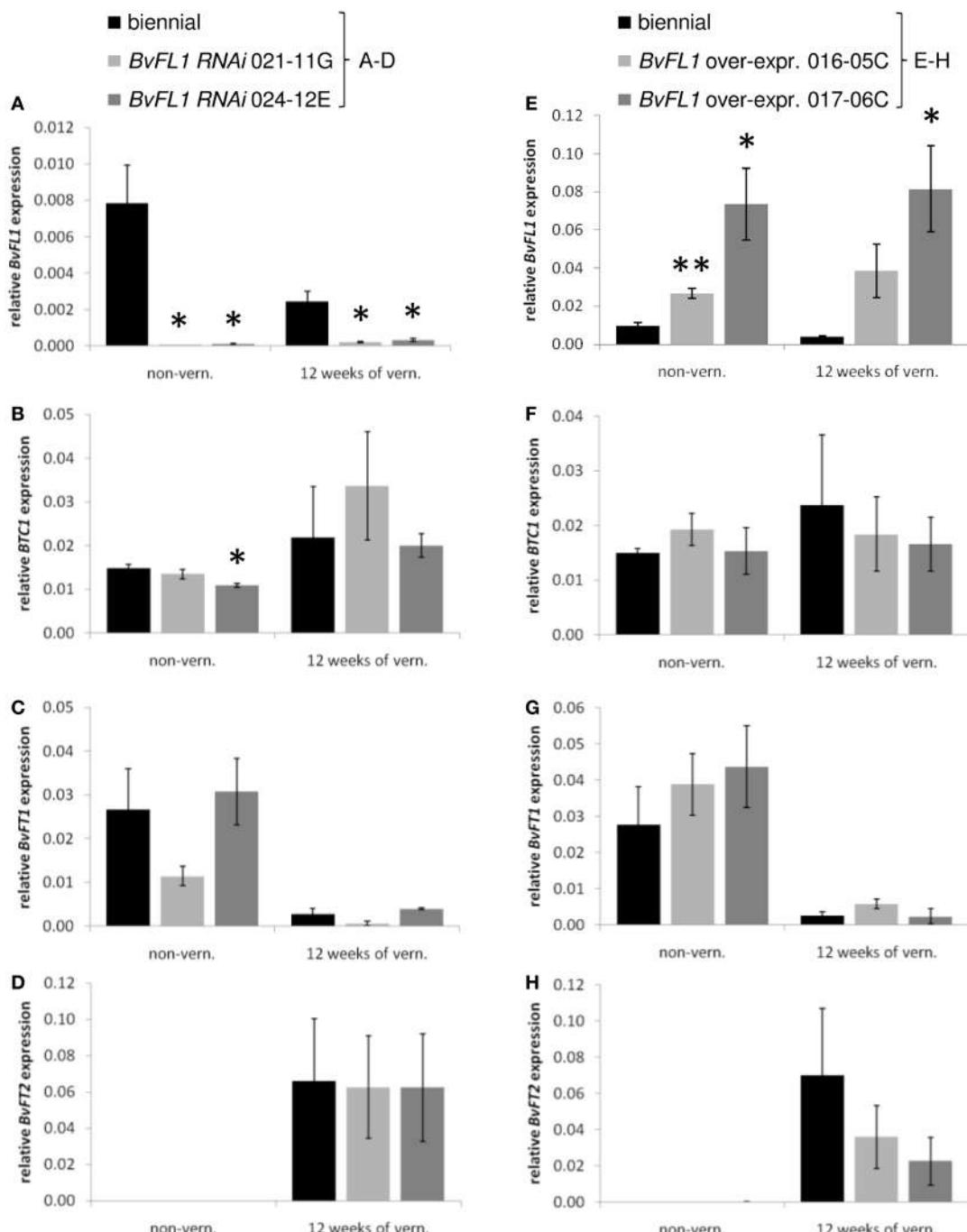
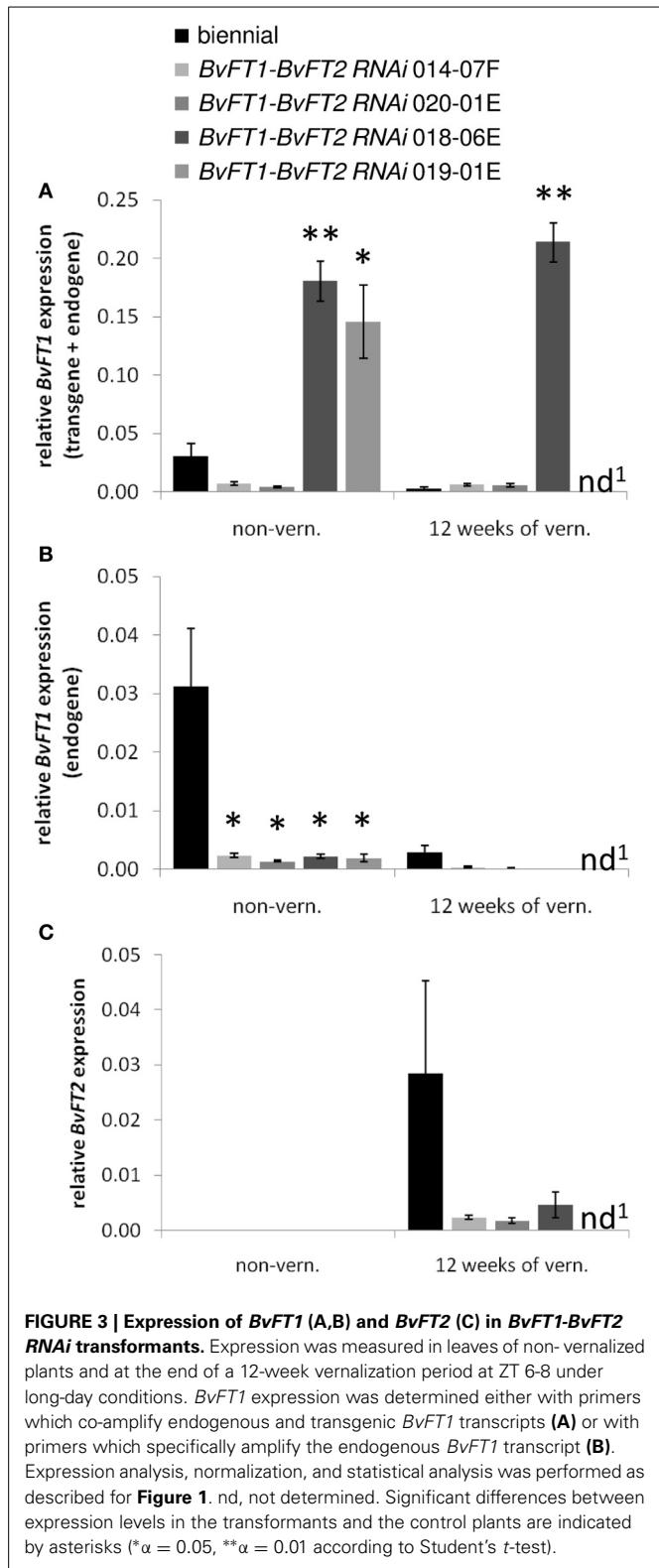


FIGURE 2 | Expression of floral regulators in *BvFL1 RNAi* (A–D) and *BvFL1* over-expression (E–H) transformants. Expression of *BvFL1* (A,E) and the floral regulators *BTCl* (B,F), *BvFT1* (C,G), and *BvFT2* (D,H) was measured in leaves of non-vernalized plants and at the end of a 12-week vernalization period at ZT 6–8

under long-day conditions. Expression analysis, normalization, and statistical analysis was performed as described for Figure 1. Significant differences between expression levels in the transformants and the control plants are indicated by asterisks (* $\alpha = 0.05$, ** $\alpha = 0.01$ according to Student's *t*-test).

that *BvFL1* itself is diurnally regulated. Over-expression of *BvFL1* resulted in strongly increased transcript accumulation during the entire course of the day (Figure 4B). Transcript accumulation was not constant but peaked at ZT 12. Diurnal fluctuations of similar amplitude in expression from a CaMV 35S promoter

in transgenic plants were observed before (Millar et al., 1992; Lu et al., 2011). *BvLHP1* transcript accumulation exhibited two peaks in the early afternoon (ZT 10) and in the middle of the night (ZT 20; Figure 4C). Over-expression of *BvFL1* correlated with a phase shift by approximately 2 h in *BvLHP1* expression



during the light cycle compared to the control plants, resulting in a peak of expression at ZT 12. *BvLHP1* expression in the dark was in phase with the control. Expression of *BvGI* (Figure 4D) and *BTC1* (Figure 4E) was similar as reported previously (Pin

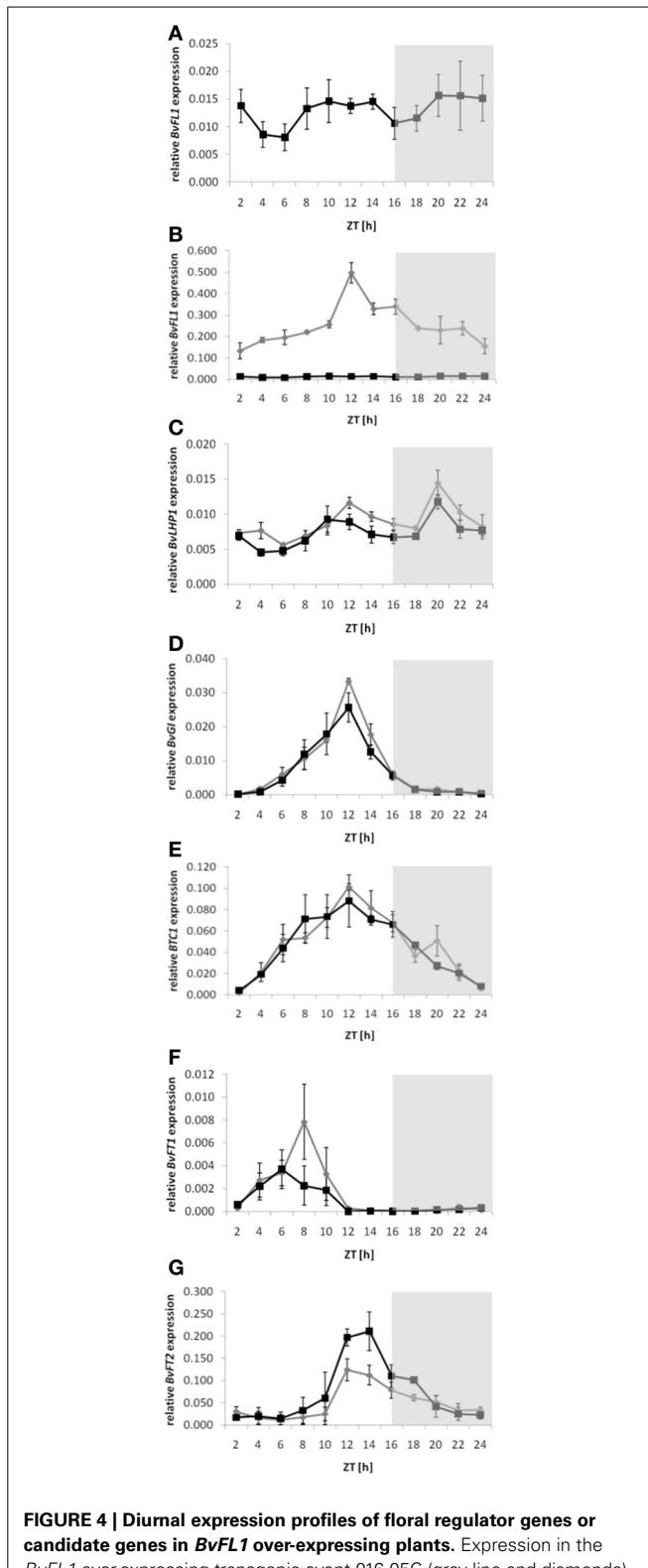


FIGURE 4 | Diurnal expression profiles of floral regulator genes or candidate genes in *BvFL1* over-expressing plants. Expression in the *BvFL1* over-expressing transgenic event 016-05C (gray line and diamonds) and the biennial control genotype (black line and squares) was determined 4 weeks after the end of vernalization under long-day conditions. **(A,B)** *BvFL1*, **(C)** *BvLHP1*, **(D)** *BvGI*, **(E)** *BTC1*, **(F)** *BvFT1*, and **(G)** *BvFT2*. Expression analysis and normalization was performed as described for Figure 1.

et al., 2012). While expression of both genes reached its maximum around mid-afternoon, the peak of expression was broader for *BTC1*. Overall, the expression profiles of both *BvGI* and *BTC1* were similar in the *BvFL1* over-expressing plants and the control plants.

As reported before (Pin et al., 2010, 2012), the floral repressor *BvFT1* is only relatively weakly expressed after vernalization but exhibits detectable transcript accumulation in the morning hours, whereas expression of the floral activator *BvFT2* peaks around mid-afternoon. Expression of these genes in the control plants of the current study were in accordance with the previous reports (Figures 4F,G). However, the *BvFL1* over-expressing plants revealed the following deviations from the regular expression patterns in the control plants: (1) *BvFT1* expression showed a sharp peak of increased expression around ZT 8, and (2) *BvFT2* showed a reduction in gene expression in the afternoon and evening when compared to the control plants.

DISCUSSION

Recent studies have revealed a central role of the *B* locus gene *BTC1* and its downstream target gene *BvFT1* in vernalization response and bolting control in beet (Pin et al., 2010, 2012). Furthermore, in contrast to Arabidopsis, where vernalization requirement and growth habit is governed by natural variation at *FLC* or its upstream activator *FRI*, life cycle control in beet is determined by allelic variants of *BTC1*. Despite the apparent differences in the genetic make-up of the core regulatory modules in Arabidopsis and beet, an *FLC* homolog has been identified in beet (Reeves et al., 2007). Complementation analysis in an early-flowering *flc* mutant in Arabidopsis showed that the *FLC*-like gene *BvFL1* was able to rescue the wild-type phenotype, but the function of *BvFL1* has not yet been analyzed in beet. The current study aimed to address the functional role of *BvFL1* and possible regulatory interactions with *BTC1* and/or the *BvFT1-BvFT2* module by transgenic analyses in beet. The main findings are that (1) down-regulation of *BvFL1* neither affects bolting time majorly after vernalization nor enables bolting without vernalization, (2) over-expression of *BvFL1* is not sufficient to prevent bolting after vernalization but can result in a moderate delay of bolting, and (3) co-silencing of the *BvFT1-BvFT2* module in *BvFT1 RNAi* transformants leads to a stronger bolting delay than *BvFL1* over-expression and high percentages of non-bolting plants in some events.

The observed lack of a floral inductive effect in *BvFL1 RNAi* transformants stands in contrast to observations in Arabidopsis, where mutation or antisense-mediated down-regulation of *FLC* strongly accelerates flowering (Michaels and Amasino, 1999; Sheldon et al., 1999, 2000) and can eliminate the very late-flowering phenotype found in winter-annual (vernalization-responsive) accessions (Michaels and Amasino, 2001). This observation corroborates the notion from the work on *BTC1* and *BvFT1* that in beet a different regulatory switch has evolved for the control of growth habit, and shows for the first time that in beet altered regulation of the *FLC*-like gene is not sufficient to promote an early-bolting (annual) growth habit. The fact that *BvFL1 RNAi* transformants are responsive to vernalization further suggests that vernalization can promote bolting

through a *BvFL1*-independent pathway. In Arabidopsis, despite the regulatory role of *FLC* in the vernalization pathway, *flc* null mutants are also vernalization-responsive, which suggested the presence of an *FLC*-independent vernalization response pathway also in this species (Michaels and Amasino, 2001). Later work implicated other MADS-box genes in *FLC*-independent regulation of vernalization response (Alexandre and Hennig, 2008). In beet, a *BvFL1*-independent vernalization response pathway is likely to involve at least in part the actions of *BTC1* and *BvFT1*.

While *BvFL1* may not have a key role in the regulation of vernalization requirement and response in beet, the moderate delay in bolting that was observed in transformants over-expressing *BvFL1* suggests that the gene has retained a functional role in the control of floral transition, and is consistent with the earlier complementation studies in Arabidopsis (Reeves et al., 2007). However, phenotypic effects of over-expression are not a definite proof of a gene's function in an endogenous biological process. For example, ectopic expression of the *A. thaliana* gene *FLC* in rice also delayed flowering despite the absence of *FLC*-like genes in rice (Tadege et al., 2003). With regard to growth habit, the biennial, vernalization-responsive sugar beet accession used in the current study is similar to winter-annual Arabidopsis accessions. Over-expression of *FLC* in winter-annual Arabidopsis accessions frequently resulted in transformants which completely failed to flower (Michaels and Amasino, 1999; Sheldon et al., 1999). In beet, however, complete suppression of bolting by *BvFL1* over-expression was not observed. The notion that *BvFL1* expression is not sufficient to prevent floral transition in beet also appears consistent with the previous finding that the temporary down-regulation of *BvFL1* during vernalization is reversed upon transfer to warmer temperatures (Reeves et al., 2007). The moderate phenotypic effects of altered *BvFL1* expression may suggest that the strong floral inhibitory effect of *BvFT1* (Pin et al., 2010) masks or overrides a possible contributory role of *BvFL1* in the repression of bolting.

In Arabidopsis, allelic variation at *FLC* was suggested to affect circadian period length (Swarup et al., 1999) and over-expression of *FLC* lengthened the circadian period by approximately 1 h (Salathia et al., 2006). Salathia et al. further argued that repression of *FLC* in response to vernalization and the resultant shorter circadian periods may reduce the critical daylength required for the photoperiod pathway to promote flowering, thus accelerating flowering in spring. In our study, expression of *BvFL1* showed diurnal oscillations both in the biennial control plants and the *BvFL1* over-expressing plants. While strong diurnal oscillations of *FLC* have not been reported in Arabidopsis (e.g., Fujiwara et al., 2010), a similar expression profile to that observed here for *BvFL1* was found by Lu et al. (2011), with peaks of *FLC* expression in the afternoon and at the end of the night. Among the putative clock-regulated genes analyzed in *BvFL1* over-expression plants, neither *BvGI* nor *BTC1* were majorly affected in their diurnal expression profiles. Expression of both of these genes and of *BvLHP1* was slightly elevated in the afternoon hours in the *BvFL1* over-expressing plants, but the differences were too subtle to be conclusive.

FLC inhibits floral transition at least in part by repression of *FT* in leaves, which involves a direct interaction of *FLC* protein

with *FT* chromatin (Helliwell et al., 2006; Searle et al., 2006). Our data for beet tentatively suggest that *BvFL1* over-expression leads to a reduction of *BvFT2* expression, which is apparent both at the end of vernalization (**Figure 2H**) and in the diurnal expression profile 4 weeks after vernalization (**Figure 4G**). *BvFT2* expression in *BvFL1* over-expressing transformants rises more slowly in the mid-day hours and is reduced compared to the control plants during the afternoon and evening hours. This suggests that the observed bolting delay may also be mediated by negative regulation of *BvFT2* by *BvFL1* in beet. However, although *BvFT2* down-regulation is consistent in all samples and is apparent at multiple consecutive time points in the diurnal expression profile, the differences are not statistically significant in pairwise comparisons with the respective controls. Similarly, the analysis of *BvFT1* in *BvFL1* over-expression plants showed an increase in *BvFT1* expression which however was not statistically significant. Thus, it remains speculative whether changes in *BvFT1* and/or *BvFT2* expression mediate the observed bolting delay in *BvFL1* over-expression plants.

The current study also revealed phenotypic effects of co-silencing of *BvFT1* and *BvFT2*. *BvFT1* and *BvFT2* share 80% sequence identity at the nucleotide sequence level within the 361 bp region of the coding sequence that was used for RNAi vector construction, including a 23 bp tract of perfect sequence identity, suggesting that down-regulation of *BvFT2* is due to off-target effects. Down-regulation of *BvFT1* by RNAi had not been achieved previously (cf. Pin et al., 2010). Although down-regulation of *BvFT1* in the RNAi transformants investigated here was accompanied by down-regulation of *BvFT2*, the data provide new evidence for the critical role in bolting control of the *BvFT1-BvFT2* module in beet, and show that the concomitant down-regulation of both activities inhibits rather than promotes bolting. The data also suggest that *BvFT1* expression before vernalization as it is typical for biennial beets is not necessary for pre-vernalization development and that the main function of *BvFT1* is its role in the control of vernalization response. This notion is consistent with the apparent lack of *BvFT1* expression in annual beets throughout development (under long-day conditions; Pin et al., 2010). All *BvFL1 RNAi*, *BvFL1* over-expression and *BvFT1-BvFT2 RNAi* transformants investigated here were grown and analyzed side-by-side with each other as well as with the *btc1 RNAi* transformants described by Pin et al. (2012), thus facilitating a comparative view. The strong phenotypic effect of altered regulation of the *BvFT1-BvFT2* module in *BvFT1 RNAi* transformants when compared to *BvFL1 RNAi* or over-expression point at the predominant role of the *FT* genes in bolting control in beet. Finally, it is also interesting to note that among all the *BvFL1*, *BvFT1-BvFT2*, and *btc1 RNAi* transformants, it was clearly the *btc1 RNAi* transformants which showed the strongest suppression of bolting, with multiple transgenic events in which bolting was completely suppressed until the end of the experiment 6 months after the end of vernalization (Pin et al., 2012).

Perhaps noteworthy, the strongest inhibitory effect on bolting was found in two transgenic events (018-06E and 019-01E) in which the *BvFT1* transgene was highly expressed despite a strong reduction in accumulation of the endogenous *BvFT1* transcript. A large number of plants derived from these events failed to bolt after vernalization [11 out of 15 plants (73%) and 7 out of 16

plants (44%), respectively], while the remaining plants of these events bolted very late and showed a stunted phenotype similar to *btc1 RNAi* transformants (Pin et al., 2012). The concomitant accumulation of the transgene transcript and silencing of the endogenous transcript may suggest that in these transformants transgenic and endogenous transcripts are not co-suppressed, but that the transgene transcript may trigger RNAi of the endogenous transcript without itself being a target of (efficient) RNAi-mediated transcript degradation. Because the two transformants carry multiple copies of the transgene it is conceivable that at least one of these copies carries the complete *BvFT1* inverted repeat cassette and effects RNAi, whereas another copy may have integrated only partially and escaped silencing. The cDNA fragment used for RNAi transgene construction spans ~67% of the full-length coding sequence and covers 88% of the central PEBP domain, including the functionally important amino acids in the fourth exon (Pin et al., 2010). The putative translation product, starting with the first in-frame ATG codon downstream of the 35S promoter, is predicted to contain 92 amino acids (~51%) of the full-length protein and ~67% of the PEBP domain. Thus, it is conceivable that expression of a partial *BvFT1* protein at least contributes to the particularly strong inhibition of bolting observed in these transformants. In this scenario, the protein sequence outside the 92 amino acid region would appear to be dispensable for repression of bolting by *BvFT1*.

In conclusion, our data show that *BvFL1* is not a major regulator of vernalization response in beet. A comparison with phenotypic data from *BvFT1-BvFT2 RNAi* plants and our previously described *btc1 RNAi* transformants further suggests that in beet the *BvFT1-BvFT2* module and its upstream regulator *BTC1* have evolved a more dominant role in the control of vernalization response and bolting time. Future comparative studies between both species may help to uncouple the contributions of *FLC* and *FLC*-like genes to floral regulation through direct effects on *FT* genes or upstream interactions between vernalization- and photoperiod responsive flowering time control mechanisms. From an evolutionary perspective, knowledge of conservation and divergence of floral control mechanisms between model species and the phylogenetically distant dicot species *B. vulgaris* is casting an increasingly interesting light on one of the best studied developmental processes in plants.

AUTHOR CONTRIBUTIONS

Sebastian H. Vogt designed and performed experiments and wrote the manuscript. Guy Weyens and Bettina Bork designed and performed experiments. Marc Lefebvre and Axel Schechert designed and supervised experiments. Andreas E. Müller designed and supervised the project and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00146/abstract>

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Memory of the vernalized state in plants including the model grass *Brachypodium distachyon*

Daniel P. Woods^{1,2,3}, Thomas S. Ream^{1,2†} and Richard M. Amasino^{1,2 *}

¹ Department of Biochemistry, University of Wisconsin-Madison, Madison, WI, USA

² U.S. Department of Energy-Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI, USA

³ Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI, USA

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Iain Robert Searle, The University of Adelaide, Australia

***Correspondence:**

Richard M. Amasino, Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706-1544, USA
e-mail: amasino@biochem.wisc.edu

†Present address:

Thomas S. Ream, Monsanto Company, 700 Chesterfield Parkway West, Chesterfield, MO, USA

Plant species that have a vernalization requirement exhibit variation in the ability to “remember” winter – i.e., variation in the stability of the vernalized state. Studies in *Arabidopsis* have demonstrated that molecular memory involves changes in the chromatin state and expression of the flowering repressor *FLOWERING LOCUS C*, and have revealed that single-gene differences can have large effects on the stability of the vernalized state. In the perennial *Arabidopsis* relative *Arabis alpina*, the lack of memory of winter is critical for its perennial life history. Our studies of flowering behavior in the model grass *Brachypodium distachyon* reveal extensive variation in the vernalization requirement, and studies of a particular *Brachypodium* accession that has a qualitative requirement for both cold exposure and inductive day length to flower reveal that *Brachypodium* can exhibit a highly stable vernalized state.

Keywords: vernalization, flowering, *Brachypodium*, epigenetics, life history

INTRODUCTION

Specific timing of flowering is an important adaptive trait that ensures flowering occurs when conditions are favorable. In many plant species, flowering takes place during a particular time of year in response to the sensing of seasonal cues, such as changing day lengths and/or temperature. One adaptation to the seasonal changes that occur in temperate climates has been the evolution of a vernalization response (Amasino, 2010; Andrés and Coupland, 2012). Vernalization is the process by which exposure to the prolonged cold of winter results in the ability to flower in the next growing season (Chouard, 1960). Cold exposure alone is typically not sufficient to induce flowering, but it often must be coupled to an additional inductive cue such as the increasing day lengths experienced during spring and summer months (Lang, 1952). Although satisfying the vernalization requirement often permits flowering after exposure to inductive cues, the key adaptive value of a vernalization requirement is that it prevents flowering in the fall season, thus ensuring that flowering does not commence as winter begins (Amasino, 2010).

CONCEPT OF MEMORY

An interesting component of the vernalization response in some plant species is that the acquisition of the ability to flower from cold exposure is mitotically stable after plants resume active growth in warm conditions (Amasino, 2004). This “memory of winter” is readily demonstrated in species for which there is a qualitative requirement for an additional cue to flower, such as inductive photoperiods, after the cold requirement has been satisfied. One

of the first studies on the stability of the vernalization state (or thermoinduced state as it was sometimes referred to) was done with a biennial strain of henbane (*Hyoscyamus niger*) that has an obligate requirement for a vernalizing cold exposure followed by long days (LD); i.e., vernalized henbane plants will not flower when grown in non-inductive short days (SD). In henbane, the vernalized state is “remembered” because vernalized plants grown in SD for long periods of time readily flower after they are shifted to inductive LD (Lang and Melchers, 1947).

MOLECULAR BASIS OF THE MEMORY OF WINTER

A considerable amount of information regarding the molecular nature of the vernalization pathway is known from studies in the eudicot model *Arabidopsis thaliana* (Brassicaceae; Kim et al., 2009; Amasino, 2010; Song et al., 2013). The vernalization requirement is largely due to the expression of a MADS-box-containing transcription factor, *FLOWERING LOCUS C* (*FLC*), which is an effective flowering repressor (Michaels and Amasino, 1999; Sheldon et al., 1999). The level of *FLC* expression is determined by an extensive regulatory network that includes components involved in small RNA metabolism (Swiezewski et al., 2009; Heo and Sung, 2011; Song et al., 2013) as well as a protein complex that appears to have evolved specifically for *FLC* activation (the FRIGIDA complex; Choi et al., 2011; Lee and Amasino, 2013). The *FLC* protein prevents flowering at a molecular level by binding to the promoters of specific genes and blocking their expression (Hellmell et al., 2006; Searle et al., 2006); these genes include *FT*, which encodes the mobile “florigen” signal in leaves as well as

SUPPRESSOR OF CONSTANS 1 (SOC1) and *FD* in meristems. *FD*, *FT*, and *SOC1* encode proteins that activate a suite of floral homeotic genes such as *APETALA1 (AP1)* that specify floral organs (Kim et al., 2009).

Vernalization results in the silencing of *FLC* expression (Michaels and Amasino, 1999). During winter, exposure to prolonged cold results in a polycomb-like, chromatin-modifying complex initiating the modification of *FLC* chromatin, transforming it from an active euchromatic state into a stably repressed heterochromatic state that remains repressed for the rest of the life cycle (for reviews see Amasino, 2010; Andrés and Coupland, 2012; Zografos and Sung, 2012; Song et al., 2013). During cold, a unique, cold-specific polycomb component known as *VERNALIZATION INSENSITIVE 3 (VIN3)* is induced; the VIN3 protein is necessary for polycomb-mediated *FLC* silencing (Sung and Amasino, 2004; Wood et al., 2006; De Lucia et al., 2008). The polycomb complex adds methyl groups to histone 3 (H3) at lysine 27 (K27) residues to form trimethylated H3 (H3K27me3), and increased H3K27me3 of *FLC* chromatin appears to be one of the first chromatin changes accompanying *FLC* silencing (Bastow et al., 2004; Sung and Amasino, 2004; Finnegan and Dennis, 2007; De Lucia et al., 2008; Angel et al., 2011). Vernalization-mediated *FLC* silencing is also associated with increased lysine 9 (K9) trimethylation at H3 (H3K9me3; Sung et al., 2006a,b), and H3K9me3 appears to be required for the memory of winter at *FLC*.

The H3K27me3 modification at *FLC* spreads and persists after the cold exposure is over (Finnegan and Dennis, 2007; De Lucia et al., 2008; Angel et al., 2011), and H3K9me3 is likely to spread as well. Because the vernalized state and *FLC* chromatin modification persist through mitotic cell divisions in meristem cells after cold treatment ends, it is reasonable to think of vernalization as an environmentally induced epigenetic switch (Amasino, 2004; Schmitz and Amasino, 2007). Although *FLC* repression is maintained throughout the plant's life cycle, the repressed state of *FLC* becomes reset to an active state in the following generation, resulting in the re-establishment of the vernalization requirement (Amasino, 2004; Schmitz and Amasino, 2007). The stable repression of *FLC* is consistent with the annual life history of *Arabidopsis* which involves the conversion of all shoot meristems to flowering which maximizes the number of progeny in a single cycle of reproduction (Amasino, 2009).

TO HAVE OR NOT TO HAVE MEMORY

In contrast with annual plants such as *Arabidopsis*, perennials live for many years and flower repeatedly throughout their lives. For perennials to persist for multiple growth cycles, it is critical that not all of the shoot meristems become irreversibly floral; rather, some meristems need to be reserved for next season's growth (Amasino, 2009; Turck and Coupland, 2013). Recently, aspects of the molecular basis of the perennial life history trait have been studied in *Arabis alpina*, a relative of *Arabidopsis* in the Brassicaceae (Wang et al., 2009; Bergonzi et al., 2013; Turck and Coupland, 2013). Like many accessions of *Arabidopsis*, *A. alpina* also requires vernalization in order to flower. However, unlike *Arabidopsis*, in *A. alpina* vernalization does not result in the flowering of all shoot meristems (Wang et al., 2009). Only

certain meristems (those that were most actively growing before cold exposure commenced) produce flowers, whereas other meristems produce only vegetative shoots following cold exposure (Wang et al., 2009).

In *A. alpina*, not all shoot meristems become floral at least in part because vernalization is "forgotten." In many (and probably all) Brassicaceae, a vernalization requirement results from flowering repression mediated by *FLC* or an *FLC* ortholog. In *A. alpina*, the *FLC* ortholog is known as *PERPETUAL FLOWER1 (PEP1)*; Wang et al., 2009). The *PEP1* expression pattern is similar to that of *FLC* before and during cold; specifically, it is highly expressed prior to cold exposure and is down-regulated during cold exposure. However, a key difference is that after cold exposure ends, *FLC* repression is maintained in *Arabidopsis*, whereas *PEP1* repression is forgotten: after cold exposure ends, *PEP1* mRNA levels begin to rise and eventually reach pre-vernalization levels (Wang et al., 2009). Furthermore, although the repressive chromatin mark, H3K27me3, increases during cold, it does not persist after cold exposure ends (Wang et al., 2009). Thus, the difference in the stability of chromatin modifications in *PEP1* versus *FLC* contributes to the perennial versus annual life history trait of *A. alpina* and *Arabidopsis*. As discussed below, single-gene mutations in *Arabidopsis* can result in *PEP1*-like behavior at the *FLC* locus including a transient, cold-specific increase in H3K27me3 (Sung and Amasino, 2004).

This perennial strategy "works" in *A. alpina* because in this species (as well as in *Arabidopsis*) once flowering commences it is irreversible and no longer subject to *FLC/PEP1*-mediated repression. Some of the shoot meristems of *A. alpina* become irreversibly committed to flowering before post-vernalization *PEP1* levels rise, whereas the post-vernalization resumption of *PEP1* expression appears to prevent flowering in other meristems thus "reserving" those meristems for the next growing season (Wang et al., 2009). Irreversible *FLC*-independent flowering results, at least in part in *Arabidopsis*, from a positive feedback loop involving the floral meristem-identity genes *LEAFY* and *APETALA1* which activate each other's expression (Liljegren et al., 1999), and the feedback loop is not subject to *FLC* repression.

GENETIC VARIATION FOR MEMORY

Interestingly, the stability of the vernalized, repressed state of *FLC* in *Arabidopsis* is easily perturbed through single-gene mutations (Levy et al., 2002; Sung et al., 2006a; Schmitz et al., 2008; Kim et al., 2009). For example, in *vrn1*, *prmt5*, or *lhp1* mutants, cold-mediated *FLC* repression is transient similar to that in *A. alpina* – i.e., *FLC* is repressed during cold exposure, but expression rises after plants resume growth in warmer conditions (Levy et al., 2002; Sung et al., 2006a; Schmitz et al., 2008). *VRN1*, *PRMT5*, and *LHP1* do not encode components of the polycomb complex; rather, they encode other types of chromatin-modifying proteins involved in epigenetically "locking in" the repressed state of *FLC* such that polycomb-initiated repression becomes mitotically stable in warm conditions (Kim et al., 2009). This mitotic stability appears to require an increased level of H3K9 trimethylation at *FLC*, as well as H3K27 trimethylation (Bastow et al., 2004; Sung and Amasino, 2004; Sung et al., 2006a,b). Thus, a single-gene change can determine the difference between a memory of winter or lack thereof,

and it might be expected that families of plants in addition to the Brassicaceae would contain vernalization-requiring members that had a memory of winter and members that did not depending upon the life history strategies they have evolved.

MEMORY IN A GRASS MODEL

Vernalization-requiring species exist in many plant groups spanning angiosperm diversification (Preston and Sandve, 2013). There are additional examples of species (or varieties within a species) in which the vernalized state is not stable in non-inductive SD conditions such as sugar beet (Caryophyllales; Margara, 1960), primrose (Myrtales; Chouard, 1960), carrot (Apiales; Bernier et al., 1981; Bernier, 1988), wheat (Poales; Evans, 1987), and *Cheiranthus* (Brassicaceae; Barendse, 1964). Thus, many plant species that have a vernalization requirement do not have the ability to “remember” prior cold.

We sought to determine if the small, temperate grass *Brachypodium distachyon* has the ability to “remember” prior cold. Recently, we and others have characterized natural variation in the vernalization response in many *Brachypodium* accessions, and we found considerable variation in flowering behavior, ranging from accessions that exhibit rapid flowering without prior cold exposure in inductive LD to accessions that have an obligate vernalization requirement (Schwartz et al., 2010; Ream et al., 2012, 2014; Colton-Gagnon et al., 2013; Tyler et al., 2014). Furthermore, among the accessions that have an obligate vernalization requirement, the amount of cold needed to saturate the vernalization response ranges from 2 weeks to greater than 16 weeks (Ream et al., 2014). With respect to the requirement for inductive photoperiods, none of the *Brachypodium* accessions tested flower after several months of outgrowth in SD (8-h day length) even if vernalized extensively (Ream et al., 2014) i.e., like biennial henbane, many *Brachypodium* accessions have an obligate requirement for both vernalization and inductive photoperiods in order to flower. Thus, there are *Brachypodium* accessions that can be used to investigate whether or not *Brachypodium* can remember prior cold with an experimental design similar to the classic work first done in henbane by Lang and Melchers (1947).

To determine if the vernalized state is mitotically stable in *Brachypodium*, we grew vernalized plants in non-inductive photoperiods before shifting to inductive photoperiods (experimental design is outlined in **Figure 1A**). Briefly, we first vernalized the accession Bd29-1 as imbibed seed for 8 weeks, which is a saturating vernalization treatment (Ream et al., 2014). After the vernalization treatment, we placed the vernalized seeds (and non-vernalized controls at the same stage of development) in either non-inductive SD or inductive 20-h LD. Some of the vernalized as well as non-vernalized plants grown in SD for 70 days were then shifted to LD. The shift of the vernalized plants from SD to LD revealed that the vernalized state was robustly maintained in SD because the time to flowering after the shift to LD was the same as that for plants directly moved from cold exposure into inductive photoperiods (**Figure 1B**). Specifically, the vernalized plants directly moved into LD as well as the vernalized plants first moved into SD flowered in fewer than 30 days forming only four leaves in inductive LD prior to flowering (**Figure 1B**, leaf data not shown). This indicates

that, as is the case in henbane, there are accessions of *Brachypodium* in which the vernalization response is mitotically stable and that vernalization provides only the competence to flower, given none of the SD-only controls flowered during the duration of the experiment.

There are several controls for this experiment. One is to shift non-vernalized plants from SD to LD. The reason for this control is that short days are able to substitute for vernalization in some accessions of *Brachypodium* (Schwartz et al., 2010; Ream et al., 2014) as well as in accessions of other grass species such as wheat and rye (Purvis and Gregory, 1937; Evans, 1987; Heide, 1994). However, the Bd29-1 accession was chosen for this study because growth in SD does not have any effect on the vernalization requirement (**Figure 1B**). An additional control is the growth of both non-vernalized and vernalized plants in SD or LD for the duration of the experiment. None of the SD-only control plants or the LD-non-vernalized controls flowered during the duration of the experiment [170 days; **Figure 1A**; several SD-only control plants were also dissected after 170 days of growth and all meristems were vegetative (data not shown)]. The SD-only controls demonstrate that indeed growth in SDs does not permit flowering even in vernalized plants. The LD-only controls were chosen to ensure that the robust flowering observed in the vernalized plants was indeed due to the prior vernalization treatment and not simply due to the age of the plant when shifted into inductive LD. As expected for a species with a monocarpic life history, vernalized plants grown in LD senesced rapidly after seed fill whereas non-vernalized controls were green and still actively producing leaves throughout the duration of the experiment (**Figures 1B,C**).

We also determined whether the quantitative aspect of the memory of vernalization is maintained in SD – i.e., is there a memory of the duration of cold exposure in *Brachypodium* when prolonged growth in SD separates cold exposure from a shift into inductive LD? Accordingly, imbibed seeds of Bd29-1 were exposed to varying lengths of cold (4, 6, 8, and 10 weeks), and then transferred to SD for 120 days prior to transfer to inductive LD (**Figure 2**; note this “memory test” is longer than the 70-day SD treatment presented in **Figure 1**). Cold exposures of 4 and 6 weeks are sub-saturating for vernalization in Bd29-1 (Ream et al., 2014). In this study, controls were similar to those presented in **Figure 1**: vernalized plants did not flower in SD and growth in continuous LD without prior vernalization also did not result in flowering illustrating the obligate nature of the vernalization requirement. A key control was the transfer of plants directly to inductive LD after cold exposure, which enabled a comparison of the efficacy of different durations of cold exposure with and without an interlude between cold exposure and photoperiodic induction of flowering. Even sub-saturating durations of cold exposure were effectively “remembered” during this long SD interlude as shown by the similar time to flowering after exposure to LD commenced in the plants exposed to 4 or 6 weeks of cold and then shifted immediately or after 120 days to LD (**Figure 2**). Saturating cold exposures of 8 and 10 weeks were also fully “remembered” during the 120-day SD treatment (**Figure 2**).

Information about the vernalization systems in Pooideae has largely been derived from studies of existing allelic variation in

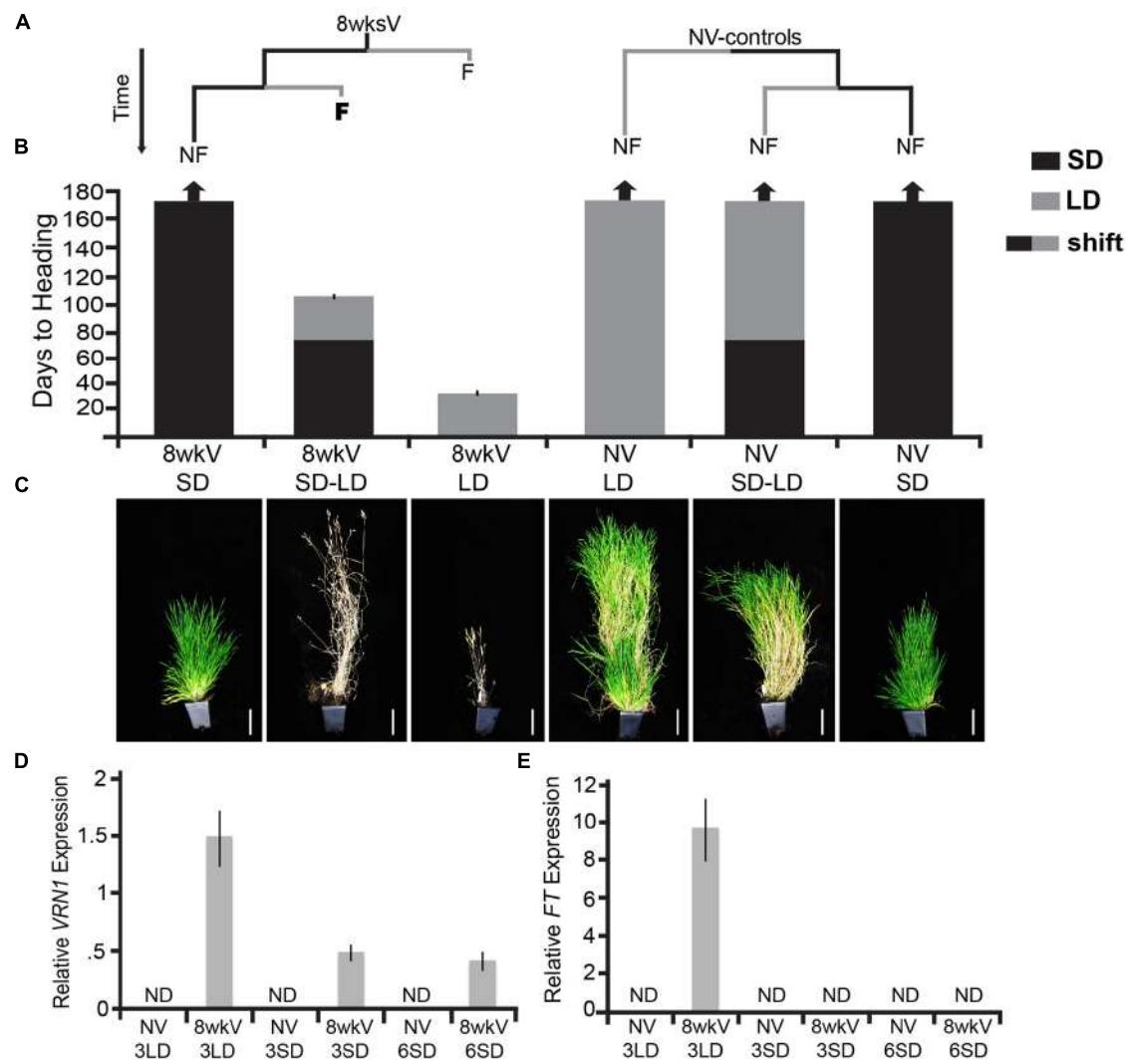


FIGURE 1 |The vernalization response is mitotically stable in *Brachypodium distachyon* (Bd29-1). **(A)** Plants were exposed to either 8 weeks of cold to saturate the vernalization response (8wksV) or were non-vernalized (NV-controls). Both the 8wksV and NV treated plants were placed either into inductive 20-h long days (LD; indicated by the gray bar) or non-inductive 8-h short days (SD; indicated by the black bar). After 70 days, some of the plants that had been in SD were shifted into LD, and, as a control the remainder of the SD-grown plants were kept in SD for the duration of the experiment. Only vernalized plants flowered in LD indicated by the letter F (flower); plants that did not flower are indicated by NF (non-flowering). **(B)** Days to heading was measured as the number of days to first spikelet emergence as done in Ream et al. (2014). Note that time during vernalization treatment is excluded. Arrows at the top of the bar graph indicate plants that did not flower for the duration of the experiment (170 days). Black bars represent SD-grown plants; gray bars represent LD-grown plants and bars with black and gray represent those plants that were first exposed to SD for

70 days followed by a shift into LD (SD-LD). Bars represent the days to heading average of six plants (experiment was repeated with similar results). **(C)** Photographs of representative plants at the end of experiment. Plants were grown and scored as described in Ream et al. (2014). Scale bar = 9 cm. **(D,E)** *VRN1* and *FT* expression in a newly expanded 3rd leaf and 6th leaf of Bd29-1. Imbibed seeds in soil were exposed to either a saturating cold treatment (5°C for 8 weeks) or no cold. At the end of cold treatment, non-vernalized and cold-treated plants were grown in SD and LD for ~3 weeks (3LD, 3SD) and SD for an additional ~6 weeks (6SD) at 22°C during the light phase and 18°C during the dark phase until plants reached the third leaf stage and the sixth leaf stage (Note vernalized 29-1 in LD had flowered by 5 weeks so did not sample 6LD). *VRN1* and *FT* transcript levels were determined by RT-qPCR as described in Ream et al. (2014) and normalized to *UBIQUITIN-CONJUGATING ENZYME18*. ND denotes no expression detected. Bars represent the average of four biological replicates ± standard deviation (three leaves per replicate).

wheat and barley. Such studies are consistent with a flowering model in which three genes, *VERNALIZATION1* (*VRN1*), *VERNALIZATION2* (*VRN2*), and *VERNALIZATION3* (*VRN3*) form a regulatory loop in leaves that responds to vernalization and photoperiod (Greenup et al., 2009; Distelfeld and Dubcovsky, 2010). *VRN3* is orthologous to *FT* (Yan et al., 2006) and hereafter will

be referred to as *FT*. Prior to cold exposure, *VRN2* represses *FT* expression and thus prevents flowering, whereas during and after cold *VRN2* expression decreases thus permitting flowering (Yan et al., 2004; Sasani et al., 2009; Distelfeld and Dubcovsky, 2010). Therefore, *VRN2* occupies a position in the flowering “circuitry” analogous to that of *FLC* (both are inhibitors of flowering that are

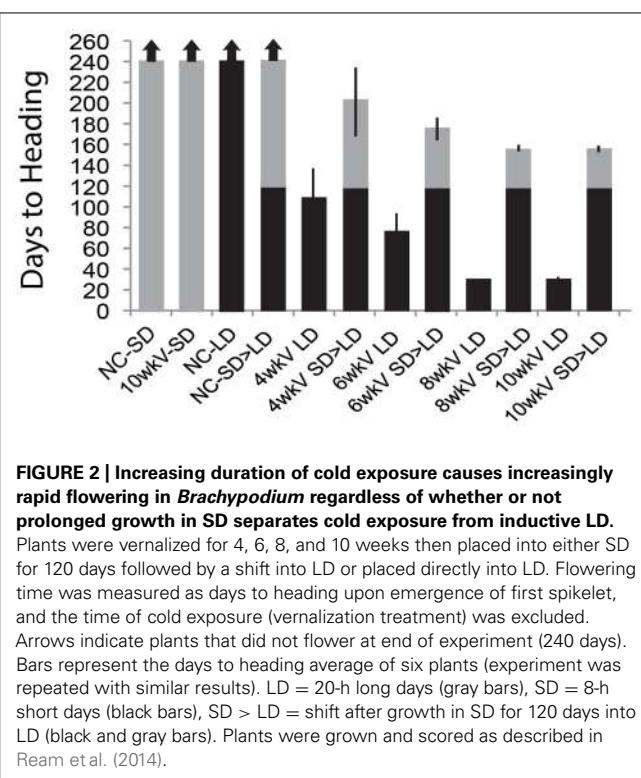


FIGURE 2 | Increasing duration of cold exposure causes increasingly rapid flowering in *Brachypodium* regardless of whether or not prolonged growth in SD separates cold exposure from inductive LD.

Plants were vernalized for 4, 6, 8, and 10 weeks then placed into either SD for 120 days followed by a shift into LD or placed directly into LD. Flowering time was measured as days to heading upon emergence of first spikelet, and the time of cold exposure (vernalization treatment) was excluded. Arrows indicate plants that did not flower at end of experiment (240 days). Bars represent the days to heading average of six plants (experiment was repeated with similar results). LD = 20-h long days (gray bars), SD = 8-h short days (black bars), SD > LD = shift after growth in SD for 120 days into LD (black and gray bars). Plants were grown and scored as described in Ream et al. (2014).

repressed by cold), although *VRN2* encodes a protein not related to *FLC* – it is a CCT domain-containing transcription factor and part of the type VI *CO-like* family of genes (Yan et al., 2004). Unlike, *FLC* in which chromatin-level suppression is the basis of memory, *VRN2* suppression is not likely to be the primary event in memory for two reasons. One is that no changes in chromatin marks have been observed around the *VRN2* locus during or after cold (Oliver et al., 2009). The other is that in *Brachypodium* *VRN2* mRNA levels are the same before and after cold exposure and this expression pattern is not consistent with *VRN2* acting as part of an evolutionarily conserved memory system in grasses (Ream et al., 2014).

VRN1, however, does exhibit cold-mediated chromatin changes. *VRN1* is a repressor of *VRN2* and it is up-regulated in leaves by cold exposure and its increased expression is maintained in warm post-vernalization conditions (Yan et al., 2004; Sasani et al., 2009; Distelfeld and Dubcovsky, 2010; Chen and Dubcovsky, 2012). The activation of *VRN1* by cold is accompanied by a decrease in the repressive chromatin modification H3K27 methylation and an increase in activating H3K4 methylation in a presumed regulatory region of its first intron (Oliver et al., 2009, 2013). The level of *VRN1* expression is proportional to the amount of cold experienced and thus correlates with the quantitative nature of the vernalization response in wheat, barley and *Brachypodium* (Yan et al., 2003; Sasani et al., 2009; Ream et al., 2014). Furthermore, the “henbane-like” behavior of certain *Brachypodium* accessions such as 29-1 enabled us to determine that increased *VRN1* expression is maintained after cold exposure regardless of whether or not the plants are shifted to inductive photoperiods (Figure 1D). That *VRN1* expression is maintained after

cold exposure in SD – conditions in which *FT* is not expressed and flowering does not occur – demonstrates that vernalization causes a stable *VRN1* “on state” that is independent of *FT* expression (Figures 1D,E). This day length-independent stability of the on state of *VRN1* after cold exposure is consistent with a role for stable *VRN1* activation to contribute to the memory of the vernalized state in *Brachypodium*. That *VRN1* mRNA levels are higher in LD than in SD after vernalization is likely to be a result of *FT* enhancing *VRN1* expression as shown in other cereals (Yan et al., 2006; Sasani et al., 2009; Shimada et al., 2009; Distelfeld and Dubcovsky, 2010).

There are other candidates for genes that may have a role in the memory of winter in grasses. For example, Huan et al. (2013) recently identified several hundred genes in *Brachypodium* for which the expression patterns changed during cold and the changes are maintained after 7 days post cold. It will be interesting to determine which *Brachypodium* genes maintain vernalization-mediated expression changes during a “long-term memory test” of prolonged exposure to SD after vernalization. Recently *FLC-like* genes have been identified in monocots, but whether or not these *FLC-like* genes have a role in flowering in the grass lineage remains to be experimentally determined (Ruelens et al., 2013). There remains much to learn about the molecular basis of vernalization in temperate grasses.

AUTHOR CONTRIBUTIONS

Daniel P. Woods, Richard M. Amasino, and Thomas S. Ream designed the experiments. Daniel P. Woods performed the experiments. Daniel P. Woods and Richard M. Amasino wrote and edited the manuscript.

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Florigen and anti-florigen – a systemic mechanism for coordinating growth and termination in flowering plants

Eliezer Lifschitz¹*, Brian G. Ayre² and Yuval Eshed³

¹ Department of Biology, Technion – Israel Institute of Technology, Haifa, Israel

² Department of Biological Sciences, University of North Texas, Denton, TX, USA

³ Department of Plant Sciences, Weizmann Institute of Science, Rehovot, Israel

Edited by:

George Coupland, Max Planck Society, Germany

Reviewed by:

Steve M. Mount, University of Maryland, College Park, USA
Rita Sharma, Jawaharlal Nehru University, India

***Correspondence:**

Eliezer Lifschitz, Department of Biology, Technion – Israel Institute of Technology, Technion City, Haifa 3200003, Israel
e-mail: lifs@tx.technion.ac.il

Genetic studies in *Arabidopsis* established *FLOWERING LOCUS T* (*FT*) as a key flower-promoting gene in photoperiodic systems. Grafting experiments established unequivocal one-to-one relations between *SINGLE FLOWER TRUSS* (*SFT*), a tomato homolog of *FT*, and the hypothetical florigen, in all flowering plants. Additional studies of *SFT* and *SELF PRUNING* (*SP*, homolog of *TFL1*), two antagonistic genes regulating the architecture of the sympodial shoot system, have suggested that transition to flowering in the day-neutral and perennial tomato is synonymous with “termination.” Dosage manipulation of its endogenous and mobile, graft-transmissible levels demonstrated that florigen regulates termination and transition to flowering in an *SP*-dependent manner and, by the same token, that high florigen levels induce growth arrest and termination in meristems across the tomato shoot system. It was thus proposed that growth balances, and consequently the patterning of the shoot systems in all plants, are mediated by endogenous, meristem-specific dynamic *SFT/SP* ratios and that shifts to termination by changing *SFT/SP* ratios are triggered by the imported florigen, the mobile form of *SFT*. Florigen is a universal plant growth hormone inherently checked by a complementary antagonistic systemic system. Thus, an examination of the endogenous functions of *FT*-like genes, or of the systemic roles of the mobile florigen in any plant species, that fails to pay careful attention to the balancing antagonistic systems, or to consider its functions in day-neutral or perennial plants, would be incomplete.

Keywords: flowering time, florigen, mobile growth terminators, *SFT/SP* regulatory hierarchy, shoot architecture, sympodial tomato

EVOLUTION OF THE FLORIGEN EXPERIMENTAL PLATFORM AND THE SFT/SP REGULATORY PARADIGM IN TOMATO

The florigen hypothesis emerged from elegant grafting experiments in photoperiod-sensitive plants (Chailakhyan, 1936a,b). Extensive experiments in the following 40 years, using a variety of photoperiod-sensitive plants, supported the florigen paradigm and established its core physiological parameters. These were critically evaluated in the superb compendium directed by Zeevaart (1976) and can be summarized as follows: (A) Changing light regimes induces systemic florigenic signals in cotyledons and leaves, which are transported, primarily via the phloem, to the apical meristems, where they induce transition to flowering. (B) While the primary environmental inductive signals may vary, the final stimulus is universal and thus, can be transmitted between species; long-day and short-day plants respond to the same florigenic signal. (C) The florigenic stimulus and the flowering response are quantitative. (D) The florigenic stimulus is balanced by systemic anti-florigenic agents.

In the domains of classic plant physiology, florigen was considered the ultimate and sometimes the sole agent for flowering. The classification of florigen as a systemic stimulant and the prevailing expectation that it is a metabolic product analogous

to auxin and other plant hormones, laid the foundation for decades of futile hunts. Florigen took on mythical proportions, and became the “Holy Grail” of plant biology: extensively sought after, but never found (Bernier, 1988; Zeevaart, 2006). With the adoption of genetic and molecular approaches in plant biology, pioneering screens for flowering genes identified five discrete flowering pathways (Koornneef et al., 1991; Simpson and Dean, 2002; Turck et al., 2008). The discovery of seemingly linear, and only partially overlapping genetic pathways, gave rise to post-florigenic interpretations of floral induction, in which florigen had no place. However, further genetic analyses in *Arabidopsis* showed that while several independent flowering inducing pathways exist, the final outputs of all pathways converge on a small number of flowering promoting genes, one notable one being *FLOWERING LOCUS T* (*FT*; Samach et al., 2000; Putterill et al., 2004).

Our encounter with the florigen odyssey was stirred by the analysis of *SELF-PRUNING* (*SP*) in tomato (Yeager, 1927; MacArthur, 1932; Pnueli et al., 1998), a perennial, day-neutral bush with a stereotypical sympodial growth habit (Figure 1). The upright growth of tomato is manifested by an apparent linear shoot consisting of consecutive sympodial units (SU), each forming three leaves before terminating in a compound inflorescence. Mutant *sp*

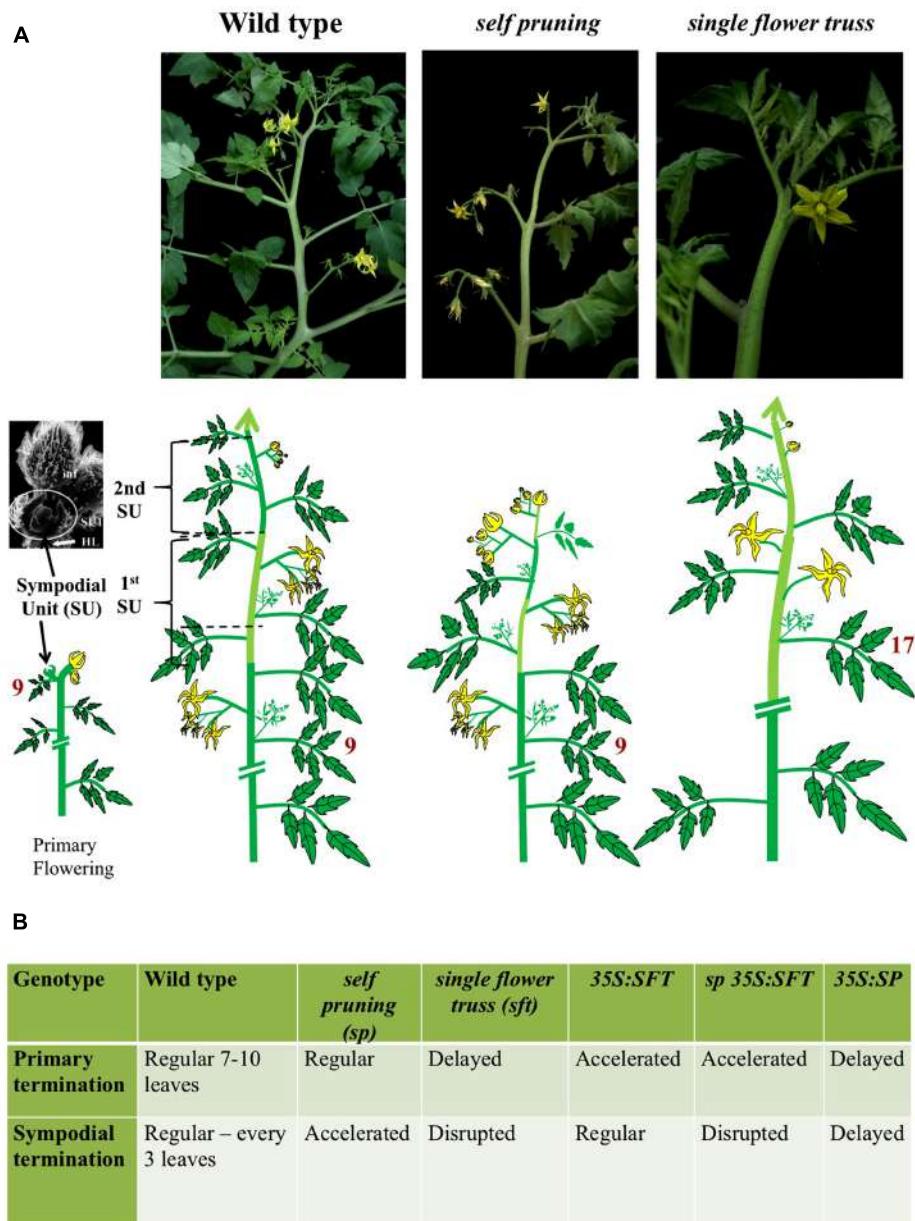


FIGURE 1 | Genetic regulation of shoot architecture in sympodial tomato. (A) Live images (top) and the corresponding schematic illustrations (bottom) of the wild type, *self-pruning* and *single flower truss* shoots of tomato. The primary shoot of wild type tomato (left) is terminated by the first inflorescence relatively early, after about 8–12 leaves. The first termination event activates the sympodial cycle (bottom left). In sympodial plants, the apparent main shoot consists of a reiterated array of sympodial units (SU). Each SU arises from the most proximal axillary meristems, and, depending on the prior regulated termination of the preceding unit, consists, in the case of tomato, of three vegetative nodes and a terminal inflorescence. The tomato inflorescence itself is a sympodial structure in which vegetative nodes are suppressed, intercalary leaves removed and each SU is terminated by a single flower. Similarly, the complexity of SUs may vary between systems being reduced to two vegetative nodes in cotton or one in Petunia. The “determinate” shoot of the *self-pruning* mutant plants (middle). A mutant *sp* gene accelerates the termination of SUs but does not change the rules of the sympodial habit as such. The result is a progressive reduction in the number of vegetative nodes between inflorescences in a pattern that depends on light intensity and genetic background. The indeterminate growth

in *sft* mutant plants (right). Unlike *SP*, the *SFT* gene targets, among its other pleiotropic functions, the sympodial branching pattern proper. In *sft*, primary termination is delayed and the terminating organ forms one flower and then proceeds as a vegetative shoot with irregular intercalary flowers. And since the formation of a new SU depends on the termination of the former one, the incomplete termination in *sft* results in the delayed formation, or complete suppression of the sympodial buds. Axillary buds release form leaves of the primary shoot or vegetative inflorescence follow the same path to generate the *sft* shoot system. Note that no full-proof loss-of-function allele of *SFT* is available and that the number of leaves formed by the vegetative inflorescence depends on the genetic background, and on the integral light doses. **(B)** Dose-dependent regulation of primary and sympodial termination by the *SFT* and *SP* genes. The *SFT/SP* paradigm is derived from the contrasting, but at the same time mutually dependent effects, of the loss and gain of functions of the two CETS genes. For examples: *sft* disrupts the sympodial habit but is epistatic to *sp* in the double mutant combination. The effects of *SFT* on the sympodial habit, stem growth, and leaf complexity depend on the dose of *SP* and in general the morphogenetic effects of mutant *SP* depend on a functional *SFT*.

plants form progressively shorter SUs, until the shoots terminate in two successive inflorescences (**Figure 1**). Therefore, in wild type (WT) and in *sp* plants, flowering is synonymous with termination (i.e., termination of the SU), with *SP* functioning as an anti-terminator, maintaining vegetative growth (i.e., production of leaves) in each SU. This basic understanding inspired the appreciation that “termination” is the prime function of florigen. In retrospect, *SP* was the first annotated flowering antagonist gene and the introduction of the recessive *sp* gene into tomato cultivars 70 years ago, facilitated mechanical harvesting, industrial production, and the irreversible flooding of the world with ketchup.

The identification of *CENTRORADIALIS* (*CEN*) of *Antirrhinum majus* and *TFL1* of *Arabidopsis* as homologous genes that maintain the indeterminate habit of the shoot apical meristems (SAMs) of monopodial plants (Bradley et al., 1996, 1997), conceptually and practically facilitated the cloning of *SP* as the third member in the *CETS* gene family (Pnueli et al., 1998). These findings were followed by the cloning of *FT*, another *CETS*-family member (Kardailsky et al., 1999; Kobayashi et al., 1999). Then, critical evidence was obtained that the proteins produced by *SP* of tomato and *FT* of *Arabidopsis* share binding partners (Pnueli et al., 2001) and that the tomato homolog of *FT* is the late-flowering *SFT* gene (Lifschitz et al., 2006). In contrast to *SP*, inactivation of *SFT* suppresses termination, consequently promoting the formation of an indeterminate vegetative inflorescence, typically consisting of one or a few flowers intervened by leaves. Furthermore, because the release of new SUs is linked to termination, *sft* concomitantly suppresses the timely formation of SUs (**Figure 1**). By regulating the periodicity of vegetative–reproductive switches, *SP* and *SFT* dictate the overall architecture of the shoot system.

Thus, genes promoting termination and flowering in monopodial, indeterminate, and photoperiod-sensitive plants congregate under a single molecular umbrella together with genes regulating the growth/termination cycling in perennial day-neutral plants. The evidence for a common molecular flower-promoting denominator in such diverged systems, and the fact that of all genes assigned to the multiple flowering pathways, only *FT/SFT* were not transcription factors, expedited our attempts to duplicate the grafting experiments that evoked the florigen hypothesis. Instead of exploiting donors induced by photoperiodic signals, we used *SFT*-overexpressing tomato plants, and instead of receptors/testers growing under non-permissive day-length conditions, we used tomato lines defective in flowering genes. These experiments showed that *SFT* generates graft-transmissible signals that substitute for external and internal flowering signals and established, for the first time, an unequivocal one-to-one relation between the elusive florigen and a single Mendelian gene (**Figure 2**; Lifschitz et al., 2006). The requirements for universality were satisfied by the mobile florigen-complementing flowering mutants independent of light regimes and were further substantiated by demonstrating that a tomato donor of *SFT* rescued flowering of the classic Maryland Mammoth tobacco grown under non-permissive conditions (Garner and Allard, 1920; Yang et al., 2007). The genetic evidence for the florigenic signal being a protein, and not RNA, is convincing and amply discussed

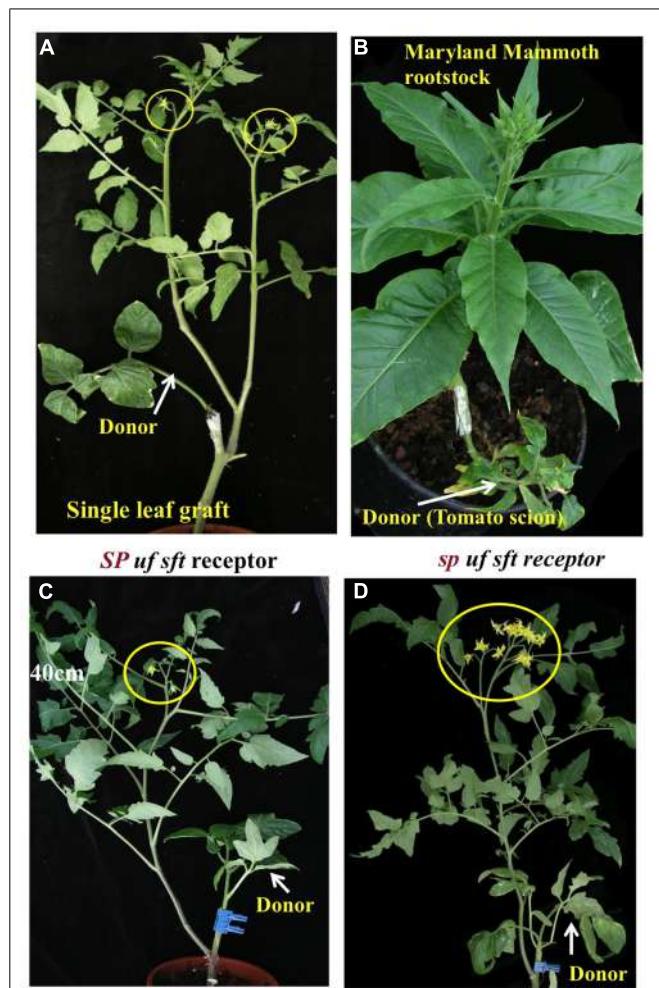


FIGURE 2 | Systemic delivery of Florigen and its local check by SP.

(**A**) A single leaf donor can stimulate flowering in an ever-vegetative *uf sft* recipient. (**B**) A Tomato florigen donor induces flowering in a long-day grown MM tobacco plant (Lifschitz et al., 2006). (**C,D**) Quantitative amelioration of Florigen impact – the regular response of ever-vegetative *uf sft* recipient shoot (**C**) is greatly enhanced in the absence of *SP* (**D**).

(Lifschitz et al., 2006; Kobayashi and Weigel, 2007; Lin et al., 2007; Mathieu et al., 2007; Turck et al., 2008), although critical details of the systemic pathway as discussed below, are still lacking or under debate.

Gene dosage analysis and epistasis tests using loss- and gain-of-function *SFT* and *SP* genes, revealed that, in addition to their effects on flowering, the two genes are involved in multi-organ pleiotropic effects, all of which can be traced to shifts in the growth-termination equilibrium. Concomitantly, grafting experiments showed that every developmental change conditioned by endogenous shifts in the *SFT/SP* ratios can be reproduced by elevated ratios imposed by graft-transmissible florigen (Shalit et al., 2009).

Integration of the results from complementary grafting and genetic experiments provided a new working hypothesis for the florigen world: endogenous *SFT/SP* ratios regulate local growth-termination equilibria in all meristems of the tomato shoot system.

A switch from growth to maturation and termination is triggered by an organ-specific shift from a low to a high SFT/SP ratio. In its refined version, and within given thresholds, the SFT/SP paradigm specifies that the mere shift, rather than the absolute levels of its components, determines the developmental outcome. Florigen originates primarily in mature leaves, which are the best exporters of florigen, but is distributed to all organs including other developing leaves. Thus, by enhancing the maturation of acceptor leaves, florigen generates an autoregulatory systemic information system. Florigen functions as a universal growth hormone (Shalit et al., 2009; Turnbull, 2011), where its role in boosting flowering reflects its fundamental function as a modifier of growth and termination across the shoot systems. In all its roles, the florigen protein emerges as both the architectural regulator and “great communicator” of the shoot systems in flowering plants.

Below, we review the impact of the mobile florigen in the context of the SFT/SP paradigm, on development, and while many pioneering molecular details associated with the endogenous functions of *FT*-like genes were formulated in *Arabidopsis*, we use tomato as the “centre of gravity” in this review.

FLORIGEN, FLOWERING PATTERNS, AND SHOOT ARCHITECTURE

Shoot and flowering systems in plants differ in their inherent potentials to respond to environmental signals and in their innately regulated growth habits, which together dictate different adjustments of, and by, the florigen system. Comparison of the florigen systems in different species facilitates the classification of its universal versus species-specific functions; however, such a comparison is only useful when the growth habits of the examined plants are well understood. In their response to photoperiods, plants are categorized as long-day, short-day, or day-neutral plants. Species can be classified by their life cycles as annual, biennial, or perennial, each with its own adaptive florigenic system, and also by their growth habits as monopodial or sympodial. In the biennial onion, the response to floral signals depends on exposure of the bulb to a cold winter (Lee et al., 2013), whereas in the carrot, storage is built up during the first year and flowering occurs in the following summer. In short-lived perennials such as *Arabis alpina*, flowering is restricted to particular branches that will flower in the next year, only after exposure to inductive conditions (Wang et al., 2011). Interestingly, a short-lived perennial, or alternatively, an annual habit, is observed in populations of monkey flowers living in near-shore habitats or in more inland populations, respectively (Lowry and Willis, 2010). Polycarpic perennial bushes with multiple flowering cycles in every growing season, such as tomato, maintain a sympodial habit in all their shoots (Figure 1), whereas others, like cotton, display a blend of developmentally regulated sympodial and monopodial shoots (Mauney and Ball, 1959; McGarry and Ayre, 2012a,b). Deciduous perennials, such as apple, maintain largely sympodial branching and regulate flowering in lateral branches by a combination of endogenous and external cues (Hallé et al., 1978; Costes and Guèdon, 2012).

How are the different components of the florigen system, which includes biosynthesis, cellular compartmentalization, export,

phloem transport, and targeting, adapted to regulate such diverse patterns of vegetative and reproductive cycles?

The annual monopodial and day-length-sensitive plants are best represented by *Arabidopsis* (Meyerowitz, 1989; Koornneef et al., 1991). The apical meristem of *Arabidopsis* is indeterminate throughout its life cycle and all appendages are laterals, with leaves and flowers being the only determinate organs. The annual life cycle of *Arabidopsis* must match the seasons, and requires a timely, and thereby inducible, transition to flowering. This occurs once during the life cycle, affects both apical and axillary apices and is manifested by a clear distinction between vegetative and reproductive phases. Accordingly, in order to serve its role in flowering, florigen may reach the primary apex only once in a lifetime, within a narrow time window. Such an initial day-length signal is mostly sufficient to activate flowering in lateral shoots (Corbesier et al., 1996).

The inducible flowering systems of annual plants, such as *Arabidopsis* and rice, are also regulated, in part, by internal signals, but primarily by signals generated by light quality and periodicity (Putterill et al., 2004). Central to the upstream activating program of *FT*-like genes in photoperiod-sensitive annual plants, is the circadian clock output transmitted by the *GIGANTEA-CONSTANS* (*GI-CO*) pathway (Suarez-Lopez et al., 2001; Mizoguchi et al., 2005). CO lacks a DNA-binding domain and is recruited to CCAAT binding sites of *FT* by NF-YC, a member of the trimeric CBF family (Ben-Naim et al., 2006; Wenkel et al., 2006; Kumimoto et al., 2008). The role of CO in regulating *FT* in day-length plants was adequately demonstrated by its contrasting effects on the *FT* genes of *Arabidopsis* and rice (Putterill et al., 1995; Hayama et al., 2003), but variations on these themes have been reported. For example, expression of tomato CO failed to modify flowering time in tomato, *Arabidopsis*, and tobacco (Ben-Naim et al., 2006). In the short-day *Pharbitis nil*, the daily expression profiles of the two flowering-promoting *FT* paralogs are uncoupled to those of CO, suggesting that *FT* in this species might be regulated by other transcription factors (Hayama et al., 2007). Therefore, mechanistic claims based solely on expression profiles of the seasonal CO-*FT* module in other plants, particularly deciduous trees, should be considered with caution because, unlike in *Arabidopsis* and *Pharbitis*, such claims have not been backed by rigorous genetic tests (Ballerini and Kramer, 2011). Direct conditional repressors of *FT* in *Arabidopsis* include the MADS genes *FLC* and *SVP* (Li et al., 2008), *SMZ* (Mathieu et al., 2009), *PIF4* (Kumar et al., 2012), epigenetic transcription regulators (Turck et al., 2007; Adrian et al., 2010), and more. But because florigen activates flowering in response to multiple environmental and endogenous signals, regulatory relations discovered in *Arabidopsis* should only be taken as a lead for system-specific studies.

THE TOMATO SYMPODIAL SHOOT SYSTEM AND THE SFT/SP RATIO

The sympodial system of tomato, with its polycarpic and poly-cyclic habits, captures the most typical features of deciduous perennials, such as grape vines and apple trees, or of perennial bushes like roses, cotton, potato, or black nightshade (*Solanum nigrum*; Bell, 1992). The sympodial shoot system of tomato is a

conglomerate of three branching habits: (A) Axillary meristems of the primary shoot, which are first arrested by apical cues but are gradually released from dormancy after floral termination of the apical meristems above them (**Figure 1**). (B) Sympodial branching, involving an axillary meristem, hosted by the third leaf of each SU that is not subjected to apical dominance and grows out in response to signals generated by the terminating SAM, with no intervening dormancy to form the next SU (Lifschitz and Eshed, 2006). When the termination of the sympodial apex is delayed or accelerated, the formation of the next SU will be affected in a similar manner. Thus, “termination,” floral transition, phyllotaxis, and branching in each SU must be coordinated in the apical bud, within a distance of 10–50 cells. (C) Regulated branching of the two remaining basal axillary meristems of each SU, from which only the second is regularly activated after being released from apical dominance. Because each SU is a replicate of its predecessor, their formation requires a self-perpetuating mechanism, with regularly cycling flowering and anti-flowering messages. Notably, the inflorescence shoot itself is a sympodium from which nodal leaves are removed and in which each flower represents the terminating organ of the previous SU (Lifschitz and Eshed, 2006; Lippman et al., 2008; Park et al., 2014; Périlleux et al., 2014).

SFT/SP RATIOS IN SHOOT MERISTEMS

In the tomato apex, while *sp* accelerates termination of SU in a progressive, age-related manner, the primary shoots of *SP* and *sp* isogenic plants terminate after the same number of leaves. Conversely, overexpression of *SP* delays flowering of both primary and sympodial apices (Pnueli et al., 1998). A similar delay in the termination of the primary apices occurs in *sft* plants and results in an indeterminate inflorescence shoot with a mixture of leaves and solitary flowers that substitute for the regular compound inflorescence. Overexpression of *SFT* induces extreme premature termination of the primary shoot, but, in contrast to *sp*, the shoot continues to form regular SUs. When *SFT* is overexpressed in the *sp* background, the sympodial system collapses, as manifested by termination of the primary apex with only 1–3 flowers, arrest of the sympodial meristems and their failure to support the formation of both a compound shoot, and formation of 1–3 leaves on the 3–4 axillary meristems of the primary shoot before terminating with a “blind” apex (Shalit et al., 2009). Termination induced by high *SFT* concentrations is therefore sensitive to *sp*, particularly in the SUs, which are resistant to high *SFT* levels under functional *SP*.

Termination and flowering in cultivated tomato are not sensitive to day length but are extremely sensitive to integral light doses (Kinet, 1977). Under low light conditions, primary flowering is delayed: first inflorescences tend to abort at their primordial stage, mature inflorescences turn partial leafy, and flowering within the SU is extended from three to five or six leaves. *sp* plants are less sensitive to low light intensity, while *sft* plants are much more sensitive than their WT siblings. Overexpressors of *SFT*, in WT or *sp* backgrounds, are virtually insensitive to low-light intensities. Likewise, *sp* accelerates sympodial of flowering in every examined recessive late-flowering background. Therefore, both *sp* and high *SFT/SP* ratios can substitute for light in tomato.

In agreement with their responses to the *SFT/SP* ratio, the sympodial meristems are also sensitive to intermediate levels of *SP* and *SFT*. When *SFT* is expressed in *sp*/+ heterozygous plants, regular SUs, with two instead of three leaves, dominate the shoots (Shalit et al., 2009). But the reproductive differentiation of the shoot apex is also sensitive to dose changes in *sft*/+ plants. Krieger et al. (2010) reported that tomato plants heterozygous for *SFT* and growing under wide spacing conditions, produced a much higher fruit yield as compared to other heterozygous lines, and attributed this effect to a single gene heterosis. More recently, dedicated measurements and appreciation of the fact that the heterozygous plants were also homozygous for *sp*, led to the conclusion that a dosage response to *SFT*, i.e., the *SFT/SP* ratio, tunes shoot architecture in a quantitative manner, and in particular field stands, such tuning may result in higher yields (Jiang et al., 2013).

Primary and sympodial tomato meristems are, as mentioned above, differentially sensitive to similar *SFT/SP* ratios. In the mature inflorescence shoots of monopodial *Arabidopsis*, buds in the axils of bracts terminate with a flower, with no intervening vegetative phase, while the primary SAM remains indeterminate. The SAM is “protected” from florigen activity by *TFL1*, as shown by *tfl1* primary shoots, which are terminated by a flower while their flowering time is only marginally affected (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). However, lateral shoots of *tfl1* plants flower after the formation of 0–2 leaves instead of 2–5 leaves in WT. Therefore, flowering and the role of *SFT/SP* (*FT/TFL1*) ratios in both annual *Arabidopsis* and perennial tomato, must be considered in the framework of the two flowering systems, one for the primary shoots and the other for lateral shoots, sympodial in tomato and the regular laterals in *Arabidopsis* (Lifschitz and Eshed, 2006).

Importantly, all meristematic activities in the shoot systems can be terminated via elevated *SFT/SP* ratios. In particular, arrest of lateral expansion and secondary growth of the stems, most likely by attenuated cambial activity, typical to *SFT* overexpressing plants, is maintained throughout growth even if the sympodial cycle is completely recovered. Under the same *SFT* level, as noted above, sympodial meristems of the same shoots, maintain a regular, even if accelerated, 3-leaf cycle. The quantitative, meristem-specific impacts of the *SFT/SP* ratio are further illustrated through the response of the compound leaf to high *SFT/SP* ratios.

COMPOUND LEAVES VIEW THE 1:1 RATIOS OF *SFT/SP* AND *sft/sp* AS EQUALLY INFORMATIVE

A correlation between leaf growth and flowering has been frequently observed in many plants but has generally been attributed to secondary effects of flowering. Our results implied that transition to flowering and reduced leaf-growth represent two facets of the same developmental process. The formation of a compound leaf in tomato requires the activation of lateral leaflet meristems along the primary rachis and of additional rounds of ramification along secondary leaf rachises (Hareven et al., 1996; Efroni et al., 2010). The complexity of the leaf is marginally reduced in *sp* plants, but plants overexpressing *SP*, e.g., bearing a low *SFT/SP* ratio, show excessive activity of the plate meristem in the lamina (Shalit et al., 2009). *sft* leaves have excess intercalary leaflets (foliolles, Molinero-Rosales et al., 2004), while leaves overexpressing

SFT form smaller blades and lack folioles. However, when *SFT* was overexpressed in *sp* plants, a dramatic reduction in complexity was observed and leaves became progressively simple (**Figure 3**). All these features have also been obtained via graft-transmissible florigen. Thus, both flowering and the simplification of leaves reflect a shift from growth to termination via changing *SFT/SP* ratios.

Simplification of the compound leaves is also effective in *sp* plants, if the system is sensitized by other genes, such as *TRIFOLIATE (TF)*. Mutant *tf* (a MYB factor, Naz et al., 2013) leaves form only one pair of leaflets (Hareven et al., 1996) and *tf* plants have enhanced apical dominance. In contrast, *tf sp* plants (high endogenous *SFT/SP* ratios) flower earlier and their leaves become progressively simple with the advance of flowering, mimicking *sp* plants overexpressing *SFT*. Conversely, *tf sft* leaves (low endogenous *SFT/SP* ratios) carry additional leaflets. However, the regular trifoliated phenotype was rescued when *sft* was inactivated as well, as in *tf sp sft*. Thus, when one of the two genes is functional, the *SFT/SP* ratio determines contrasting morphogenetic consequences, but as long as functional and dysfunctional *SFT* and *SP* maintain a one-to-one ratio, the morphogenetic consequences are identical (Shalit et al., 2009). This indicates that the ratio of *SFT* to *SP*, and not only their actual levels, determines the morphogenetic response. Significantly, all phenotypic changes induced by modified endogenous *SFT/SP* ratios, were recapitulated by graft-transmitted (systemic) florigen. In addition to rescuing the regular sympodial pattern of *sft* plants, mobile florigen, fully complemented all other pleiotropic defects of *sft*, i.e., the extended adaxial sepal, suppression of sympodial buds, defective sympodial patterning and the extra folioles, by leading to recovery of the correct *SFT/SP* ratios. Likewise, mobile florigen also substituted for high endogenous *SFT*, inducing the collapse of the sympodial pattern and the simplification of the leaves in *sp* plants, conditioning slim stems in receptor WT shoots, and stimulating leaf simplification in *tf* plants. The mobile florigen also rescued the size and structure of defective abscission zones in the floral pedicles of mutant *macrocalix* and *blind* plants (**Figure 3**; Shalit et al., 2009), and the normal flowering pattern in *miR156*-overexpressing plants (unpublished).

Are these characteristics of the *SFT/SP* ratio paradigm universal or unique to tomato? Accumulating observations indicate that growth and termination in reproductive and vegetative meristems in other plants are also dictated by the ratio of *SFT* and *SP* homologs. In *Arabidopsis*, termination of the inflorescence by *tfl1* is associated with simple, smaller, and more oblique leaves in long days with photoperiodic induction of *FT* (i.e., high *FT/TFL1* ratio); all of these characteristics can be reversed when *FT* levels are reduced by short days, low light, cold temperatures, or their combinations (lower *FT/TFL1* ratio; our observations). Suppression of flowering in the *Maryland Mammoth* tobacco grown under long-day conditions is associated with giant leaves. Likewise, the leaf complexity gradually declined in distal compound leaves of different plants approaching flowering, as seen in roses (Efroni et al., 2010). In maize, reduction of florigen or increase in *SP*-like (ZCN2) levels resulted in larger leaves, more branched tassels and, as in tomato, thicker stems (Danilevskaya et al., 2011). In cotton, vegetative shoots are monopodial, while reproductive fruiting branches are sympodial. High *FT* levels induced

promiscuous arrest of the sympodial branches, small and lanceolate leaves, rather than deeply lobed leaves, and thinner stems (McGarry and Ayre, 2012a,c). In potato, florigen promoted the formation of tubers – which are determinate structures – at the tips of stolons (Navarro et al., 2011), while in grapes, misexpression of an *SP*-like gene confers a box-like architecture, instead of an inverted pear-shape architecture on Carignan fruit clusters (Fernandez et al., 2010). An *FT* homolog (not in the form of a mobile florigen) of *Populus tremula* was shown to regulate bud set and growth (Böhnenius et al., 2006). However, since *Populus* has two *FT* paralogs, with non-overlapping seasonal expression pattern (Hsu et al., 2011), it remains unknown which of the two triggers the flowering response. Finally, the significance of the *SFT/SP* ratio paradigm is well illustrated in the short-lived perennial *A. alpina* and long-lived perennial Rosaceae. In these cases, early and reoccurring flowering, small shoots, and simpler leaves are associated with suppression of their corresponding *SP*-like genes (Wang et al., 2011; Iwata et al., 2012; Kurokura et al., 2013).

THE ANTAGONISTIC COMPONENT OF THE SFT/SP BALANCE

Florigen and antiflorigen are equally important constituents of floral induction (Chailakhyan, 1936a,b; Zeevaart, 1976; Lang et al., 1977). The *SFT/SP* ratio paradigm implies that genes belonging to the *CEN*, *TFL1*, and *SP* branch of the CETS may function as universal antagonists for florigen. But it also implies that regulated inactivation of *SFT* as such, is sufficient to generate an antagonistic developmental response.

The origin of the current florigen system in flowering plants is debated. It has been proposed that florigen endows early flowering plants, having improved conductive systems and fast-growing meristems, with faster and versatile capabilities to adapt to new habitats and respond more efficiently to different environmental signals. But because flowering is synonymous with termination and because high, unchecked florigen levels are detrimental, we speculated that the founder *SP*-like antagonist coevolved with florigen to alleviate its harmful effect (Shalit et al., 2009). Indeed, a thorough phylogenetic analysis (J. Bowman, in Shalit et al., 2009, and Supplementary information therein) showed that genes of the *CEN/TFL1/SP* branch are missing from non-flowering plants. An additional analysis confirmed these results but suggested that a duplication of an ancient *MFT* gene gave rise to an intermediate florigenic *FT/TFL1-like* gene in extant gymnosperms, while additional duplications gave rise to the *FT/SFT* and *CEN/TFL1/SP* branches of angiosperms (Karlgren et al., 2011). In contrast to the situation in extant gymnosperms, there are only five CETS in the genome of *Amborella*, the only known living species from the earliest branch in the angiosperm lineage (Amborella Genome Project, 2013): three being *MFT*-like, one a classic *FT/SFT* and one a classic *SP/TFL1* gene.

Expression of the *FT/TFL1-like (FTL)* genes of gymnosperms in *Arabidopsis* resulted in delayed flowering, implying a function more similar to that of *TFL-1* (Klintenäs et al., 2012). However, the temporal and spatial expression of *PaFTL2* and *PaFTL1* implied roles in late-season bud sets (Karlgren et al., 2013) and when *PaFTL2*, under a heat shock promoter, was expressed in Norway

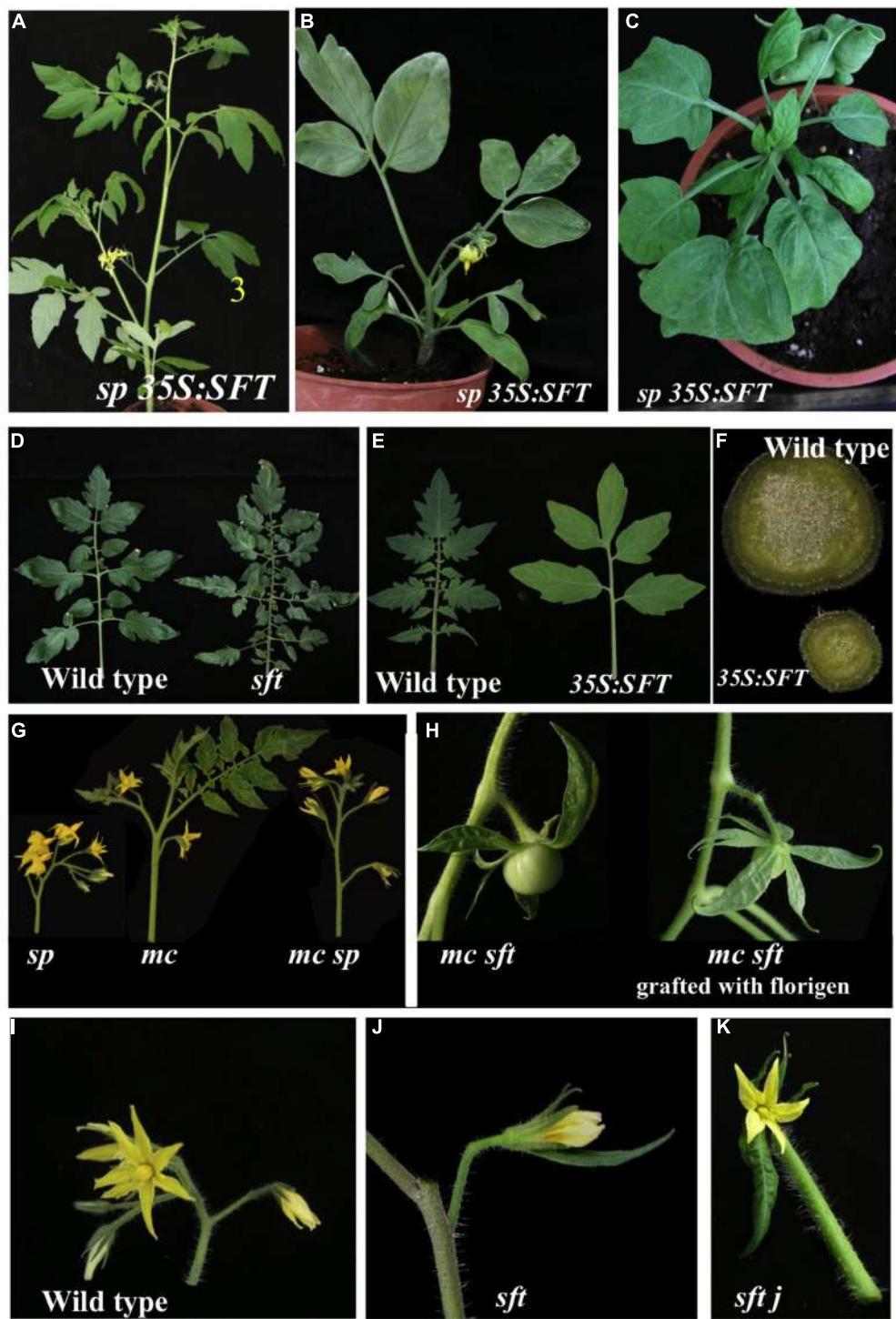


FIGURE 3 | Diverse developmental systems are regulated by the SFT/SP ratio. (A) Tomato plants expressing the *35S:SFT* gene flower very early but maintain normal sympodial cycling. (B,C) In the absence of *SP* high SFT suppresses axillary meristems (B) or even vegetative and inflorescence apical meristems. (D) *sft* leaves generate extra leaflets and folioles (E,F) *35S:SFT* plants produce simpler leaves with entire margins. (E) High SFT induces

thinner stems by arresting secondary growth. (G) Loss of *MACROCALYX* (*mc*, a putative homolog of *AP1*) results in partially leafy inflorescence that is rescued by further loss of *SP*. In contrast, the incomplete abscission zone of *mc* is completely eliminated in *mc sft* but is fully restored by a mobile florigen (H). (I–K) Within the flower, *sft* (J) conditions the formation of a larger adaxial sepal which is greatly enhanced in *jointless1* (*j1*) background (K).

spruce (*Picea abies*), vegetative growth transitioned to terminal bud set and growth cessation, both being steps toward dormancy and winter hardiness. Therefore, despite the observations in *Arabidopsis*, the *TFL1* clade in gymnosperms functions more analogously to *FT* in angiosperms by regulating patterns of perennial growth.

Much like the odyssey of florigen (Sparks et al., 2013), the course of understanding the role of the *Arabidopsis TFL1* was quite convoluted before being determined as a universal antagonist of florigen. Mutations in *TFL1* were initially recognized because their inflorescence shoots terminated with a single flower (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992); the gene was appropriately assigned a role in maintaining the indeterminate state of the inflorescence meristem (Ratcliffe et al., 1998, 1999). But depending on growth conditions, *tfl1* also displayed modestly early flowering, condensed stem nodes and smaller and more oblique leaves (Bradley et al., 1997). Overexpression of *TFL1* induced late flowering, excessive branching of vegetative inflorescence shoots and expanded rosette leaves (Ratcliffe et al., 1998). But these features were always discussed in relation to floral and inflorescence meristem fate and to general regulation of the vegetative and reproductive phases, rather than in relation to floral transition proper. Any generalization with respect to flowering time was further hindered by the effects of the *CEN* gene of *A. majus*, which, unlike *TFL1*, did not impact flowering time when inactivated. The findings that *TFL1* forms a regulatory loop with *API* and *LEAFY* (Liljegren et al., 1999; Ratcliffe et al., 1999) implicated *TFL1* in regulation of the “identity” of SAMs (Ratcliffe et al., 1998). But since no place was found for *TFL1* along the linear day-length flowering pathways, its role in regulating flowering in *Arabidopsis* was largely ignored. This perspective, namely that *FT* is involved in flowering time, while *TFL1* is involved in the distinctively separate function of meristem identity, is interestingly reflected by the complete disappearance of *TFL1* from recent authoritative models and reviews on flowering (Putterill et al., 2004; Kobayashi and Weigel, 2007).

By contrast, the humble *SP* was viewed, from the outset, as a legitimate flowering suppressor and its recessive allele as a flowering-promoting gene. This was mostly due to the original assignment of *SP* as a regulator of the “determinate” vs. “indeterminate” modes of sympodial branching (Yeager, 1927; MacArthur, 1932). Like *CEN*, *SP* plays no role in primary flowering, yet, its inactivation induces accelerated termination without disrupting sympodial branching *per se*, and its overexpression delays both primary and sympodial termination. We thus regarded the gene as a legitimate component of flowering and a part of the florigen system (Pnueli et al., 1998, 2001; Lifschitz and Eshed, 2006). An important developmental consideration is that while inactivation of *TFL1*-like genes accelerates primary flowering in some plants, it always promotes flowering in secondary branches. Growing genetic and molecular evidence, from *Arabidopsis*, tomato, and other plants, provides a reasonable basis for the analysis of antagonism in the framework of the SFT/SP ratio and the endogenous and systemic functions of the *CETS* genes. Below, we consider this critical issue from various perspectives, while leaving several questions wide open.

IT'S ALL IN THE FAMILY

CETS genes form a small family common to all plants, with a complexity ranging from 5 in *Amborella*, 6 in *Arabidopsis*, 12 in tomato, and 25 in maize (Danilevskaya et al., 2008, 2011). *CETS* genes belong to a family of genes encoding mammalian phosphatidylethanolamine binding proteins (PEBP; Schoentgen et al., 1987), of which the *Raf kinase inhibitor protein* (RKIP), which functions via direct interaction with a 14-3-3 protein is best understood (Yeung et al., 1999; Granovsky and Rosner, 2008). *CETS* have been classified into three major clades (Ahn et al., 2006; Shalit et al., 2009; Karlsgren et al., 2011), named after the corresponding *Arabidopsis* genes, *TFL1*, *FT*, and *MFT* (Mother of *FT* and *TFL1*). Thus far, *MFT* genes forming the ancestral clade, have no clear relation to flowering.

Structural analysis of RKIP revealed a small [\sim 180 amino acids (AA)] globular protein with a putative binding pocket for phosphorylated ligands (Banfield et al., 1998; Serre et al., 1998). 3D analysis of the *CEN* protein confirmed the universal structural aspects of PEBPs, but also identified a unique, unstructured, external loop comprised of 14 AA (Banfield and Brady, 2000) common to all *CETS* proteins. Both the binding pocket and the external loop proved critical for the floral-promoting and floral-suppressing functions of *CETS* genes in *Arabidopsis* (Hanzawa et al., 2005; Ahn et al., 2006). A Y85H mutation, at the entrance of the putative binding pocket, or swapping the external loop of *FT* for that of *TFL1*, were each sufficient to convert *FT* to a flowering suppressor. However, reciprocal alterations failed to convert *TFL1* to a flowering promoter. In addition, the 14-AA external loop has been shown to be conserved among *FT*-like genes, while those of *TFL1* and *MFT*-like genes are widely divergent. A recent comprehensive study identified only four additional AA that were critical for converting *FT* to a functional *TFL1* (Ho and Weigel, 2014).

Natural conversion of an *FT*-like gene to a floral suppressor was found in domesticated sugar beet, where a single Y to N conversion in the external loop of an *FT*-like paralog generated an *FT* antagonist (Pin et al., 2010). It will be interesting to explore the role of the authentic *SP/TFL1*-like gene in this species. However, as more *CETS* genes and their functions in different plants are reported (Meng et al., 2011; Hecht et al., 2011), it is becoming increasingly difficult to specify the number of AA alterations required to provide the external loop with a universal repressing role. Interestingly, *CEN* and *SP* independently antagonize flowering in *Arabidopsis*, but *TFL1* is inert in tobacco and tomato (Amaya et al., 1999; Ahn et al., 2006). As *CETS* genes can counteract florigen via various mechanisms, sequence-based functional predictions can be misleading by missing the critical residues and the underlying mode of action.

An additional mechanism, generating antagonistic *FT*-like gene products by posttranslational modification may involve the ligand binding pockets. Nakamura et al. (2014) reported the association of phosphatidylcholine by the ligand-binding pocket of *FT*, suggesting the possibility other phosphorylated ligands, or a yet unknown post-translation modification, function as auto-antagonists.

In tomato, tobacco, and *Arabidopsis*, the natural systemic antiflorogens candidates were *SP*, *CET2/4*, and *TFL1*, respectively

(Zeevaart, 2006), genes that, unlike *FT* paralogs, are preferentially expressed in the SAM. Short-range, cell-to-cell movement via plasmodesmata was shown for TFL1 in *Arabidopsis* (Conti and Bradley, 2007) and such movement may be a general feature of CETS genes. Nevertheless, it was recently reported that one of two *TFL1-like* genes in *Chrysanthemum seticuspe* expressed in leaves, *CsAFT*, suppresses flowering under inductive short-day conditions, and also induces late flowering via grafting (Higuchi et al., 2013). It is possible that in other species, other antagonistic CETS are preferentially expressed in leaves and systemically translocated to the apex. In tomato, *SP* is predominantly expressed in the apex, but *SP5G*, a potential promoter of indeterminacy, is predominantly expressed in the leaves. Thus, while florigen was the first protein hormone characterized in plants, other members of the family, being functional homologs or antagonists, are also likely to function at long range.

CETS PROTEINS FUNCTION AS PARTNERS IN TRANSCRIPTION COMPLEXES

The most likely mechanism by which *TFL1*-like genes antagonize their flowering-promoting homologs is via formation of competitive or antagonistic transcription complexes. The first candidates for a core functional complex of CETS factors were discovered as SP-Interacting Proteins (SIP, Pnueli et al., 2001), and included the 14-3-3 adaptor proteins, SPGB (SP-associated G-box), a bZIP transcription factor homolog of FD, an SP-associated NIMA-like kinase (SPAK, Osman et al., 1988), and a 99-amino acid-long polypeptide called SIP4. All SIPs independently interacted with the 14-3-3s and in addition, SPGB interacted with the *Arabidopsis* FT. SP and FT bind different 14-3-3s with different affinities and SPAK and SIP4 are, most likely, SP-specific (Pnueli et al., 2001). The plasticity, flexibility, and diversity of protein–protein interactions suggest that CETS proteins, in analogy with the 14-3-3 adaptors, function as hubs in signaling systems, with the potential to integrate a wide variety of environmental cues (Pnueli et al., 2001).

In a recent milestone discovery, Taoka et al. (2011) presented a 3D structure of the hetero-hexameric complex consisting of two molecules each of the rice FT homolog (Hd3a), 14-3-3 and FD (OsFD1), and named it florigen activation complex (FAC). In this assembly, the 14-3-3 protein forms a bridge between Hd3a and OsFD1, which otherwise do not directly interact. It was therefore postulated that the 14-3-3s are the primary intercellular receptors for mobile florigen, and that the successful interactions between bZIPs (FD clade) and FTs in yeast are actually mediated by BMH1 and BMH2, the two endogenous yeast 14-3-3s. But, as logical as these conjectures are, direct experimental support for both is lacking. Taoka et al. (2011) reported that other proteins, such as KANADI and WAVE-DAMPENED2 homologs, which share the SAP (Ser-Ala-Pro) domain required for binding with 14-3-3s, also recognized Hd3a. We identified a calmodulin binding protein of the IQD type (Abel et al., 2005), which similarly interacts with 14-3-3 proteins in a similar fashion. Ho and Weigel (2014) discovered several TCP transcription factors that differentially interact with FT and TFL1. However, different TCP binding partners were reported for the same *Arabidopsis* CETS proteins (Liu et al., 2012; Hiraoka et al., 2013; Ho and Weigel, 2014). In our experiments,

SP interacted with a TCP (AF175965) that was later shown to be encoded by *LANCEOLATE* (Ori et al., 2007) and similar TCP factors suspiciously interacted with BELL, KN, or CO. Thus, the relevance of TCB, and similarly the specificities and redundancies of the 14-3-3s to the regulatory functions of florigen require further study.

Both SFT and SP of tomato bind in yeast 14-3-3 and bZIP (SPGB) proteins, but also have their own specific binding proteins (Pnueli et al., 2001). In *Arabidopsis*, FT and TFL1 bind FD and FDP (Abe et al., 2005; Wigge et al., 2005), but the intercellular localization of TFL1 is questionable (Conti and Bradley, 2007; Sohn et al., 2007). The likely inference is that antagonistic proteins of the CETS family compete for a position in the same core functional complexes, or that stoichiometric relations permit the formation of two overlapping but nonidentical transcription complexes. Under either condition, the two complexes may compete for common targets or each may activate distinct antagonistic processes.

TARGET COMPETITION

One candidate target system for FT-TFL1 (SFT-SP) antagonism involves the *FT*-targeted *API-SOC1-LEAFY* network, which is partially regulated by *TFL1* (Ratcliffe et al., 1999) and activates inflorescence and floral identity genes (Kobayashi and Weigel, 2007; Turck et al., 2008). However, the definition of *API* as a primary target of *FT* and a pivotal flowering gene is problematic. Schmid et al. (2003) showed that activation of *API* by long days is delayed until after *SOC1* and *FUL* have responded. Yamaguchi et al. (2009) assigned the *API* output to the meristem identity rather than to the reproductive transition. High activity of *LFY*, *API*, or even *FUL* indeed induce early flowering, but it is equally important to note that, unlike *ft* or *soc1*, inactivation of *LFY* or *API* has no, or only a marginal, effect on flowering time (Ruiz-García et al., 1997). Likewise, in tomato, inactivation of *FAL-SIFLORA* (*FALS*), the *LEAFY* homolog, or *MACROCALYX* (*MC*), the *API* homolog, does not appreciably delay flowering. Significantly high *SFT* levels are epistatic to the “vegetative” genes *fals*, *mc*, and *j1* and to all their double combinations [*J1*, *JOINTLESS* is a *MADS* gene which interacts genetically with *SP* (Pnueli et al., 1998; Szymkowiak and Irish, 2006) and the resulting early terminating organs maintain the authentic mutant phenotypes (Shalit et al., 2009)]. While it is still unknown if constitutive overexpression of *FT* is epistatic to the various combinations of *ap1* with the *soc1* and *agl24* late-flowering mutants, flowering of *soc1 agl24* double mutants is delayed in short days (Li et al., 2008). Additional modifications in the current schemes for flowering transition are required to include the phenotypic interactions of *svp* with the double suppressor of flowering *soc1-2 ful-2*, as reported by Torti et al. (2012).

Additional inconsistencies in present models must be considered too. *fdp*, *fd*, or *fd fdp* double-mutant *Arabidopsis* plants flower, where the double mutant flowering is not as late as in *ft* and certainly not as late as in *ft tsf* (Yamaguchi et al., 2005; Jaeger et al., 2013). Tomato *sft* plants also flower but, unlike with *ft*, full proof null alleles are not available. Interestingly, unlike *Arabidopsis* or wild tomato species, the cultivated tomato genome carries no other functional *SFT* homologs.

Obviously, difficulties in reaching a coherent model arose from the ever-increasing complexity of the genetic interactions and from the lack of clarity at numerous levels: the relations between termination, inflorescence specification, and floral differentiation, between phase transition and meristem fate and between determinate and indeterminate shoots. In the indeterminate and monopodial shoots of *Arabidopsis*, only axillary meristems terminate to form flowers, whereas in tomato, the determinate and sympodial meristems themselves terminate and subsequently differentiate to form an inflorescence. Further understanding requires that the molecular interactions of the systemic flowering antagonists with the FT-dependent MADS target network be properly appreciated. Furthermore, to fully understand the florigen hormone and the SFT/SP regulatory hierarchy, their end users, i.e., cellular systems such as cell cycle, or cytoskeleton, not just transcription networks, should be identified.

GENERAL CONSIDERATIONS RELATING TO THE SYSTEMIC PATHWAY OF FLORIGEN

Classic grafting experiments supported the hypothesis that florigen uses the phloem track to move from leaves to the shoot apices (Zeevaart, 1962, 1976). This premise acquired molecular support when CO, regulated by companion cell-specific promoters, *AtSUC2* from *Arabidopsis* and *CmGAS1* of *Cucumis melo*, successfully induced flowering under non-permissive conditions (An et al., 2004; Ayre and Turgeon, 2004). It was also shown that *FT* is predominantly expressed in the vasculature and that *FT*-derived polypeptides were detected in the phloem sap of several species. It should be noted that three phloem tracks (primary, secondary and an additional centrally located phloem) are open for the mobile florigen in Solanaceae and Cucurbitaceae (Esau, 1969), but no particular mechanistic preference has been reported. At the cellular level, most tagged *FT* and *SFT* proteins were located in the nucleus (Abe et al., 2005; Lifschitz et al., 2006), but the cytoplasmic compartments hosting the rest of the protein were not identified.

Reciprocal grafting experiments in tomato demonstrated that, in these plants, florigen moves upward and downward from *SFT*-overexpressing source shoots, and enters axillary buds (sinks) of the recipient stems, as was inferred from the classic analysis of florigen (Zeevaart, 1976). In a standard grafting experiment in tomato, a donor scion overexpressing *SFT* is grafted onto a non-flowering *sft uf* (*uniflora*, Dielen et al., 2004) recipient tester with 3–4 basal leaves and their transiently dormant axillary buds. Flowering is then recorded in the out-growing laterals of the non-flowering tester (stalk). To induce flowering in this setup, florigen must translocate from the donor leaves of the scion downward to the activated axillary shoots. In a reciprocal graft, florigen is moved upward from leaves of the donor stalk to organs and apices of the recipient *sft uf* scion.

The cellular mechanisms regulating the intercellular migration of florigen have not yet been ascertained. As a globular protein within the size exclusion limits of plasmodesmata, florigen may enter and exit the translocation stream by a non-selective process. Recently, a newly discovered *FT*-interacting protein (FTIP1; Liu et al., 2012), which is associated with the endoplasmic reticulum, was suggested to mediate the exit of *FT* from phloem

companion cells to sieve elements in *Arabidopsis*. However, the impact of FTIP1 on flowering is relatively mild and, more importantly, it does not bind TFL1. If the claimed specificity of FTIP1 (Liu et al., 2012) holds true, it follows that each CETS protein may require its own exit chaperon. Recently, experiments combining *FT*-mutant genes, grafting tests, and a viral expression system in *Cucurbita moschata*, identified mutant *FT* variants that cannot exit the phloem. It was suggested, that the *FT* protein enters the phloem translocation stream through either the selective or non-selective pathway. But active uploading from the phloem to the (unknown) target cells in the SAM (Yoo et al., 2013) requires short-distance active cell-to-cell movement, via plasmodesmata.

In *Arabidopsis*, *FD* is expressed exclusively in the SAM (Abe et al., 2005; Wigge et al., 2005) and it has therefore been rightfully accepted, that florigen must reach the apex in order to induce flowering. However, due to low levels of the traveling protein and poor resolution, direct evidence for a tagged mobile florigen reaching the target cells in the SAM proper remains to be obtained. All reported cases, in *Arabidopsis*, rice, or potato, recorded florigen close to, but not in, the SAM (Corbesier et al., 2007; Tamaki et al., 2007; Navarro et al., 2011). Another line of evidence, namely that *FT* proteins are found in the phloem sap, is required but conceptually problematic. This is because thousands of proteins have been detected in the phloem sap of several species but, with the exception of few, their functional significance in this critical compartment is poorly understood.

Interestingly, in tomato, all members of the *FD* clade are expressed in leaves and at levels higher than *SFT* and in addition florigen enter leaves in which *SFT* is already expressed (Shalit et al., 2009). If, as we propose, the critical impact of florigen is on regulation of preexisting SFT/SP ratios in all organs, it is likely that, in some species, *FT-like* genes are also expressed in the SAM to maintain a local pre-flowering ratio as in tomato leaves. Indeed, we found that *SFT* is expressed in the primary SAM of tomato from its early inception (A. Shalit, personal communication).

Florigen was the first protein in plants demonstrated to function long range and to promote growth attenuation in all above-ground meristems, with flowering being its most visual output. The reiterated phase transition, the perennial evergreen nature of the tomato shoot system and the day-neutral flowering response require that the distribution of, and the response to florigen, be regulated on a daily basis. Mature leaves are the primary source of florigen and the removal of young tomato leaves accelerates flowering (Leopold and Lam, 1960). Florigen produced by the mature parts of the compound leaf is distributed to younger leaflets and subsequently to all organs, including leaves where *SFT* is already expressed. Thus, on the whole-plant level, by regulating leaf maturation, florigen controls its own levels and mode of distribution (Shalit et al., 2009).

CONCLUSION

While the core SFT/SP ratio paradigm initially provided a conceptual framework for the analysis of the growth versus termination equilibrium in the reproductive and vegetative meristems of tomato, supporting observations from other flowering systems conferred it broader and more general significance. It presents the

best platform for further exploration of the seemingly unrelated vegetative/reproductive phenomena in several plants.

The complex interactions among *CETS* genes, the rapid expansion and decline in the size of this gene family, and their species-specific incorporation into environmental sensing-programs, have facilitated evolution of a range of flowering modes, from simple, fast-cycling annuals to complex perennials, where different branches display different autonomous flowering modes.

Transition to flowering, inflorescence differentiation, and leaf morphogenesis are quantitative and cumulative processes. The cellular mechanisms underlying the response of meristems to changing SFT/SP ratios remain unknown and it is still premature to determine the basis for growth/termination, not only vegetative/reproductive, shifts, in each meristem, or more so, in different flowering plants. Notably, the dramatic enlargement and doming of the vegetative apical meristem presents a common indication of floral transition (Bernier, 1988). Thus, SFT/SP targets must include regulators that modify the rate and orientation of cell divisions enforcing a redistribution of local signaling systems.

Adaptation of plants to different environments, an obvious consequence of domestication by humans, requires changes in flowering regulation, as well as in shoot architecture, with an unpremeditated exploitation of the SFT/SP ratio playing a seminal role in this process. As described here, these attributes were first recognized by the exploitation of a mutant *SELF PRUNING* gene, where accelerated termination of the WT regular sympodial cycles facilitated rapid breeding of mechanically harvested tomatoes (Rick, 1978; Pnueli et al., 1998). In retrospect, the same genetic system was exploited during the domestication, via selection of naturally occurring *CETS* alleles, to revolutionize a wide range of crop plants, or even to turn an exotic plant into a crop. These include, in addition to tomato, direct exploitation of changes in SP-like genes in soybeans (Tian et al., 2010), beans (Kwak et al., 2008), roses, strawberries (Iwata et al., 2012), or even in barley (Comadran et al., 2012). Likewise, mutations converting duplicated *FT*-like genes into floral antagonists were selected during domestication of sunflowers (Blackman et al., 2010), and sugar beet (Pin et al., 2010). Allelic variations in SFT/FT-like genes or in their upstream regulation have been reported for rice (Kojima et al., 2002; Ogiso-Tanaka et al., 2013), wheat (Bonnin et al., 2008), and potato (Kloosterman et al., 2013). Tuning of the SFT/SP ratio constitutes one of the most important processes behind crop domestication.

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Mutations in single *FT*- and *TFL1*-paralogs of rapeseed (*Brassica napus* L.) and their impact on flowering time and yield components

Yuan Guo, Harloff Hans, Jung Christian and Carlos Molina *†

Plant Breeding Institute, University of Kiel, Kiel, Germany

Edited by:

Dorothee Staiger, Bielefeld University, Germany

Reviewed by:

Mingsheng Chen, Chinese Academy of Sciences, China

Franziska Katharina Turck, Max Planck Society, Germany

Rod J. Snowdon, Justus Liebig University, Germany

***Correspondence:**

Carlos Molina, Plant Breeding Institute, University of Kiel, Olshausenstrasse 40, D-24098, Kiel, Germany
e-mail: c.molina@plantbreeding.uni-kiel.de

†Present address:

Carlos Molina, E-nema GmbH, Schwentinental, Germany

Rapeseed (*Brassica napus* L.) is grown in different geographical regions of the world. It is adapted to different environments by modification of flowering time and requirement for cold. A broad variation exists from very early-flowering spring-type to late-flowering winter cultivars which only flower after exposure to an extended cold period. *B. napus* is an allopolyploid species which resulted from the hybridization between *B. rapa* and *B. oleracea*. In *Arabidopsis thaliana*, the PEBP-domain genes *FLOWERING LOCUS-T* (*FT*) and *TERMINAL FLOWER-1* (*TFL1*) are important integrators of different flowering pathways. Six *FT* and four *TFL1* paralogs have been identified in *B. napus*. However, their role in flowering time control is unknown. We identified EMS mutants of the *B. napus* winter-type inbreed line Express 617. In total, 103 mutant alleles have been determined for *BnC6FTb*, *BnC6FTa*, and *BnTFL1-2* paralogs. We chose three non-sense and 15 missense mutant lines (M_3) which were grown in the greenhouse. Although only two out of 6 *FT* paralogs were mutated, 6 out of 8 *BnC6FTb* mutant lines flowered later as the control, whereas all five *BnC6FTa* mutant lines started flowering as the non-mutated parent. Mutations within the *BnTFL1-2* paralog had no large effects on flowering time but on yield components. F_1 hybrids between *BnTFL1-2* mutants and non-mutated parents had increased seed number per pod and total seeds per plant suggesting that heterozygous mutations in a *TFL1* paralog may impact heterosis in rapeseed. We demonstrate that single point-mutations in *BnFT* and *BnTFL1* paralogs have effects on flowering time despite the redundancy of the rapeseed genome. Moreover, our results suggest pleiotropic effects of *BnTFL1* paralogs beyond the regulation of flowering time.

Keywords: *Flowering Locus-T*, *Terminal Flower-1*, *TILLING*, rapeseed, allopolyploid, differential function

INTRODUCTION

Rapeseed (*Brassica napus* L.) is a major oil crop accounting for more than 60 million tons of seed and more than 20 million tons of extracted oil worldwide (<http://www.worldoil.com/>). This crop is widely cultivated in several temperate regions of the world such as northern Europe, Canada, China and Australia. Adapting flowering time to regional environmental conditions has been a major target of rapeseed breeding. A broad variation exists from very early-flowering spring-type to late-flowering winter cultivars that only flower after exposure to an extended cold period, a process known as vernalization (Iniguez-Luy and Federico, 2011). In rapeseed, flowering time and yield are closely linked to each other. Several genomic regions have been reported to contain major QTL for both traits. In a doubled haploid (DH) population derived from a cross between the Chinese semi-winter line Ningyou7 and the European winter-type Tapidor, at least four flowering time QTL were suggested as indicator QTL for yield (Long et al., 2007; Shi et al., 2009). Genetic variation within the different rapeseed types is relatively small, implying a need for

wide crosses between non-adapted ecotypes to introduce traits of interest into elite materials (Girke et al., 2012). However, the introgression of genes from non-adapted ecotypes into elite cultivars is difficult due non-adapted flowering time. Therefore, it is of great interest to measure the effects of different paralogs on flowering and other characters.

Brassica napus and its close relatives *Brassica oleracea* and *Brassica rapa* belong to the family Brassicaceae which also includes the model plant *Arabidopsis thaliana*. Rapeseed is an allotetraploid species originating from the spontaneous hybridization between *B. rapa* (AA) and *B. oleracea* (CC) less than 5000 years ago (Ziolkowski et al., 2006; Wang et al., 2011). These two progenitor species are ancient polyploids that underwent genome triplication between the *Brassica-Arabidopsis* split (~13 MYA) and their actual divergence event (~two MYA). Comparative mapping between the *Arabidopsis* and *Brassica* genomes revealed numerous homologous regions arranged in highly syntenic chromosome blocks. Many *Arabidopsis* genes are represented in the *B. napus* genome by at least three paralogs

(Schranz et al., 2006). Due to their close phylogenetic relationship and the high economic importance of rapeseed, knowledge transfer from the model species *Arabidopsis* to the complex *Brassica* genomes constitutes a worthwhile challenge for genomics research.

In *Arabidopsis*, four pathways controlling flowering time have been extensively studied (Amasino, 2010). All these pathways converge at the *CO/FT* regulon (Andres and Coupland, 2012). Under long day (LD) conditions, the CONSTANS (CO) protein accumulates in leaves and induces expression of the floral integrator gene *FLOWERING LOCUS T* (*FT*) in the phloem companion cells (Moon et al., 2003; Turck et al., 2008). *FT* is the long-sought “florigen” and it is reported to be a strong mobile signal triggering activation of floral identity genes in the *Arabidopsis* shoot apical meristem (Andres and Coupland, 2012). The *FT* protein is transported via the sieve tubes to the shoot apex, where it forms a heterodimer with the FD (*FLOWERING LOCUS D*) protein (Abe et al., 2005; Wigge et al., 2005). Interestingly, a very closely related gene, *TERMINAL FLOWER 1* (*TFL1*) plays an *FT*-antagonistic role by competing for FD, leading to a repression of floral transition (Valverde, 2011; Andres and Coupland, 2012). In *Arabidopsis*, *FT*-overexpressing plants and *TFL1* non-sense mutants show the same early-flowering phenotype and produce terminal flowers in the shoot apex. *TFL1* represses transcription of genes which are activated by *FT* (Hanano and Goto, 2011). In *Arabidopsis*, these two highly similar polypeptides belong to a family of six members characterized by the phosphatidylethanolamine-binding domain (PEBP) (Kardailsky et al., 1999). Substitutions of crucial amino acids from the *FT* and *TFL1* exon II, as well as the exchange of the exon IV led to contrasting protein functions for both polypeptides (Hanzawa et al., 2005; Ahn et al., 2006).

Apart from their major role to control flowering time, *FT* and *TFL1* orthologs have been shown to alter a variety of phenotypic characters. In tomato, the *SINGLE FLOWER TRUSS* (*FT* ortholog) and *SELF PRUNING* (*TFL1* ortholog) genes impact fruit yield heterosis. *F₁* hybrids generated by crosses between loss of function *SFT* mutants and tomato wild type (WT) plants of the non-mutagenized line M82 have shown strong increment in fruit production (Molinero-Rosales et al., 2004; Krieger et al., 2010). *FT/TFL1* gene orthologs have been characterized in diploid crops such as rice (Kojima et al., 2002), pea (Hecht et al., 2011), barley (Faure et al., 2007), poplar (Böhnenius et al., 2006), and sugar beet (Pin et al., 2010) and in two polyploids, wheat (Yan et al., 2006) and potato (Navarro et al., 2011). The characterization of the *FT/TFL1* gene orthologs in polyploid plants is a special challenge because duplicated genes can build new regulation networks leading to sub- or neo-functionalization (Pin and Nilsson, 2012).

Rapeseed has six *BnFT* paralogs (*BnA2FT*, *BnC2FT*, *BnC6FTa*, *BnC6FTb*, *BnA7FTa*, and *BnC7FTb*) sharing high sequence similarity (92–99%) in their four exons (Wang et al., 2009). It has been shown that the *BnC2FT* copy is silenced in *B. napus* and *B. oleracea* due to the insertion of a miniature inverted-repeat transposable element (MITE) in its promoter region, whereas the remaining five copies are detectable in *B. napus*, *B. rapa* and *B. oleracea* (Wang et al., 2012a). There are at least four *TFL1* paralogs in the *B. napus* genome (Mimida et al., 1999). Among them,

the *BnTFL1-2* paralog shares high homology with the *B. rapa* ortholog on chromosome A10, whereas *BnTFL1-1*, *BnTFL1-3* are highly similar to their *B. oleracea* counterparts. The *BnC6FTa* and *BnC6FTb* paralogs were co-located to a major flowering time QTL detected in nine winter-cropped environments which could support their function as flowering time regulators in *B. napus* (Qiu et al., 2006; Shi et al., 2009). Until now, *B. napus* *FT/TFL1* homologs have not been functionally characterized.

This study had two major aims. First, we aimed to uncover the role in flowering time control of different *FT* and *TFL1* paralogs in *B. napus* by analyzing EMS-treated offspring with missense and splice-site mutations within selected paralogs. We demonstrate that single mutations can change the onset of flowering in *B. napus* despite the redundancy of its allopolyploid genome. Moreover, we postulated that *BnTFL1* mutations also affect seed yield components in rapeseed. We found increased seed yield in *F₁* plants carrying a mutated *BnTFL1* allele on the Express 617 background. Our data suggest that EMS-generated alleles may constitute a new resource to broaden the genetic basis of rapeseed breeding.

MATERIALS AND METHODS

PLANT MATERIALS AND GREENHOUSE EXPERIMENTS

Seedlings of *M₃* lines and Express 617 (controls) were grown in the greenhouse at constant temperature (22°C) under long days (LD, 16 h light/8 h dark) for 4 weeks. Express 617 is an inbreed line (*F₁₁*) originated from the rapeseed winter-type cultivar Express (Harloff et al., 2012). Subsequently, plants were vernalized for 8 weeks at 4°C under LD conditions in a cold chamber. Of each *M₃* line, 30 plants were grown. After vernalization, plants were returned to the initial greenhouse conditions and transplanted to 11 × 11 cm pots. *M₃* plants and Express 617 controls were arranged in randomized blocks. Plant positions on the greenhouse were indexed and linked to randomly generated numbers using the Microsoft Excel software. Selected *M₃* lines were crossed with the male sterile (MS) line MSL007 (NPZ, Hohenlieth, Germany) using homozygous *M₃* plants as a pollinators. *F₁* plants and Express 617 controls were grown in the greenhouse under the conditions mentioned above. *F₂* populations were produced by crossing *M₃* homozygous plants from a selected *BnC6FTb* mutation (*BnC6FTbG2154A*) and non-mutagenized Express 617 plants. In each greenhouse experiment, the following phenotypic characters were measured according to the BBCH scale (<http://www.jki.bund.de/en/startseite/veroeffentlichungen/bbch-codes.html>): first non-cotyledonal leaves (NCL, BBCH10), rosette plant (BBCH30), visible floral buds (BBCH50), first open flower (BBCH60), and end of flowering (BBCH69). Plants that did not grow beyond NCL (BBCH 10) were excluded from the experiment. Plant height, number of branches, initial flowers, filled pods, seed number and seed weight were recorded for each plant separately.

MUTATION SCREENING

A total of 3488 *M₂* plants of the Express 617 EMS-population were screened by TILLING as described by Harloff et al. (2012). Gene specific primers were designed for *BnC6FTa* (FJ848915.1), *BnC6FTb* (FJ848917.1), and *BnTFL1-2* (AB017526.1)

(Supplementary Table S1). For primer design and comparative analysis, *B. rapa* and *B. oleracea* genome sequences were downloaded from (<http://brassicadb.org/brad/downloadOverview.php>) and (<http://ocri-genomics.org/bolbase/>), respectively. Plant genomic DNA arrayed in two dimensional 8-fold pools was amplified by direct or nested PCR. Forward and reverse primers were 5'-end labeled with 700 nm (DY-681) or 800 nm (DY-781) IRD fluorescence dyes, respectively (Biomers, Ulm, Germany, www.biomers.net). PCR amplifications with labeled oligos were done using the following profile: 95°C 5 min; 35 cycles of 95°C 30 s, 60°C 45 s, 72°C 90 s, 72°C 10 min. Heteroduplex-specific restriction endonuclease *CEL1* was extracted from celery and stored at -80°C as reported by Frerichmann et al. (2013). Labeled fragments were separated by a LI-COR 4300 DNA analyzer (LI-COR Biosciences) for 3:15 to 4:15 h at 1,500 V, 40 mA and 40 W. Gel images were analyzed using the software GelBuddy (<http://www.proweb.org/gelbuddy/>). After positive pools had been identified, single plant DNA was amplified with unlabeled oligos and sequenced for SNPs confirmation. Sequences were analyzed with the CLC-bio Main Workbench sequence alignment tool (CLC bio, Aarhus, Denmark).

DNA ISOLATION AND GENOTYPING

Total DNA was extracted from young leaves using a CTAB protocol (Morjane et al., 1994). Total DNA was treated with RNase I (Fermentas, www.fermentas.de), and DNA concentration was determined by spectrometry (NanoDrop, www.nanodrop.com). DNA quality was checked by 1% agarose gel electrophoresis. For genotyping mutant lines, genomic DNA from single plants was amplified by PCR using unlabeled primers. PCR was done essentially as described in the previous paragraph. Five micro litter of each PCR product were loaded on 1% (w/v) agarose gels. Upon band size confirmation, the remaining 25 µl of PCR product were sequenced via Sanger capillary sequencing. The sequences were analyzed with the CLC-Bio software (CLC bio, Aarhus, Denmark) using the sequence assembly viewer tool.

TISSUES SAMPLING AND RT-qPCR

Young leaves of M₃ plants and Express 617 controls were sampled at four developmental stages, as described above. Genomic DNA sequences from the different flowering time genes analyzed were retrieved from the non-redundant NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov>). Individual sequences were loaded to the CLC-bio main workbench version 6.0 (<http://www.clcbio.com>), and groups of paralogs were aligned with the help of the internal alignment routine. Two main strategies were applied for expression analysis: (i) primers were designed in conserved regions within groups of paralogs for detecting joint gene expression, and (ii) copy-specific primers were designed for the members of selected paralog genes (Supplementary Table S1). Total RNA was extracted using the RNeasy kit (QIAGEN, www.qiagen.com) according to the manufacturer's protocol. The RNA concentration was determined by spectrometry (Nano Drop; Thermo Scientific, Wilmington, USA) and quality was checked by agarose gel electrophoresis. Total RNA was treated with DNase I (Fermentas Inc., Maryland, USA). First-strand cDNA was synthesized using

Oligo(dT)₁₈ primers and the M-MuLV Reverse Transcriptase (Fermentas).

Quantitative real-time RT-PCR (RT-qPCR) was performed with SYBR qPCR Super mix w/ROX (Invitrogen Corporation, Carlsbad, USA) using a CFX96 Real-Time System (Bio-Rad Laboratories GmbH, München, Germany). Reactions were performed in a total volume of 15 µl containing 100 nM of each primer and 2 µl of diluted cDNA templates, and amplified using the following cycling conditions: 95°C for 3 min, 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, followed by 95°C for 10 min. A melting curve was generated using a temperature range from 65°C to 95°C with increments of 0.5°C every 5 s. For each sample at least three technical replications were performed. For data analysis, the mean *C_t* value of the target gene was normalized against the average *C_t* value of two housekeeping genes (*BnGADPH-3* and *BnB-Tub*). Calculation of relative expression values was carried out following Pfaffl (2001) after extracting main *C_t* values via CFX manager software (Bio-Rad Laboratories GmbH, München, Germany). In each analysis, the relative expression value for the reference sample has been set to 1. Normalized expression was averaged over two biological replicates and three technical repetitions in each case. Standard curves for the target and housekeeping genes are based on dilution series of purified cloned fragments for each gene.

SEQUENCE DIVERSITY ANALYSIS

For analyzing sequence diversity within the *BnC6FTb* and *BnTFL1-2* genes, genomic DNA from one-hundred accessions of the *B. napus* ASSYST panel was amplified with paralog-specific primers and sequenced via Sanger method. We selected 117 lines from the *B. napus* ASSYST diversity set (Bus et al., 2011) including winter, semi-winter and spring types which had been phenotyped in several environments worldwide (Supplementary Table S2). Lyophilized leaf samples harvested from young plants were used for DNA isolation with the NucleoSpin Plant II DNA isolation kit (Macherei & Nagel, Germany), following the manufacturer's instructions. PCR amplifications were carried out with paralog-specific primers as follows: 95°C 5 min; 35 cycles of 95°C 30 s, 60°C 45 s, 72°C 90 s, 72°C 10 min. Sequences resulting from single band amplicons were assembled and aligned using the CLC-bio main workbench software (CLC bio, Aarhus, Denmark) and the resulting FASTA alignment was loaded into the software TASSEL (<http://www.maizegenetics.net>) for identification of polymorphic SNPs.

RESULTS

PARALOG-SPECIFIC EXPRESSION OF FIVE *BnFT* GENES

We carried out a RT-qPCR experiment to measure the paralog specific expression of six *BnFT* paralogs in leaves of the winter-type inbred line Express 617 during the transition to reproductive stages. Samples were taken from greenhouse-grown plants at three different stages of development (BBCH30 before and after vernalization and BBCH50). Relative expression values for each paralog were calculated after *Ct* normalization using *BnGAPDH* as a reference gene. Leaf samples at BBCH30 before vernalization (preV) were used as reference samples for relative expression calculations. At BBCH30 before

vernification (BBCH30-preV), four *BnFT* paralogs (*BnC6FTA/b* and *BnC6FT7a/b*) were weakly expressed (Figure 1), whereas two transcripts were not expressed (*BnC2FT* and *BnA2FT*). Moreover, *BnA2FT* was only highly expressed at BBCH60 after floral transition (data not shown), whereas *BnC2FT* showed no expression at all. After vernalization (BBCH30-postV), *BnC6FTA/b* and *BnA7FTA/b* expression was higher in rosette plants, but differences between paralogs were obvious. *BnC6FTb* showed the largest relative expression level (~9-fold). At BBCH50 (visible floral buds), the *BnC6FTb* and *BnA7FTb* paralogs showed the largest relative expression levels (~13-fold) (Figure 1). In leaves at BBCH 60 (first flower open), all paralogs with exception of *BnC2FT* showed very high relative expression levels (>2000-fold), where *BnC6FTA* showed the highest relative expression (data not shown).

EMS MUTATIONS IN *BnFT* AND *BnTFL1* PARALOGS

We screened our EMS population to measure the flowering time effect of mutations within the *BnFT* paralogs *BnC6FTA* (FJ848915.1) and *BnC6FTb* (FJ848917.1). In Arabidopsis, apart from FT, other PEBP proteins such as TERMINAL FLOWER-1 (TFL1) regulate flowering by competing with FT for its binding targets (Mimida et al., 2001). Therefore, we developed primers for the *BnTFL1-2* (ABO17526) gene assigned to *B. rapa* chromosome A10. In total, 3488 M₂ plants were screened by TILLING for EMS-induced mutations in *BnC6FTA/b* and *BnTFL1-2*.

We generated paralog-specific PCR amplicons covering between 50% (*BnC6FTA*) and 100% (*BnC6FTb* and *BnTFL1-2*) of the open reading frames. The *BnC6FTA* fragment covered exon I and intron I. The two *BnC6FTb* fragments covered exon I / intron I and exons III / IV (Figure 2). The *BnTFL1-2* fragment covered all four exons. We identified 55, 14, and 34 single nucleotide mutations in the *BnC6FTb*, *BnC6FTA*, and *BnTFL1-2* genes, respectively. Forty-three mutations are located in introns, 19 are silent mutations, and three are located within the UTRs (Table 1). Mutation rates ranged between 1/72 kb and 1/24 kb per 1000 plants. The names of the mutant alleles contain the nucleotide substitution and nucleotide position (Table 2).

We identified one non-sense mutation in exon III of the *BnC6FTb* gene (*BnC6FTb*_{G1968A}) leading to a truncated protein by substitution of a tryptophan by a stop codon (position 88). Another mutation (*BnC6FTb*_{G2009A}) resulted in a splice-site deletion leading to a truncated protein by interrupting the junction between exons III and IV. The *BnTFL1-2*_{C965T} mutation in Exon III results in the substitution of a glutamine by a stop codon (position 146). Moreover, we detected numerous missense mutations in *BnC6FTA* (15), *BnC6FTb* (16), and *BnTFL1-2* (10).

We decided to focus on splice site- and missense-mutations which are most likely to affect the protein function. All observed missense mutations were compared to the SIFT database (<http://sift.jcvi.org>) in order to evaluate the impact of the amino

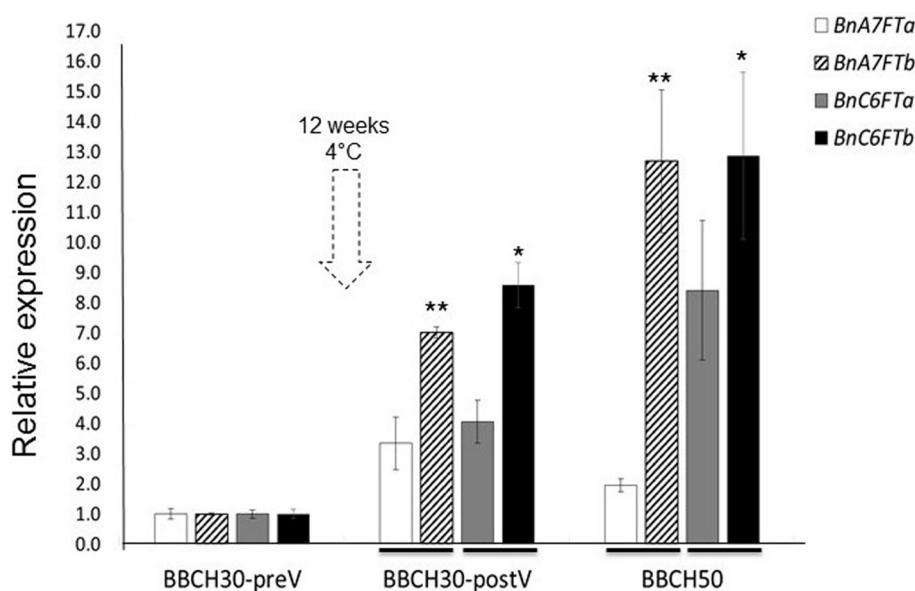


FIGURE 1 | Relative expression of five *BnFT* paralogs in Express 617 plants at three developmental stages before and after vernalization (dotted arrow). Plants at BBCH30 were analyzed before vernalization (preV) and after vernalization (postV). The time point BBCH30-preV was taken as reference for calculation of relative expression in all target genes. Two biological replicates and three technical repetitions were analyzed for each time point. Error bars indicate the standard error of the mean of the relative expression values. Expression levels of target *BnFT* genes were normalized against *BnGAPDH* total expression. Ct values of the paralogs *BnA2FT* and

BnC2FT were below the detection threshold at BBCH30 and BBCH50. Significant differences ($P < 0.05$) are depicted by asterisks. Differences in relative expression between the *BnC6FTA* / *BnC6FTb* (*) and *BnA7FTA* / *BnA7FTb* paralogs (**) at each time point were tested via t-test. Lines at the base of the bars indicate the comparison pairs. All samples were taken between zeitgeber 11 h and 12 h in each developmental stage. Express 617 plants reached BBCH30-preV ~30 days after sowing. BBCH30-postV was registered ~90 days after sowing. BBCH50 was registered ~107 days after sowing.

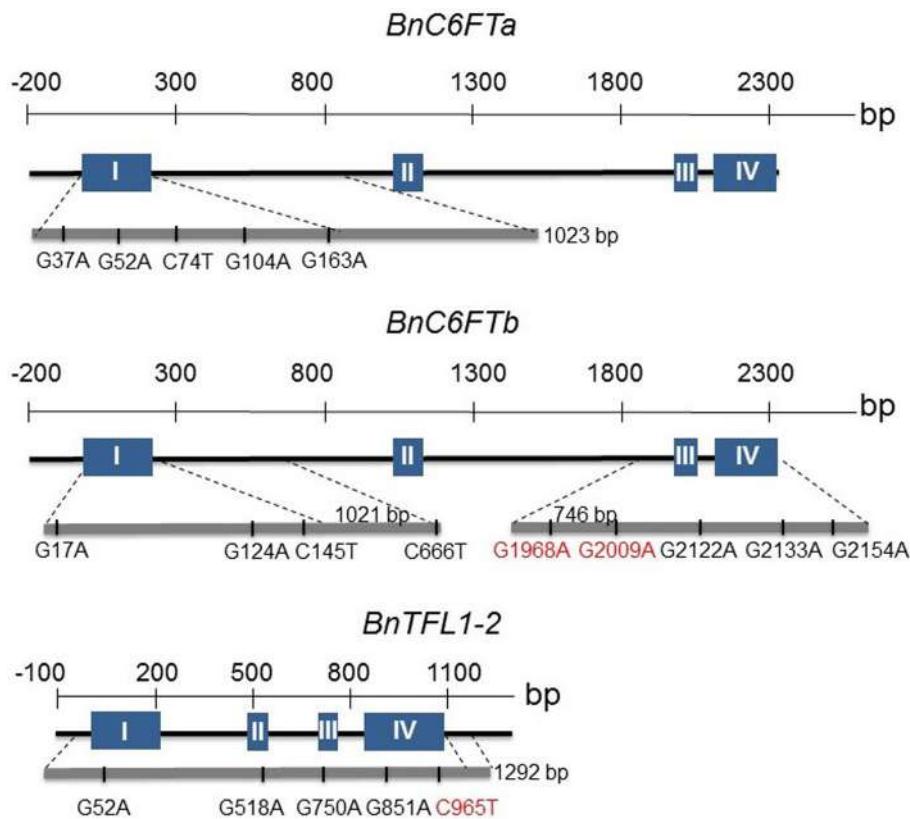


FIGURE 2 | Graphical presentation of 18 point mutations in two *FT* (*BnC6FTa*, *BnC6FTb*) and one *TFL1* (*BnTFL1-2*) paralog of the *B. napus* winter-type cultivar Express 617. The exon (blue boxes) - intron (black lines) structure of each gene is shown. Locations of

SNPs are depicted by the gray bar below. Numbers refer to the position of the mutations from the START codon. Only STOP/splice-mutations (red) and selected missense mutations are shown.

Table 1 | EMS mutations in three flowering time genes detected by TILLING of the winter-type inbred line Express 617.

| | <i>BnC6FTa</i> | <i>BnC6FTb</i> | <i>BnTFL1-2</i> |
|--------------------------------------------------|----------------|----------------|-----------------|
| Number of paralogs in rapeseed | 6 | 6 | 4 |
| Total number of M ₂ plants screened | 3488 | 3488 | 2092 |
| Sequence screened by TILLING (bp) | 1023 | 1767 | 1292 |
| Nonsense mutations | 0 | 1 | 2 |
| Missense mutations | 6 | 15 | 10 |
| Splice site mutations | 0 | 1 | 1 |
| Total number of mutations | 14 | 55 | 34 |
| Mutations/kb | 72 | 30 | 24 |
| M ₃ families selected for phenotyping | 5 | 9 | 5 |
| Total number of mutations | 103 | | |

The *BnC6FTb* paralog was screened by two different fragments, 1021 bp and 746 bp in size.

acid substitutions on the protein function (data not shown). According to this analysis, we selected 18 M₃ lines for growth experiments in the greenhouse (5 *BnC6FTa*, 8 *BnC6FTb*, and 5 *BnTFL1-2* mutations) (Supplementary Table S3) (Figure 2).

PHENOTYPIC CHARACTERIZATION OF *BnFT* AND *BnTFL1-2* MUTANTS

First, we confirmed the genotype of each selected M₂ plant by Sanger sequencing. Then, M₃ lines were grown in the greenhouse together with non-mutagenized Express 617 plants as a control. The phenological development of *BnC6FTa* and *BnC6FTb* lines was clearly different. All five *BnC6FTa* mutants flowered as the control, whereas six out of eight *BnC6FTb* mutants flowered later (Figure 3). The *C6FTb*_{G1968A} mutants (stop mutation) showed a flowering delay of ca. 18 days, while *C6FTb*_{G2009A} splice-site mutants flowered 29 days later as the control. Interestingly, 40 and 54% of the *C6FTb*_{G1968A} and *C6FTb*_{G2009A} M₃ mutants, respectively, did not bolt at all (Figure 4). *BnC6FTb* missense mutants started flowering 7 days (*BnC6FTb*_{C2122A}) up to 26 days (*BnC6FTb*_{G17A}) later as the control. Apart from flowering time, reduced fertility was also apparent, mostly in *BnC6FTb* M₃ plants (Supplementary Figure 1).

To evaluate the effect of background mutations on flowering time, we produced an F₂ population by crossing *BnC6FTb*_{G2154A} M₃ plants with non-mutagenized Express 617. *BnC6FTb*_{G2154A} M₃ missense mutants gave higher hybrid seed yield as the stop mutants and they flowered 15 days later as the control. *BnC6FTb*_{G2154A} M₃ plants showed a reduced number of initial flowers in comparison to other M₃ mutants, however most

flowers were fertile. A total of 26 F₂ plants encompassing all three genotypic classes were grown in the greenhouse together with Express 617. In agreement with M₃ observations, homozygous F₂ mutants (*ft ft*) flowered 13 days later than F₂ siblings

Table 2 | Nucleotide position and amino acid changes due to EMS mutations in 18 missense/non-sense mutations in three *B. napus* flowering time regulators.

| Gene | Mutation | Exon | Amino acid substitution | Mutant code |
|-----------------|----------|------------|-------------------------|--------------------------------|
| <i>BnC6FTa</i> | G37A | Exon I | Gly13Arg | <i>C6FTa</i> _{G37A} |
| | G52A | Exon I | Val17Le | <i>C6FTa</i> _{G52A} |
| | C74T | Exon I | Ser25Leu | <i>C6FTa</i> _{C74T} |
| | G104A | Exon I | Arg35Lys | <i>C6FTa</i> _{G104A} |
| | G163A | Exon I | Glu55Lys | <i>C6FTa</i> _{G163A} |
| <i>BnC6FTb</i> | G17A | Exon I | Arg6Lys | <i>C6FTb</i> _{G17A} |
| | G124A | Exon I | Asp42Asn | <i>C6FTb</i> _{G124A} |
| | C666T | CArG Box | CArG Box | <i>C6FTb</i> _{C666T} |
| | G1968A | Exon III | Trp88Stop | <i>C6FTb</i> _{G1968A} |
| | G2009A | Intron III | Splice site | <i>C6FTb</i> _{G2009A} |
| | G2122A | Exon IV | Arg112Lys | <i>C6FTb</i> _{G2122A} |
| | G2133A | Exon IV | Gly116Arg | <i>C6FTb</i> _{C2133T} |
| | G2154A | Exon IV | Val123Met | <i>C6FTb</i> _{G2154A} |
| <i>BnTFL1-2</i> | G52A | Exon I | Val18Le | <i>TFL1-2</i> _{G52A} |
| | C518T | Exon II | Pro83Ser | <i>TFL1-2</i> _{C518T} |
| | G750A | Exon III | Gly105Arg | <i>TFL1-2</i> _{G750A} |
| | G851A | Exon IV | Val108Met | <i>TFL1-2</i> _{G851A} |
| | C965T | Exon IV | Gln146Stop | <i>TFL1-2</i> _{C965T} |

homozygous for the wild-type allele (*FT FT*) which did not show any significant differences in flowering time as compared to non-mutagenized Express 617 (Supplementary Figure 2).

The stop mutation *BnTFL1-2*_{C965T} did not lead to a major delay in flowering time. In contrast, the missense mutants *BnTFL1-2*_{G52A} and *BnTFL1-2*_{G750A} flowered ~10 days later than the control (Figure 3). Since, the stop mutation is close to the end of the *BnTFL1-2* gene, a functional protein may still arise after translation. Furthermore, *BnTFL1*_{G750A} mutants exhibited modifications in plant architecture which gave us a reason to select them for crossing experiments. *BnTFL1*_{G750A} mutants developed normally during the early growth phase until reaching BBCH50 (visible floral buds). The internode elongation phase was much longer as compared to Express 617, as a consequence, mutant plants were not able to stand by themselves after BBCH50. In this M₃ line, the flower development limited the continuous growth of the floral meristem, whereas side branches continued flowering.

THE EMS MUTATIONS IN *BnC6FTb* AND *BnTFL1-2* PARALOGS ARE LOCATED IN HIGHLY MONOMORPHIC REGIONS OF EXON III AND EXON IV

To investigate the genetic structure of those *BnFT* and *BnTFL1* paralogs with paramount impact on flowering time, we analyzed the sequence diversity of *BnC6FTb* and *BnTFL1-2* in *B. napus* by sequencing their complete exons III and IV in 117 *B. napus* inbreed lines from different geographic origins and growth types. Sequences selected for analysis in each gene after quality trimming are deposited in Supplementary Table S2. While *BnC6FTb* exon III turned out to be highly conserved, exon IV exhibited larger sequence diversity. Within 41 bp exon III of the

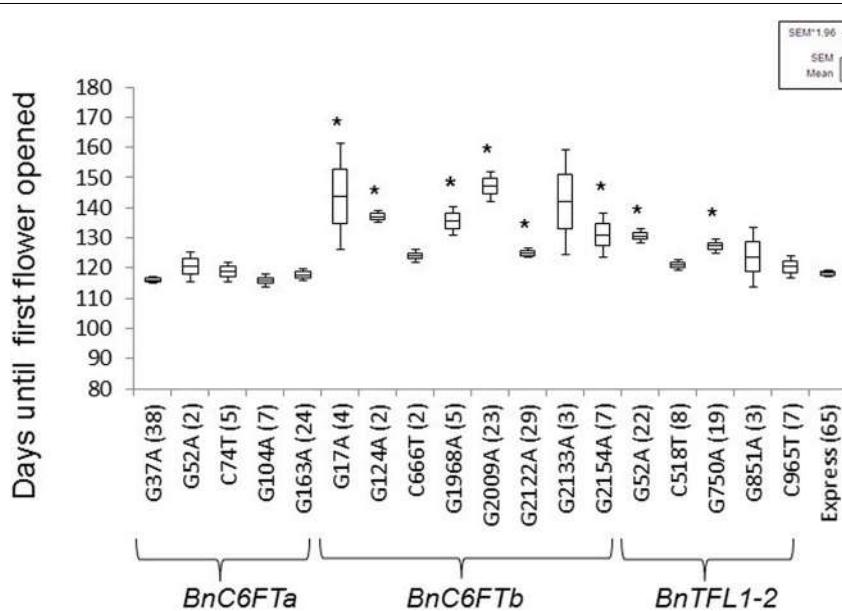


FIGURE 3 | Flowering time point of 18 *BnC6FT*- and *BnTFL1-2* mutants grown in the greenhouse at constant temperature (22°C), and LD (16 h light) after vernalization (4°C, 16 h light, 8 weeks). Days to flowering (BBCH 60) was measured in M₃ plants homozygous for the EMS allele. The

non-mutagenized donor line Express 617 was used as a control. The number of plants analyzed is written in brackets. Differences in flowering time between homozygous mutants and control plants were tested via t-test. Significant differences ($P < 0.05$) are depicted by asterisks.

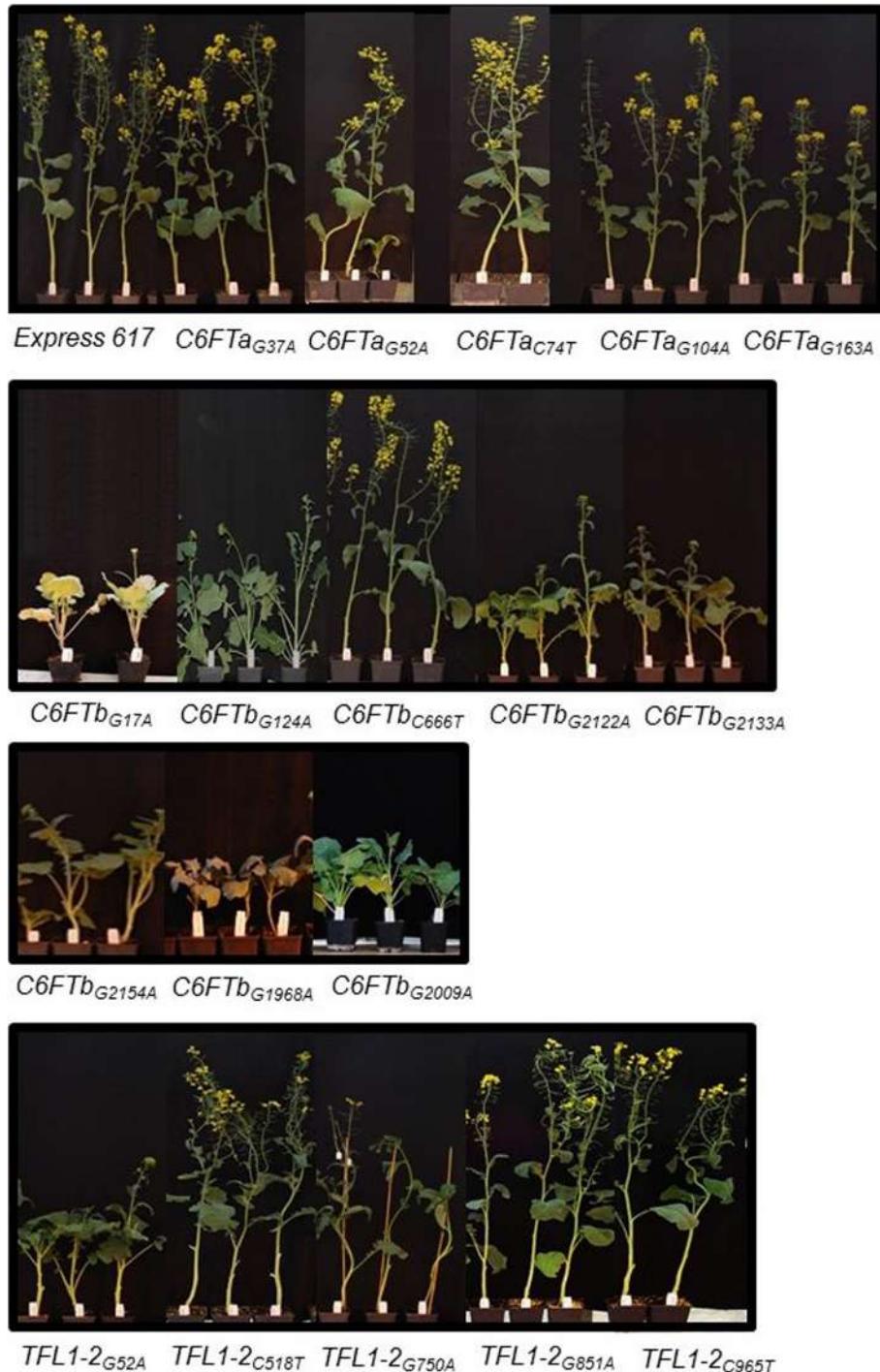


FIGURE 4 | Phenotypes of 18 *B. napus* *BnC6FTa/b* and *BnTFL1-2* EMS M₃ lines. Photos were taken as the non-mutagenized Express 617 plants started flowering (top left). Plants were grown in the greenhouse at constant temperature (22°C), and LD (16 h light) after vernalization (4°C, 16 h light, 8 weeks).

BnC6FTb gene, only a single polymorphism was found at position 2004 which corresponds to an allele frequency of 1%. The EMS mutation G1968A resides within a sequence domain which is monomorphic among all accessions investigated. For *BnC6FTb* (exon IV), six polymorphic regions were found with minor

allele frequencies of <5.0%. The EMS-generated alleles (positions G2122, G2133, and G2154) are residing in monomorphic sequences (Supplementary Figure 3).

In contrast to *BnFT* genes, a higher variability in *BnTFL1-2* exon III than in exon IV was found. In exon III the SNP

showing the largest variation was a C insertion at position 731. The EMS mutation G750A which is also located within this exon, was located in a fully monomorphic domain. For *BnTFL1-2* exon IV, only a T/C polymorphism was found at position 1030. In conclusion, there is a high degree of sequence conservation within the analyzed sequences. Our EMS treatment created novel sequence variations within these highly conserved regions (Supplementary Figure 4). FASTA-formatted sequences for each gene are deposited in Supplementary File 1. All sequences have been submitted to NCBI (www.ncbi.nlm.nih.gov) (accession numbers KJ533546 - KJ533625 and KJ533626 - KJ533728).

A *BnC6FTb*-SPlice-SITE MUTATION IMPACTS THE EXPRESSION OF OTHER FLOWERING TIME GENES IN LEAVES

We reasoned that a loss of function of the *BnC6FTb* paralog directly impacts the transcriptional activity of other major flowering time regulators downstream of *BnFT*. To test this hypothesis, we selected the *BnC6FTb_{G2009A}* mutant because, first the G2009A SNP causes a splice-site mutation that leads to a truncated protein, and second, *C6FTb_{G2009A}* M₃ plants are characterized by a marked flowering delay of about 29 days compared to the Express 617 control (Figure 3).

We chose *BnAPI* and *BnSOC1* as putative downstream targets of *BnFT* genes based on our knowledge from Arabidopsis (Yoo et al., 2005; Kaufmann et al., 2010). We measured their expression in leaves. *BnC6FTb_{G2009A}* M₃ plants were grown in the greenhouse under constant temperature and LD conditions. For expression analysis, young leaves of three different plants were taken at stages BBCH30 (pre and post-vernalization), BBCH50, and BBCH60. Arabidopsis *API* and *SOC1* sequences were BLASTed against *B. oleracea* and *B. rapa*. High homology hits were aligned and primers were designed from highly conserved regions. Subsequently, joint expression of all paralogs was measured by RT-qPCR. Gene expression levels of *BnAPI* and *BnSOC1* were normalized using *BnGAPDH* and *BnB-Tub* genes.

We detected altered transcriptional activities of *BnAPI* in *BnC6FTb_{G2009A}* mutants when compared to Express 617 control plants. Control Express 617 plants at BBCH30 (preV) were used as reference sample for relative expression calculations. At rosette stages (BBCH30), *BnAPI* expression in the M₃ mutant was higher than in control plants, while at BBCH50 and BBCH60 relative expression levels were 10–40% lower (Figure 5). Before vernalization (BBCH30-preV), *BnSOC1* expression in mutants was 2.5-fold higher than in Express 617. After vernalization, we detected a reduction of *BnSOC1* in *BnC6FTb_{G2009A}* mutants compared to the control. When the first flower opened, the difference in expression between *BnC6FTb_{G2009A}* mutants and Express 617 was at its maximum. The altered expression in leaves indicates that a single *BnC6FTb* mutation may affect other major flowering time regulators. We expect that both genes are expressed in the shoot apical meristem as well.

PERFORMANCE OF F₁ HYBRIDS USING THE *BnTFL1-2* MUTANTS AS PARENTS

In tomato, mutations in *FT* and *TFL1* orthologs accounted for fruit yield heterosis in F₁ hybrids (Krieger et al., 2010). We made an initial experiment to address the question whether

B. napus orthologs might have a similar function. For producing F₁ hybrids, we selected *BnC6FTb_{G2009A}* and *BnTFL1-2_{G750A}* homozygous M₃ mutants as pollinators due to their late flowering phenotype in conjunction with an altered inflorescence (lower number of fertile flowers, Supplementary Figure 1). We crossed homozygous M₃ plants with the male-sterile (MS) line MSL007. The MSL007 line (MSL-Express) is an isogenic line of Express that carries the male sterility Lembke (MSL) genic male sterility system (Basunanda et al., 2010). Thus, no F₁ heterosis was expected, except effects due to EMS mutations.

F₁ hybrids were vernalized and grown in the greenhouse with the parental lines and Express 617. Both F₁ hybrids showed differential effects. *BnC6FTb_{G2009A}* hybrids showed no significant differences in seed number per plant and total seed weight in comparison to Express 617 as the best parent. In contrast, *BnTFL1-2_{G750A}* F₁ hybrids had significantly higher number of seeds/plant (20%) and total seed weight (40%) as compared to the best parent (Figure 6). Although the effects of background mutations cannot be ruled out, these results could indicate that *BnTFL1* mutations impact heterosis in *B. napus*.

DISCUSSION

In the present study, more than 100 EMS-alleles have been found for three flowering time genes of *B. napus*. Based on previous reports, the average EMS-mutation frequencies are expected to be lower in diploid species (~1/380 kb) than in polyploids (~1/50 kb) (Till et al., 2007; Wang et al., 2012c). In this study, mutation frequencies ranged between 1/24 kb and 1/72 kb. Using the same EMS-population, mutation frequencies ranged between 1/12 and 1/22 kb for sinapine biosynthesis genes (Harloff et al., 2012). Although the observed *BnFT*/*BnTFL1* mutation frequencies are slightly lower, our results are in the range expected for polyploid species. The present mutants will be a valuable resource to study flowering regulatory networks in polyploids and they can be introduced into breeding programs.

Our aim was to provide data about the function of *FT* and *TFL1* paralogs in rapeseed. We found that, despite the redundancy of mutations in a single gene, either non-sense or missense mutations in the *BnC6FTb* gene resulted in a marked flowering delay. This supports our hypothesis that *BnFT* paralogs contribute differently to flowering time regulation. A large plethora of Arabidopsis reports on *FT* loss-of-function mutants have established a robust correlation between *FT* mutations and flowering time delay in Arabidopsis (Andres and Coupland, 2012). Contrasting with expectations based on Arabidopsis *TFL1*-phenotypes, *BnTFL1-2* mutants showed a slight delay in flowering time. In Arabidopsis, a single amino acid change in *TFL1* (*tfl1-1*_{Gly105Asp}) led to early flowering and limited the development of indeterminate inflorescence by promoting the formation of a terminal floral meristem (Bradley et al., 1997). On the other hand, F₁ hybrids derived from crosses between *BnTFL1-2* M₃- and rape-seed MS lines showed increased seed yield compared to *BnC6FTb* F₁ hybrids and Express 617 controls. Thus, although the role of *BnTFL1-2* involving flowering time regulation is not likely to be conserved compared to its Arabidopsis ortholog, *TFL1-2* appears to be involved in yield-related traits as reported for its tomato ortholog *SP* (Jiang et al., 2013b). As we mention in the following

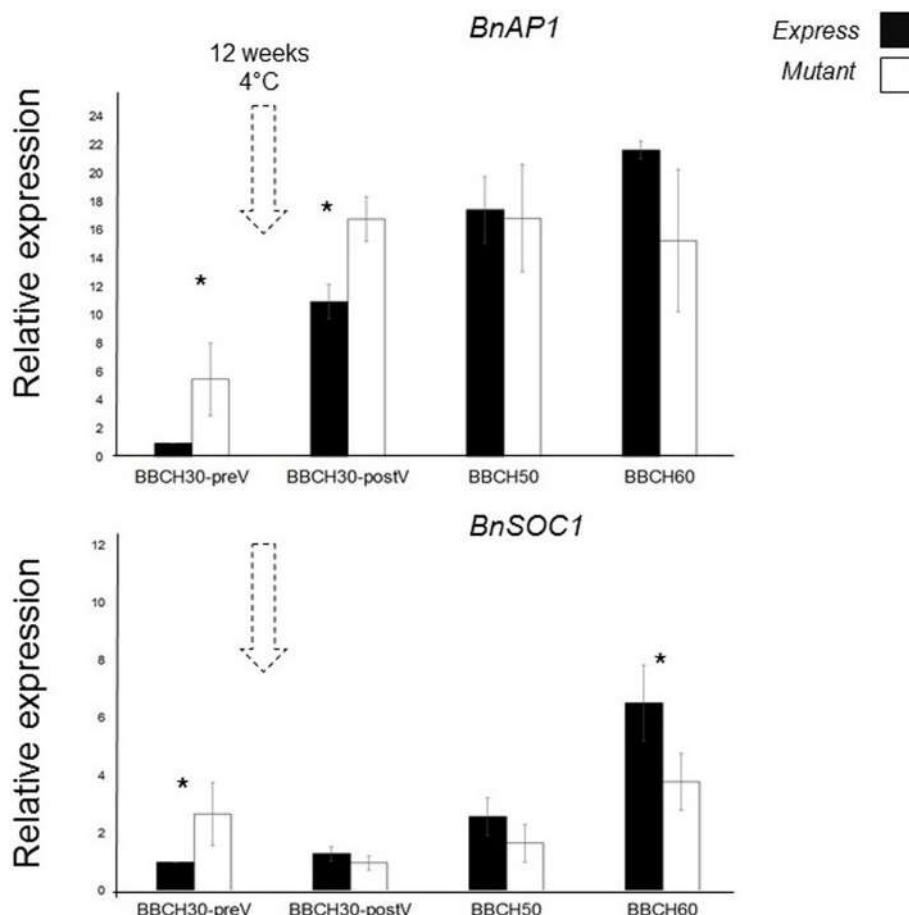


FIGURE 5 | Joined expression analysis of two *BnFT* downstream targets in the *BnC6FTbG2009* mutant (open boxes) and Express 617 (filled boxes). Four developmental stages were analyzed before and after vernalization (dotted arrows). Plants at BBCH30 were analyzed before vernalization (preV) and after vernalization (postV). Two biological replicates (M_3 plants) and three technical repetitions were analyzed for each time point. Error bars: standard error of the mean for biological

replicates. Ct of target genes were normalized against the *BnGAPDH* and *BnB-Tub* total expression. The time point BBCH30-preV in Express 617 control plants was taken as reference sample for calculation of relative expression. Differences in relative expression were pairwise tested (control Vs M_3 line) via t-test. Significant differences ($P < 0.05$) are depicted by asterisks. All samples were taken between zeitgeber 11 h and 12 h.

sections, confirming this hypothesis is a must for new research approaches.

During evolution, duplicated genes may undergo dosage adjustments (Papp et al., 2003; Conant and Wolfe, 2008), non-functionalization, or sub-/neo-functionalization (Force et al., 1999). We wanted to know whether different *BnFT* and *BnTFL1* paralogs gained different function by studying their phenotypes and their transcriptional activities. Through digital gene expression analyses, differential expression within early generations of re-synthesized- (F_1 - F_4) and natural *B. napus* accessions has been reported (Birchler and Veitia, 2010). Three highly similar genes encoding endoplasmic reticulum-bound sn-glycerol-3-phosphate acyltransferase-4 (*BnGPAT4-C1*, *BnGPAT4-C2*, and *BnGPAT4-A1*) showed different expression patterns and altered epigenetic features (Chen et al., 2011) which is in accordance with the assumption that in polyploids orthologous genes are frequently expressed in a non-additive manner (Jiang et al., 2013a). We have

also observed marked differences in the expression of six *BnFT* paralogs in support of the non/sub-functionalisation hypothesis. In regard to their position within a major flowering time QTL (Wang et al., 2009), *BnC6FTb* paralogs seem to play the most important role as flowering time regulators in winter type *B. napus*. More evidence has been given by the expression analysis of putative *FT* downstream targets *BnAP1* and *BnSOC1* in late-flowering *BnC6FTbG2009A* mutants. *SOC1* encodes a MADS-box transcription factor, acting as a floral integrator (Lee and Lee, 2010). The gene *SOC1* gene is expressed in the shoot apical meristem, and *SOC1* mutations lead to late flowering phenotype (Borner et al., 2000). However, *SOC1* is also expressed in vegetative organs (leaves) (Hepworth et al., 2002). A reduction in *SOC1* mRNA was detected in the meristem of late-flowering *ft-7* (Trp138Stop) *Arabidopsis* Ler background (Searle et al., 2006). In the future, the activities of these genes shall also be studied in the shoot apical meristem.

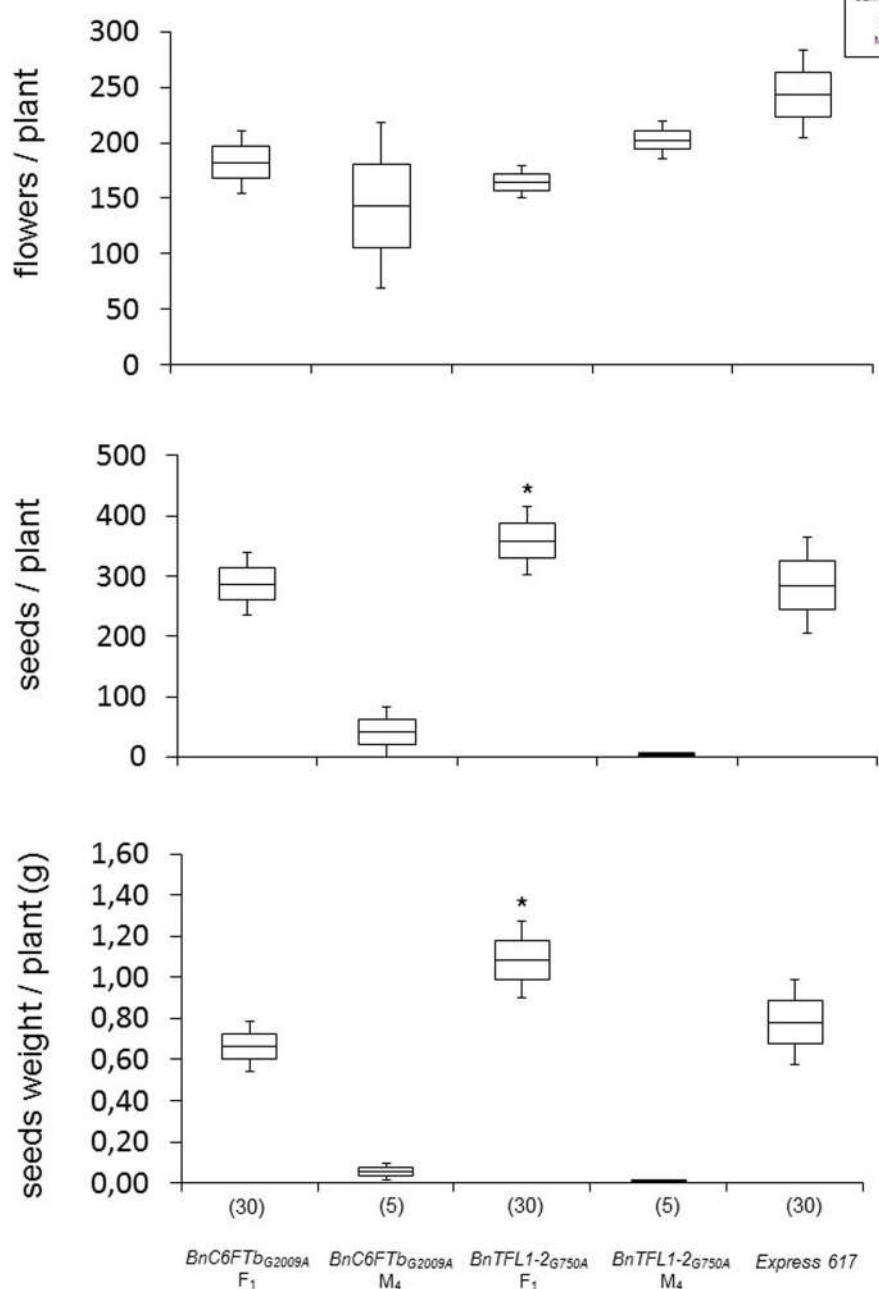


FIGURE 6 | Growth experiments with vernalized F_1 hybrids after crossing two mutants with a male sterile isogenic line (MSL007). Each F_1 was obtained from two different crossing experiments. Mutant parents (M_4 lines), and Express 617 were used as controls. Yield components were determined on single plants grown in the greenhouse. The number

of plants analyzed is given in brackets. Differences in flowers, seed number and seed weight per plant against Express 617 were tested via t-test. Significant differences ($P < 0.05$) are depicted by asterisks. Growth conditions: 22°C, 16 h light, greenhouse. Vernalization: 4°C, 16 h light, 8 weeks.

The phenotypic studies presented here gave further support to our assumption that *BnFT* paralogs do not contribute equally to flowering time regulation. In sugar beet (*Beta vulgaris*), two *FT* paralogs (*BvFT1* and *BvFT2*) were reported to antagonistically regulate flowering time (Pin et al., 2010). Knockdown of the *FT* potato paralog *StSP3D* resulted in a late flowering phenotype,

while knockdown of the second paralog *StSP6A* had no effect on flowering time but on tuberization (Navarro et al., 2011). In line with these findings, we observed differential effects of *BnC6FTA* and *BnC6FTb* mutations. The strong effect of *BnC6FTb* as a flowering time regulator in rapeseed has been confirmed. To which extend *BnC6FTA* and other *BnFT* paralogs are involved

in flowering time control needs to be investigated in the future (e.g., by using other TILLING mutants). As determined by our expression analyses, the strongest case of non-functionalization is shown by the lack of expression of the *BnC2FT* copy. This result is in full congruence with Wang et al. report (2012a) where this gene copy was neither expressed in *B. napus* nor in *B. oleracea*. A series of recent studies has demonstrated that beyond flowering time control, FT-like proteins act as mobile or cell-autonomous proteins that mediate other developmental processes, such as growth, plant architecture, and tuber formation (Carmona et al., 2007; Kinoshita et al., 2011; Navarro et al., 2011). In contrast to our *BnFT* mutant results, a previous analysis of sinapine biosynthesis mutants from the same EMS population as in our study, phenotypic or physiological effects had been observed only in double mutants (Harloff et al., 2012, Harloff, personal communication).

Although yield heterosis is regarded as a quantitative trait, single genes can contribute to heterotic effects through overdominance, such as the *Arabidopsis Erecta* locus (Moore and Lukens, 2011). As a first example of single gene overdominance, the yield of *sft-4537*± heterozygous tomato plants was increased by up to 60% in comparison to their parents after crossing high yielding M82 inbred plants with low-yielding homozygous loss of function mutants (*sft-4537*) (Krieger et al., 2010). In our work, F₁ hybrids carrying a *BnTFL1-2* mutant allele had a higher seed yield as the Express 617 parent. Our study delivers the first insights about potential *TFL1*-related heterosis in *B. napus*. In the future, experimental data are needed to verify this hypothesis. We tested our initial hypothesis by crossing mutants with the non-mutated donor line. Although, the data point at *BnTFL1* as a major gene for heterosis in rapeseed the possible impact of background mutations must be analyzed by additional hybrid combinations. Moreover, we will sequence the *BnTFL1-2* loci from rapeseed lines with high and low combining ability (Qian et al., 2007). If our preliminary greenhouse data will be confirmed by field experiments, this study will offer new perspectives for a hybrid breeding strategy which make use of *BnTFL1-2* sequence variations.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00282/abstract>

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Light quality regulates flowering in *FvFT1/FvTFL1* dependent manner in the woodland strawberry *Fragaria vesca*

Marja Rantanen¹, Takeshi Kurokura^{1†}, Katriina Mouhu¹, Paulo Pinho², Eino Tetri², Liisa Halonen², Pauliina Palonen¹, Paula Elomaa¹ and Timo Hytönen^{1,3*}

¹ Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland

² Department of Electrical Engineering and Automation, Aalto University, Espoo, Finland

³ Department of Biosciences, University of Helsinki, Helsinki, Finland

Edited by:

George Coupland, Max Planck Society, Germany

Reviewed by:

Eiji Nambara, University of Toronto, Canada

Chris Helliwell, CSIRO, Australia

***Correspondence:**

Timo Hytönen, Department of Agricultural Sciences, University of Helsinki, PO Box 27, Latokartanonkaari 7, FI-00014 Helsinki, Finland
e-mail: timo.hytonen@helsinki.fi

†Present address:

Takeshi Kurokura, Department of Agriculture, Faculty of Agriculture, Utsunomiya University, Tochigi, Japan

Control of flowering in the perennial model, the woodland strawberry (*Fragaria vesca* L.), involves distinct molecular mechanisms that result in contrasting photoperiodic flowering responses and growth cycles in different accessions. The *F. vesca* homolog of TERMINAL FLOWER1 (*FvTFL1*) functions as a key floral repressor that causes short-day (SD) requirement of flowering and seasonal flowering habit in the SD strawberry. In contrast, perpetual flowering *F. vesca* accessions lacking functional *FvTFL1* show FLOWERING LOCUS T (*FvFT1*)-dependent early flowering specifically under long-days (LD). We show here that the end-of-day far-red (FR) and blue (B) light activate the expression of *FvFT1* and the *F. vesca* homolog of SUPPRESSOR OF THE OVEREXPRESSION OF CONSTANS (*FvSOC1*) in both SD and LD strawberries, whereas low expression levels are detected in red (R) and SD treatments. By using transgenic lines, we demonstrate that *FvFT1* advances flowering under FR and B treatments compared to R and SD treatments in the LD strawberry, and that *FvSOC1* is specifically needed for the B light response. In the SD strawberry, flowering responses to these light quality treatments are reversed due to up-regulation of the floral repressor *FvTFL1* in parallel with *FvFT1* and *FvSOC1*. Our data highlights the central role of *FvFT1* in the light quality dependent flower induction in the LD strawberry and demonstrates that *FvTFL1* reverses not only photoperiodic requirements but also light quality effects on flower induction in the SD strawberry.

Keywords: flowering, *Fragaria*, FT, light spectrum, perennial, SOC1, strawberry, TFL1

INTRODUCTION

Plants monitor light intensity, duration, spectrum, and direction to adjust their growth and development. Photoperiod changes regularly throughout the year, and therefore, many plants rely on photoperiodic signals to control important phase transitions including flower induction. Furthermore, specific regions of the light spectrum have different effects on flowering. For example, far-red (FR) light, which is enriched under canopy, causes early flowering in many species (Brown and Klein, 1971; Johnson et al., 1994; Cerdán and Chory, 2003; Wollenberg et al., 2008). Plants sense changes in light by using photoreceptors. Phytochromes (Phy) are the only photoreceptors known to mediate photomorphogenic red (R) and FR signals (Takano et al., 2009; Strasser et al., 2010). In *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh.], PhyA promotes, whereas PhyB, D, and E repress flowering (Johnson et al., 1994; Reed et al., 1994; Devlin et al., 1998, 1999; Hu et al., 2013). In addition, cryptochrome (Cry) and light oxygen voltage (LOV) receptors mediate the effect of blue (B) light to control flowering (Guo et al., 1998; Mockler et al., 1999, 2003; Sawa et al., 2007).

Genes that are regulating photoperiodic flowering are conserved between annual short-day (SD) and long-day (LD) model species rice (*Oryza sativa* L.) and *Arabidopsis*, respectively, although their mode of action may differ (Hayama et al., 2003; Tsuji et al., 2011; Brambilla and Fornara, 2013). Photoperiodic flowering is explained by the external coincidence model. In *Arabidopsis*, CONSTANS (CO) mRNA is expressed rhythmically with a peak around the dusk under LD conditions and during the night under SD (Suarez-Lopez et al., 2001). Since CO is unstable in darkness, the protein accumulates only under LD when light coincides with the CO mRNA expression in the afternoon (Valverde et al., 2004). CO activates FLOWERING LOCUS T (FT) in the leaf phloem, and the FT protein moves into the shoot apical meristem (SAM) (Corbesier et al., 2007; Jaeger and Wigge, 2007; Tamaki et al., 2007). At the SAM, FT binds with the bZIP transcription factor FD, and this complex up-regulates the gene expression of MADS transcription factor APETALA1 (API) to induce flowering (Abe et al., 2005; Wigge et al., 2005). Also in SD plant rice, photoperiodic flowering occurs through external coincidence. Rice CO homolog, *Heading date 1* (*Hd1*), has similar expression pattern with CO. However, *Hd1* activates FT homolog

Heading date 3a (Hd3a) only under SD through an unknown mechanism which includes the action of PhyB (Kojima et al., 2002; Cremer and Coupland, 2003; Ishikawa et al., 2011; Tsuji et al., 2011). Furthermore, Hd3a forms a florigen activator complex with 14-3-3 proteins and OsFD1 to activate OsMADS15, a homolog of AP1, to induce flowering (Taoka et al., 2011).

Light quality affects the transcription of *CO* and *FT* and the stability of CO protein via different photoreceptors. Yanovsky and Kay (2002) showed that both Cry2 and PhyA are involved in the normal activation of *FT* mRNA expression in the photoperiodic flowering. These photoreceptors play partially redundant role to mediate B light promotion of flowering together with Cry1, although the main role of PhyA is to mediate the FR signal (Mockler et al., 2003). These photoreceptors are involved in the stabilization of CO protein, whereas PhyB promotes the degradation of CO in R light (Valverde et al., 2004). B light activated LOV-receptor, FLAVIN BINDING, KELCH REPEAT, F-BOX1 (FKF1), stabilizes CO protein specifically in the afternoon when CO promotes the expression of *FT*. FKF1 is also directly involved in the transcriptional regulation of both *CO* and *FT* (Imaiumi et al., 2003; Sawa et al., 2007; Song et al., 2012). PhyB and other light stable phytochromes have additional roles in shade avoidance conditions. Low R/FR ratio inactivates PhyB, which leads to the activation of the photoperiodic flowering pathway. An additional regulator, PHYTOCHROME AND FLOWERING TIME1 (PFT1) promotes the expression of *CO* and *FT* by repressing Phy signaling (Cerdán and Chory, 2003; Wollenberg et al., 2008).

In perennials, the molecular level studies on the light regulation of flowering have focused on *Populus* and on the woodland strawberry, *Fragaria vesca* L. that represents the model species for the economically important Rosaceae family (Böhnenius et al., 2006; Hsu et al., 2011; Koskela et al., 2012; Mouhu et al., 2013). In *F. vesca*, both seasonal flowering and perpetual flowering accessions with contrasting photoperiodic responses exist (Brown and Wareing, 1965). Seasonal flowering accessions are SD plants (Heide and Sonstebry, 2007). In perpetual flowering *F. vesca*, however, LD advances flower induction, but plants eventually flower also under SD (Sønsteby and Heide, 2008; Mouhu et al., 2009; Koskela et al., 2012). A strong floral repressor, *F. vesca* homolog of *TERMINAL FLOWER1* (*FvTFL1*) has been shown to control seasonal flowering, whereas perpetual flowering accessions have non-functional *FvTFL1* alleles with a 2 base pair deletion in the first exon (Iwata et al., 2012; Koskela et al., 2012). Also in the cultivated strawberry (*Fragaria × ananassa* Duch.), both seasonal and perpetual flowering cultivars with similar environmental responses are known (Heide, 1977; Sønsteby and Heide, 2007; Bradford et al., 2010; Kurokura et al., 2013).

Molecular analyses in *F. vesca* have revealed that homologs of *FT* and *SUPPRESSOR OF THE OVER-EXPRESSION OF CONSTANS1* (*FvFT1* and *FvSOC1*) may mediate the photoperiodic signal to control flowering through *FvTFL1*. These genes seem to form a linear pathway in which *FvFT1* promotes the expression *FvSOC1*, which leads to increased *FvTFL1* mRNA levels (Mouhu et al., 2013). Since *FvTFL1* is a strong floral repressor, the activation of this pathway under LD maintains the plants at the vegetative stage (Koskela et al., 2012). Under SD in autumn, however, the expression of *FvFT1* and *FvSOC1* decrease leading

to the down-regulation of *FvTFL1*, and consequently, the up-regulation of *FvAP1* occurs in the shoot apex in parallel with the initiation of floral development. The growth cycle continues in the next spring when determinate inflorescences emerge and produce fruits. Flowering and fruiting overlap with the next yearly growth cycle which begins with the growth of new vegetative axillary shoots with high *FvSOC1* and *FvTFL1* expression level in the spring. In perpetual flowering accession Hawaii-4 (H4), however, the lack of functional *FvTFL1* reverses the photoperiodic flowering response, and both *FvFT1* and *FvSOC1* act as floral activators (Koskela et al., 2012; Mouhu et al., 2013).

Vince-Prue and Guttridge (1973) showed that the end-of-day FR light treatment prevents flower induction in the cultivated strawberry, whereas R light has an opposite effect. To understand strawberry responses to the light quality at the molecular level, we carried out end-of-day treatments with R, FR and B light in the *F. vesca*. We report strong activation of *FvFT1* by FR light, weaker activation by B light, and almost no expression under R light. Using transgenic lines, we show evidence that *FvFT1* mediates the promotion of flowering under FR and B light treatments in the perpetual flowering accession H4, which is lacking functional *FvTFL1*. In the seasonal flowering accession, however, high *FvFT1* expression correlates with high *FvTFL1* mRNA levels, and flowering responses to different light qualities are reversed.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWING CONDITIONS

Seedlings of seasonal flowering SD accession of the woodland strawberry (*Fragaria vesca* L.) and perpetual flowering LD accession H4 (Accession numbers PI551792 and PI551572, respectively; National Clonal Germplasm Repository, Corvallis, USA) were used. Seedlings were raised in a greenhouse under non-inductive photoperiod (12 or 18 h for H4 and SD *F. vesca*, respectively) at $18 \pm 1^\circ\text{C}$ (first experiment) or at $22 \pm 1^\circ\text{C}$. High pressure sodium (HPS) lamps (Airam 400W, Kerava, Finland) were used to supplement natural light with the intensity of $150 \mu\text{mol m}^{-2}\text{s}^{-1}$. In SD conditions, darkening curtains were used to exclude any light during the 12 h night. After rooting, seedlings were transplanted to $8 \times 8 \text{ cm}$ pots. Fertilized peat supplemented with 25% (v/v) of vermiculite ($\varnothing 2 \text{ mm}$) was used as a growing media. Plants were fertilized with liquid fertilizer biweekly.

Previously reported *FvFT1* and *FvSOC1* RNAi lines in H4 background (Koskela et al., 2012; Mouhu et al., 2013), and *FvFT1* over-expression lines produced in this work (see below), were analyzed. All transgenic plants were propagated from seeds originating from the self-pollination of the primary transgenic lines. Seeds were germinated on moistened filter paper on petri dishes at room temperature for 5 days when the primary root was emerged. Since both RNAi and over-expression vectors, pK7GWIWG2(II) and p7WG2D (Karimi et al., 2002), contain green fluorescent protein (GFP) as a selectable marker, we observed GFP signal in the primary roots under the fluorescence microscope (Leica MZ FL3, Leice Microsystems, Wetzlar, Germany) and transferred GFP-positive seeds onto the soil. Transgenic seedlings were raised under SD conditions in greenhouse until the light treatments started. *FvFT1*

over-expression lines were moved to the light treatments immediately after germination in order to avoid flower induction before the treatments. Wild type control plants were raised following the same procedure with transgenic seedlings.

LIGHTING TREATMENTS

Incandescent lamps (INC; R/FR = 0.95; Philips 60W) and light emitting diodes (LED) were used for the end-of-day lighting treatments. R, FR and B LED lighting systems were built up using deep-red (LZ1-10R205; LEDEngin Inc, San Jose, USA), far-red (L735-66-60; Epitex Inc., Kyoto, Japan) and royal-blue (Z-Power D32282; Seoul Semiconductor Co. Ltd., Ansan-city, Korea) high-power LED components with measured peak wavelength emissions at 655, 740, and 455 nm, respectively. These lighting systems were used in the experiments that did not include transgenic lines. However, in the experiments with transgenic lines, Philips Green Power LED research modules (deep R, FR, and B; Philips, Amsterdam, The Netherlands) were used.

Young seedlings were subjected to the end-of-day light quality treatments in a greenhouse rooms equipped with darkening curtains during the winter season (November–March). The developmental stage of the seedlings in the beginning of the treatments is indicated in the figure legends. Plants were illuminated daily for 12 h with $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ of HPS light. After 12-h HPS illumination, the plants were subjected to low intensity ($8-15 \mu\text{mol m}^{-2}\text{s}^{-1}$, as indicated in the figure legends) end-of-day R, FR, B or incandescent light (INC) treatments for 6 h. In addition, 12-h SD was used as a control. Natural light was excluded by using darkening curtains when HPS lamps were turned off. Temperature during the treatments was 18 or $22 \pm 1^\circ\text{C}$ (indicated in the figure legends). After the treatment period of 5–8 weeks (as indicated in the figure legends), the plants were transferred to standard LD growing conditions, 18 h of HPS illumination ($150 \mu\text{mol m}^{-2}\text{s}^{-1}$) at $18 \pm 1^\circ\text{C}$, for flowering observations.

GROWTH OBSERVATIONS

Flowering time observations were carried out 2–3 times per week to record the date of first open flower. In H4, flowering time was also observed by counting the number of leaves in the primary leaf rosette before the terminal inflorescence.

GENETIC TRANSFORMATION

FvFT1 was amplified by using primers 5'-aaaaagcaggctGGATCA ATATGCCTAGGGACAGG-3' and 5'-agaaagctgggtAAAGGGT TACGATGATCTTCTC-3' (lower case letters indicate the binding site for the Gateway adapter primers), and the resulting fragment was introduced in the p7WG2D over-expression vector (Karimi et al., 2002), which includes GFP as a selectable marker, using Gateway® technology with Clonase™ II (Invitrogen, Carlsbad, USA). This construct was electroporated to the *Agrobacterium tumefaciens* strain GV3101 and transformed to the *F. vesca* accession H4 as described earlier (Oosumi et al., 2006).

RNA EXTRACTION, cDNA SYNTHESIS, AND REAL-TIME PCR

Leaf and/or shoot apex samples were collected for gene expression analyses during lighting treatments in the time points indicated in the figures and figure legends. For the leaf samples, middle leaflets of youngest fully opened leaves, and for shoot

apex samples, ~1 mm pieces containing SAM and youngest leaf initials were pooled from several plants. Three biological replicates were collected for each sample. RNA extraction was done by using pine tree method (Monte and Somerville, 2002). For cDNA synthesis (Superscript III reverse transcriptase, Invitrogen) 1 µg of total RNA was used. Real time PCR reactions were performed using SYBR Green Master Mix (Roche, Basel, Switzerland) and 3 µM primer mix (F+R) by using LightCycler 480-instrument (Roche). Real time PCR program is presented in **Supplementary Figure 1**. Three biological and three technical replicates were analyzed in each experiment. Relative expression of selected genes was measured by $\Delta\Delta\text{Ct}$ method with stable *FvMSI1* as a normalization gene (**Supplementary Figure 2**). Real time PCR primers are listed in **Supplementary Table 1**. Primer efficiencies were close to 2 for all primer pairs.

STATISTICAL ANALYSES

Flowering time results were subjected to the One-Way or Two-Way analysis of variance (ANOVA) using general linear model (GLM-procedure, SAS 9.3 Software, SAS Institute Inc., Cary, USA). Pairwise comparisons were carried out using Tukey's test ($\alpha = 0.05$ or 0.01 in one-way and two-way ANOVA, respectively).

RESULTS

END-OF-DAY FR, BUT NOT R LIGHT PROMOTES FLOWERING IN THE *F. VESCA* ACCESSION H4

Earlier studies in *F. vesca* have shown that LD advances flowering of the perpetual flowering accession H4 (Mouhu et al., 2009; Koskela et al., 2012). To analyze the effect of light quality on the photoperiodic flower induction in H4, we subjected seedlings to different end-of-day light quality treatments. Plants with one open leaf were exposed to non-inductive SD (12 h day/12 h night), or SD plus low intensity day extension (6 h) of FR, R or incandescent light (INC; R:FR ratio of 0.95; flower inductive LD control) for 5 weeks at 18°C followed by standard LD (18 h) growing conditions (see Materials and methods). In both FR and INC treatments, flower induction was advanced and resulted in terminal inflorescence after about eight leaves in the primary leaf rosette (**Figure 1A**). In contrast, plants grown under R treatment flowered after 12 leaves similarly to plants grown under SD.

We further tested the effect of light quality at higher temperature of 22°C , since earlier study showed that high temperature enhances photoperiodic responses in the perpetual flowering *F. vesca* accessions (Sønsteby and Heide, 2008). Indeed, we found stronger delay of flowering in R and SD compared to control plants grown under INC than in the experiment carried out at 18°C . Plants grown under SD and R treatments produced 8–9 leaves more than plants under INC treatment and flowered more than a month later (**Supplementary Figure 3**). We also tested the effect of B light end-of-day treatment and found that B light advanced flowering in H4, but the effect was weaker compared to INC light (**Supplementary Figure 3**).

END-OF-DAY FR LIGHT INDUCES THE EXPRESSION OF THE *FVFT1*

Koskela et al. (2012) showed that in H4, *FvFT1* expression correlated with floral induction under LD conditions as well as the expression of putative floral meristem identity genes *FvAP1* and

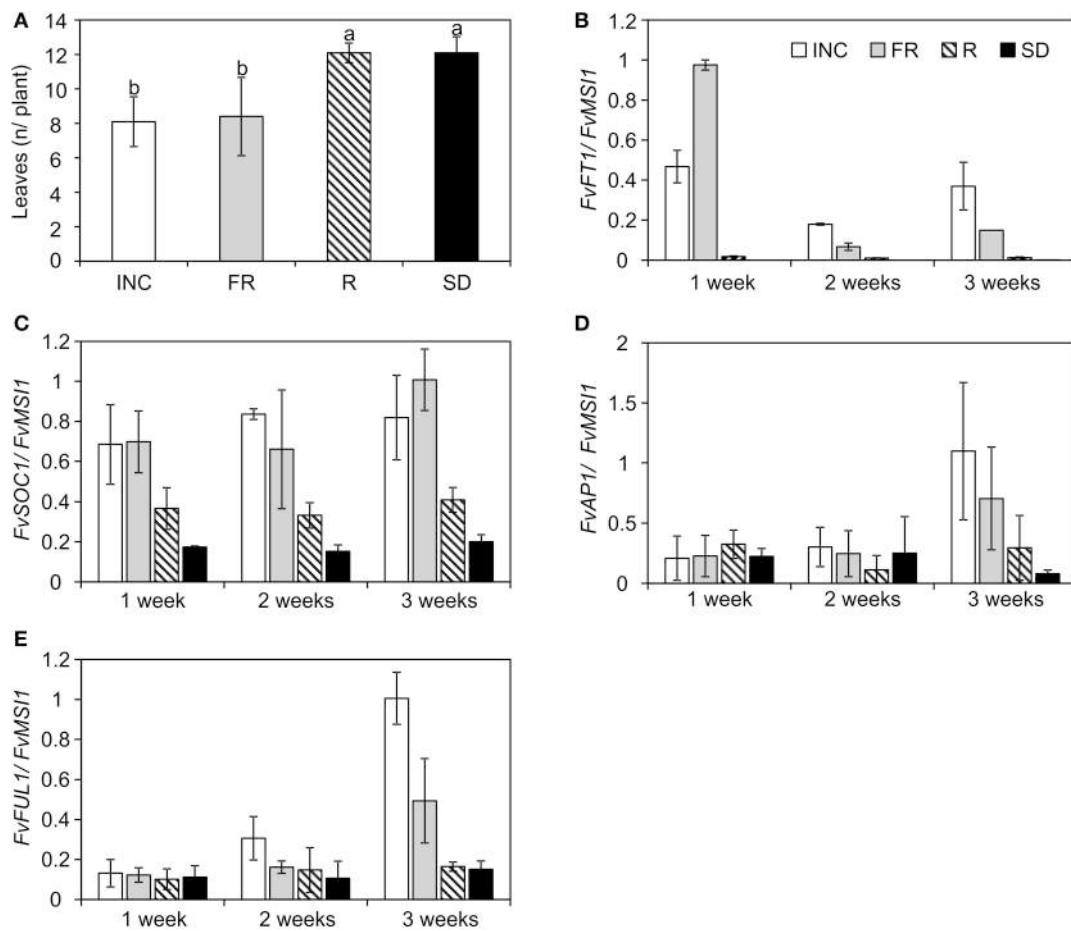


FIGURE 1 | Flowering time and the expression of flowering time genes under the end-of-day light quality treatments in the perpetual flowering *F. vesca* accession Hawaii-4 (H4). **(A)** Flowering time of the seedlings of H4 indicated as the number of leaves in the primary leaf rosette before the terminal inflorescence ($n = 8-11$). Flowering results were subjected to One-Way ANOVA (t -test), $p < 0.001$ for the treatment. Different lower-case letters indicate significant differences between the treatments according to Tukey's pairwise test, $\alpha = 0.05$. **(B)** The expression of *FvFT1* in the leaves of

H4 collected in different time points during the treatments. **(C)** The expression of *FvSOC1*, **(D)** *FvAP1*, and **(E)** *FvFUL1* in the shoot apex samples of H4 collected in different time points. Plants with one open leaf were subjected to 12-h short-day (SD) or 12-h SD plus 6-h low intensity ($8 \mu\text{mol m}^{-2}\text{s}^{-1}$) end-of-day treatment with incandescent, far-red or red light (INC, FR, and R, respectively) at 18°C for 5 weeks. For gene expression data, three biological replicates were analyzed by real-time PCR. Leaf and shoot apex samples were collected 4 h after dawn. All results are mean \pm SD.

FvFUL1. We analyzed the expression of these genes in different end-of-day light quality treatments and found a correlation between the *FvFT1* gene expression level and flowering phenotypes. In the leaves of H4, *FvFT1* was highly expressed in both flowering promoting FR and INC treatments already at 2-leaf stage, 1 week after the beginning of the treatment (Figure 1B). In contrast, we detected very low or no expression in R and SD treatments (Figure 1B), in which the plants flowered late.

In *Arabidopsis* FT activates the expression of SOC1 at the SAM (Moon et al., 2005; Yoo et al., 2005), and this regulatory connection was shown to be conserved in the *F. vesca* (Mouh et al., 2013). We found that *FvSOC1* mRNA levels in the shoot apices partially correlated with the expression of *FvFT1* in the leaves: high expression levels for both genes were detected in INC and FR end-of-day treatments in all tested time points (Figure 1C). However, *FvSOC1* mRNA was detected also in R and SD treatments in contrast to *FvFT1*, but the expression level

was lower than in FR and INC treatments. The activation of *FvFT1* and *FvSOC1* in FR and INC treatments was followed by the up-regulation of both *FvAP1* and *FvFUL1* in the shoot apex 3 weeks after the beginning of the treatments but not under SD or R (Figures 1D,E), indicating that flower induction had occurred only in INC and FR treatments at this stage.

At a higher temperature of 22°C , *FvFT1* was also strongly up-regulated in the leaves of H4 under FR light compared to R and SD treatments (Supplementary Figure 3). *FvFT1* expression was detected also in B light, but it was several times lower than under FR treatment.

FUNCTIONAL ROLE OF *FvFT1* AND *FvSOC1* IN LIGHT QUALITY RESPONSES

To confirm the functional role of *FvFT1* in different light quality treatments, we used transgenic approach. First, we overexpressed *FvFT1* under cauliflower mosaic virus 35S promoter

in H4 background and subjected two independent *FvFT1* overexpression lines to different light quality treatments. The ectopic expression of *FvFT1* led to extremely early flowering compared to non-transgenic H4 (Figures 2A–D). In addition, *FvFT1* overexpression line #7 showed no differences and line #5 minor differences in their responses to various end-of-day light quality treatments while in H4, FR and B light promoted flowering compared to R and SD treatments. Moreover, R light slightly advanced flowering compared to SD in non-transgenic H4 in this experiment. We also tested the responses of three previously reported *FvFT1* RNAi lines in H4 background (Koskela et al., 2012) to the same light quality treatments. In contrast to the wild type H4, FR and B end-of-day treatments did not advance flowering in two *FvFT1* RNAi lines compared to the R and SD treatments, while minor differences between light treatments were observed in the third line (Figure 3A). In this experiment, FR light advanced flowering in non-transgenic H4 more than B light compared to SD or R light treatment, and also R light slightly promoted flowering compared to SD.

Mouhu et al. (2013) showed that *FvSOC1* promotes flowering downstream of *FvFT1*. In line with this result, silencing of *FvFT1* abolished the up-regulation of *FvSOC1* and *FvFUL1* which was observed in wild type H4 under FR light treatment (Figures 3B,C). However, B light treatment did not clearly affect the expression of *FvSOC1* and *FvFUL1* in H4 at this time point due to differences between the observed flowering times and sampling. In this experiment, the plants that received the B light treatment flowered slightly later than those under FR treatment (Figure 3A).

To understand the role of *FvSOC1* in the light quality regulation of flowering, we also studied two independent *FvSOC1* RNAi lines in H4 background (Supplementary Figure 4) under the same light quality treatments. Interestingly, FR treatment accelerated flowering of *FvSOC1* RNAi plants similarly as in the wild type H4 while the effect of B light was absent in the transgenic lines (Figure 3A). This data suggests that FR may induce flowering through *FvFT1*, independently of *FvSOC1*, whereas *FvSOC1* is needed for early flowering in the end-of-day B light treatment. Taken together, our data on transgenic lines show that *FvFT1*, in addition to the photoperiodic flowering pathway (Koskela et al., 2012), is the central regulator in the light quality mediated flowering pathway in the perpetual flowering *F. vesca* accession H4. However, according to our data, *FvSOC1* may have more specific role in the B light regulation of flowering.

FvCO AND FvFT1 EXPRESSION PEAKS DO NOT OVERLAP IN LIGHT QUALITY TREATMENTS

In Arabidopsis, the expression of *CO* starts to increase in the afternoon, and *CO* protein activates *FT* expression in late evening specifically under LD (Suarez-Lopez et al., 2001). In addition, light spectrum affects the expression levels of both genes (Imaizumi et al., 2003; Valverde et al., 2004; Kim et al., 2008; Wollenberg et al., 2008). To get insight into the putative *CO/FT* module in *F. vesca*, we explored the expression rhythms of *F. vesca CO* and *FT* homologs in different end-of-day light quality treatments. We focused on daytime expression levels, since our earlier data showed that *FvFT1* has a minor expression peak in

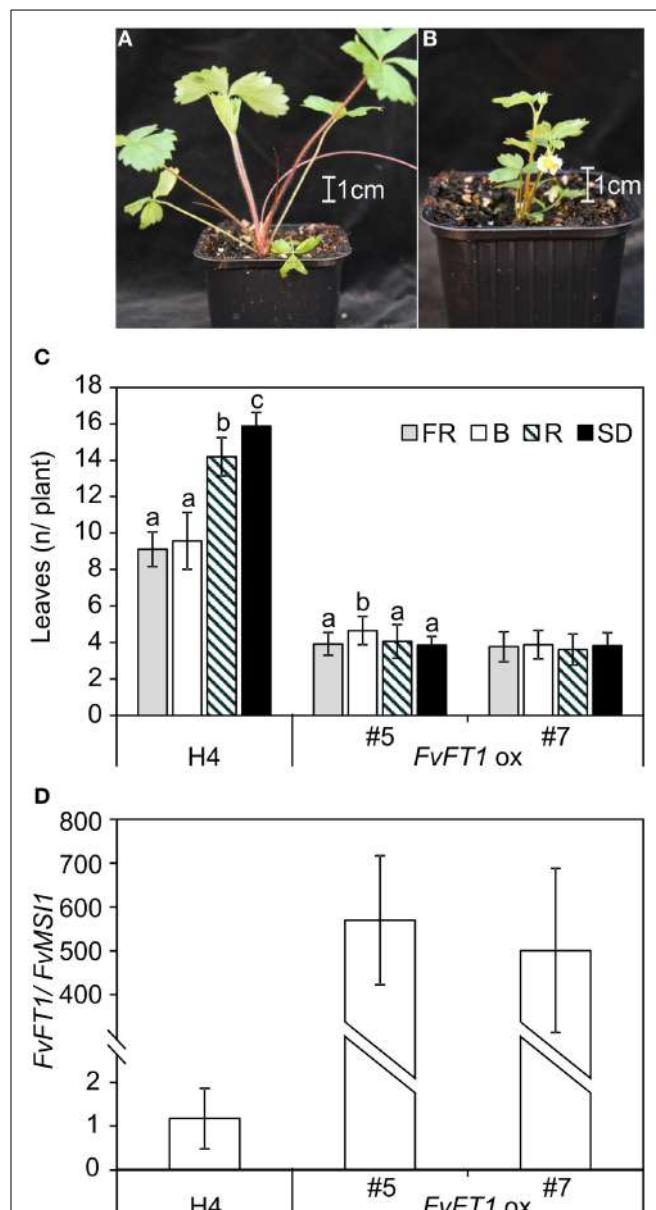


FIGURE 2 | The overexpression of *FvFT1* causes extreme early flowering in the perpetual flowering *F. vesca* accession Hawaii-4 (H4). (A) H4 wild type, and (B) 35S:*FvFT1* line #7 at the age of 8 weeks. (C) Flowering time of *FvFT1* overexpression lines in different end-of-day light quality treatments indicated as the number of leaves in the primary leaf rosette before the terminal inflorescence (#5: $n = 21\text{--}31$; #7: $n = 13\text{--}18$; wild type H4: $n = 36\text{--}38$). Flowering results were subjected to Two-Way ANOVA ($p < 0.001$ for each of treatment, genotype, and treatment \times genotype interaction). Pairwise comparisons were carried out for every genotype separately using Tukey's test, $\alpha = 0.01$. Treatments with different letters indicate significant differences within the genotype. (D) The expression of *FvFT1* in 35S:*FvFT1* plants compared to wild type Hawaii-4 under LD (18 h). T₁ and H4 seedlings with open cotyledons were subjected to 12-h short-day (SD) or 12-h SD plus 6-h low intensity ($15 \mu\text{mol m}^{-2}\text{s}^{-1}$) end-of-day treatment with far-red, red or blue light (FR, R, and B, respectively) at 22°C for 8 weeks. For gene expression data, three biological replicates were analyzed by real-time PCR. Leaf samples were collected 16 h after dawn. All results are mean \pm SD.

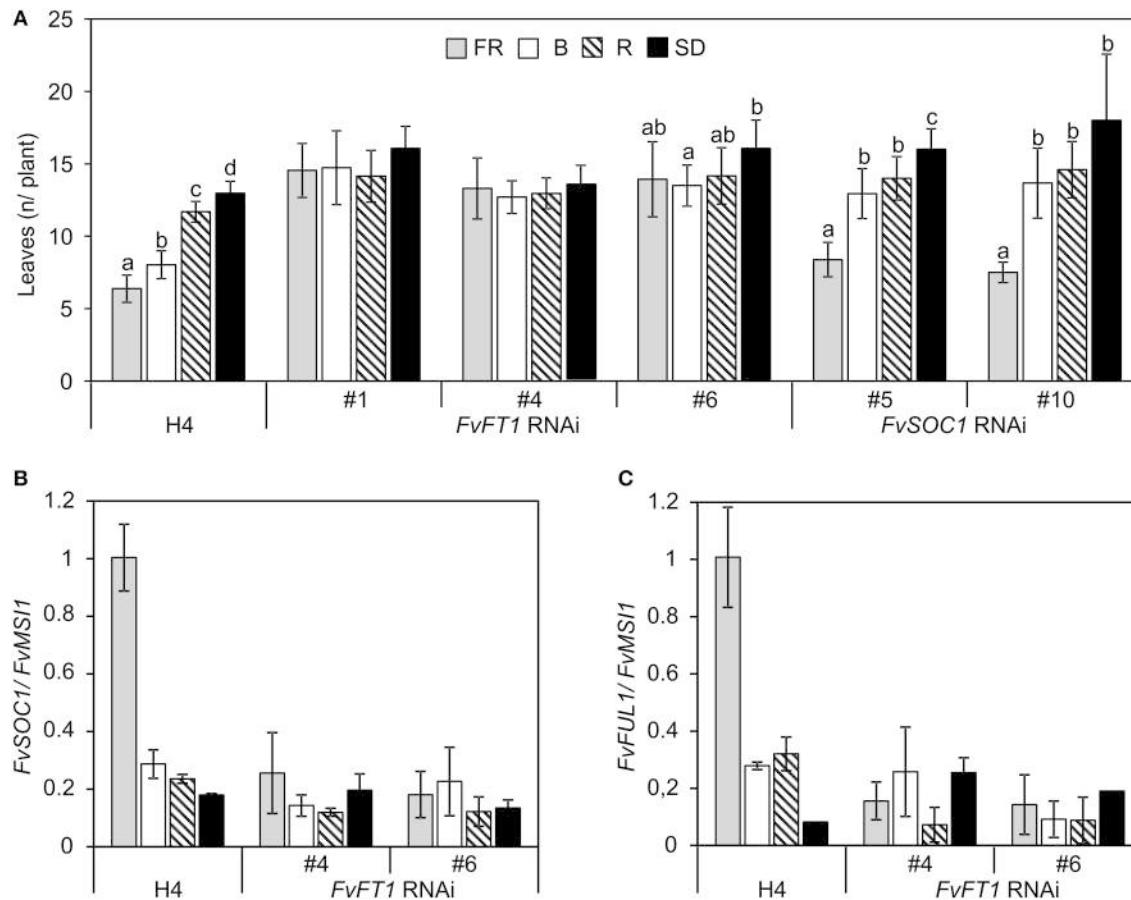


FIGURE 3 | RNAi silencing of *FvFT1* and *FvSOC1* affects the regulation of flowering by light quality in the perpetual flowering *F. vesca* accession

Hawaii-4 (H4). (A) Flowering time of H4, and *FvFT1* and *FvSOC1* RNAi lines in different end-of-day light quality treatments indicated as the number of leaves in the primary leaf rosette before the terminal inflorescence ($n = 11\text{--}23$ for *FvFT1* RNAi lines, $n = 28\text{--}30$ for H4, $n = 13\text{--}18$ for *FvSOC1* RNAi-5, $n = 5$ for *FvSOC1* RNAi-10). Flowering results were subjected to Two-Way ANOVA ($p < 0.001$ for each treatment, genotype, and treatment \times genotype interaction). Pairwise comparisons were carried out for every

genotype separately using Tukey's test, $\alpha = 0.01$. Treatments with different letters indicate significant differences within the genotype. (B) Relative expression of *FvSOC1* and (C) *FvFUL1* in the shoot apex samples of Hawaii-4 and *FvFT1* RNAi lines. T₁ and H4 seedlings with 3–4 open leaves were subjected to 12-h short-day (SD) or 12-h SD plus 6-h low intensity ($15 \mu\text{mol m}^{-2}\text{s}^{-1}$) end-of-day treatment with far-red, red or blue light (FR, R, and B, respectively) at 22°C for 7 weeks. For gene expression data, three biological replicates were analyzed by real-time PCR. Shoot apex samples were collected 4 h after dawn. All results are mean \pm SD

the morning and another peak in the late evening (Koskela et al., 2012). In the FR treatment, *FvFT1* peaked 4 h after dawn and its expression started to rise again in the evening (Figure 4A). Several times lower expression peaks were detected in B light treatment. Low morning peak (4 h) was observed also in R light treatment, but the expression level of *FvFT1* gene was almost undetectable in the evening.

Several CO homologs have been cloned in the cultivated strawberry, and according to phylogenetic analysis, one of those genes, *FrCO*, belongs to the group 1a that includes *CO*, *COL1* and *COL2* in Arabidopsis (Griffiths et al., 2003; Stewart, 2007). We searched for *F. vesca* homologs for *FrCO* from the *F. vesca* genome database (Shulaev et al., 2011; www.rosaceae.org) and found only a single gene with high sequence identity to *FrCO* at the nucleotide and protein level (gene04172-v1.0-hybrid; 97 and 96% identity at the nucleotide and protein level, respectively). We studied the daytime expression rhythm of this gene, which was previously

named as *FvCO* (Shulaev et al., 2011), in different light quality treatments. *FvCO* peaked at dawn and its expression decreased along the day (Figure 4B; Supplementary Figure 5). Very low expression level was detected in late evening when the mRNA levels of *FvFT1* were already rising. In addition, light quality treatments did not affect daytime *FvCO* gene expression pattern. These data indicates that if the CO-FT connection exists in *F. vesca*, its mode of action differs from Arabidopsis.

END-OF-DAY R LIGHT DOWN-REGULATES *FvFTL1* AND INDUCES FLOWERING IN THE SD ACCESSION

In contrast to the LD flowering H4, seasonal flowering SD *F. vesca* is induced to flower in 12 h SD, whereas 6 h day extension with INC treatment after SD prevents flower induction (Koskela et al., 2012). We subjected the SD *F. vesca* to the similar end-of-day FR, R and B treatments than H4 and found that its flowering responses to different light qualities were reversed compared to

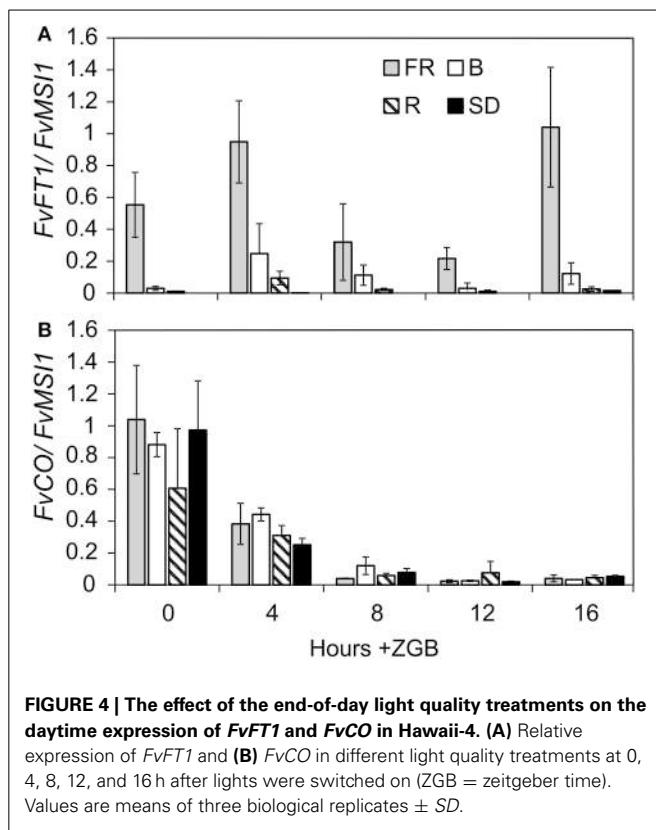


FIGURE 4 | The effect of the end-of-day light quality treatments on the daytime expression of *FvFT1* and *FvCO* in Hawaii-4. **(A)** Relative expression of *FvFT1* and **(B)** *FvCO* in different light quality treatments at 0, 4, 8, 12, and 16 h after lights were switched on (ZGB = zeitgeber time). Values are means of three biological replicates \pm SD.

H4; R induced, and FR and B prevented flowering (Figure 5A). In R and SD treatments, flowering occurred 38 and 30 days after the end of the 8-week treatment, respectively while the plants under FR and B light treatments stayed vegetative until the end of the experiment.

To understand the observed differences in flowering responses at the molecular level, we analyzed the expression of *FvFT1* in the leaves, and *FvSOC1* and *FvTFL1* mRNA levels in the shoot apex in the SD *F. vesca* 4 weeks after the beginning of the end-of-day light quality treatments. Similarly to H4, in the SD *F. vesca*, *FvFT1* was strongly up-regulated by FR light and some expression was detected in B light, whereas in R and SD treatments, the expression was hardly detected or undetectable, respectively (Figure 5B). Like *FvFT1* in the leaves, *FvSOC1* was up-regulated in the shoot apex of SD *F. vesca* in FR light and expressed only at low level in both R and SD treatments, whereas intermediate levels were observed under B light (Figure 5C). The expression of floral repressor, *FvTFL1*, closely followed that of *FvSOC1* in all light quality treatments (Figure 5D). *FvTFL1* was several-fold down-regulated in floral-inductive SD and R treatments in comparison to FR and B treatments which inhibited flower induction. Taken together, the effect of end-of-day R, FR or B treatment or SD on the expression of *FvFT1* and *FvSOC1* was similar in SD *F. vesca* as in H4, although the flowering responses were opposite. This difference is associated to *FvTFL1* which was co-regulated with *FvFT1* and *FvSOC1* by light. These data suggest that the functional *FvTFL1* reverses flowering response to different light qualities in *F. vesca*.

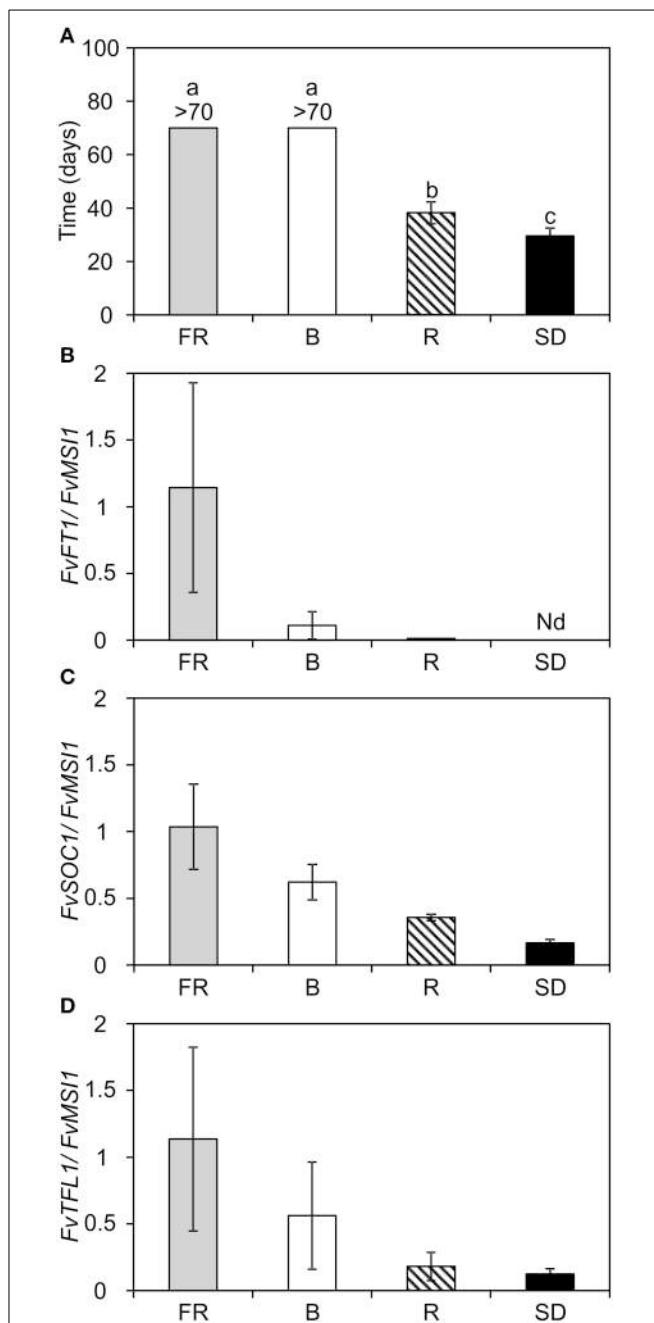


FIGURE 5 | Flowering time and the expression of flowering time genes in the SD *F. vesca* subjected to end-of-day light quality treatments. **(A)** Flowering time of the SD *F. vesca* indicated as days to anthesis after the light quality treatments ($n = 14$). Flowering results were subjected to One-Way ANOVA (t -test), $p < 0.001$ for the treatment. Different lower-case letters indicate significant differences between the treatments according to Tukey's pairwise test, $\alpha = 0.05$. **(B)** The expression of *FvFT1* in the leaf samples, and **(C)** *FvSOC1* and **(D)** *FvTFL1* in the shoot apex samples 4 weeks after the beginning of the light quality treatments. Plants with 5–6 open leaves were subjected to 12-h short-day (SD) or 12-h SD plus 6-h low intensity ($15 \mu\text{mol m}^{-2}\text{s}^{-1}$) end-of-day treatment with blue, far-red or red light (B, FR, or R, respectively) at 18°C for 8 weeks. For gene expression data, three biological replicates were analyzed by real-time PCR. Shoot apex and leaf samples were collected 4 and 16 h after dawn, respectively. All results are mean \pm SD. Nd, not detected.

DISCUSSION

FT has been considered to be a general photoperiodic signaling molecule in both SD and LD plants (Hayama and Coupland, 2004; Lagercrantz, 2009; Pin and Nilsson, 2012). Likewise, in the perpetual flowering *F. vesca* accession H4, *FvFT1* has recently been reported as an LD-induced floral activator which controls the expression of putative floral meristem identity genes *FvAP1* and *FvFUL1* (Koskela et al., 2012). Here we show evidence that *FvFT1* also mediates the effect of light quality to promote flowering in H4. However, in the seasonal flowering SD *F. vesca* with a functional *FvTFL1*, the effects of the light spectra on flowering are reversed.

END-OF-DAY FR AND B LIGHT PROMOTE FLOWERING IN H4

Flowering of H4 was advanced by the end-of-day treatment of FR or FR-rich incandescent light whereas R light had no effect or very weak effect compared to the SD control. This is a typical response of various LD plants to light quality (Meijer, 1959; Brown and Klein, 1971; Holland and Vince, 1971; Downs and Thomas, 1982; Martinez-Zapater and Somerville, 1990). Since phytochromes are the sole R/FR receptors mediating photomorphogenic and photoperiodic responses (Takano et al., 2009; Strasser et al., 2010), we suggest that these photoreceptors have a major role in the control of flowering also in strawberries. However, further studies are needed to confirm which phytochrome(s) mediate the R/FR responses observed in H4.

B light has also been shown to promote flowering in Arabidopsis (Bagnall, 1996; Mockler et al., 2003). We found that the end-of-day B light treatment promoted flowering in H4. However, the effect of B light on flowering was weaker than the effect of the FR or INC light in two out of three experiments reported here. Although these results further suggest that phytochromes are major photoreceptors regulating flowering in *F. vesca*, also B light receptor(s) likely have a role in the control of flowering. In Arabidopsis, cryptochromes and the LOV receptor FKF1 are involved in the B light regulation of flowering (Guo et al., 1998; Imaizumi et al., 2003; Valverde et al., 2004). PhyA, however, can also absorb B light, and it mediates B light signal to control flowering at least in the *cry1cry2* double mutant (Mockler et al., 2003).

FvFT1 AND *FvSOC1* HAVE DISTINCT ROLES IN THE LIGHT QUALITY REGULATION OF FLOWERING

In Arabidopsis, light signals mediated by different photoreceptors control the expression of *FT* that promotes flowering (Imaiizumi et al., 2003; Mockler et al., 2003; Valverde et al., 2004; Song et al., 2012). Consistent with the up-regulation of *FT* by FR light or low R/FR ratio (Cerdán and Chory, 2003; Mockler et al., 2003; Wollenberg et al., 2008), we found that the end-of-day FR and INC light with R/FR ratio of 0.95 strongly up-regulated *FvFT1* in the leaves of H4, whereas *FvFT1* expression level was very low under R and SD treatments. Also the end-of-day B light treatment somewhat up-regulated *FvFT1* compared to SD. However, consistent with later flowering under B light compared to FR light, several times lower *FvFT1* expression level was detected under B light treatment. The analysis of transgenic lines confirmed the role of *FvFT1* in the light quality regulation of flowering in H4.

The overexpression of *FvFT1* in H4 caused extreme early flowering independently of the end-of-day light treatment, whereas RNAi silencing of *FvFT1* abolished the FR and B light promotion of flowering. These results indicate that *FvFT1* does not only activate flowering under LD (Koskela et al., 2012) but also controls flowering according to light quality signals perceived by phytochromes and B light receptors.

FT is a positive regulator of *SOC1* in Arabidopsis (Yoo et al., 2005; Torti et al., 2012), and this regulatory link is present also in the *F. vesca* (Mouhu et al., 2013). In this study, we found highest *FvSOC1* expression levels in FR treatment, where the *FvFT1* expression level was also highest. Furthermore, the silencing of *FvFT1* prevented the up-regulation of *FvSOC1* by FR light indicating that *FvFT1* mediates at least the FR light regulation of *FvSOC1*. However, *FvFT1* can control flowering independently of *FvSOC1* under FR light, since the silencing of *FvSOC1* did not affect flowering time under FR light. This is in line with the observation that *FT* and *SOC1* act redundantly to promote flowering under FR enriched light in Arabidopsis (Kim et al., 2008). Although the end-of-day B light treatment advanced flowering compared to SD in non-transgenic H4, this did not occur in *FvSOC1* RNAi lines suggesting that *FvSOC1* is needed specifically for the B light promotion of flowering. Taken together, both *FvFT1* and *FvSOC1* are involved in the control of flowering by the end-of-day B treatment, whereas the promotion of flowering by the FR treatment can occur independently of *FvSOC1*.

FUNCTIONAL *FvTFL1* REVERSES THE END-OF-DAY LIGHT QUALITY RESPONSES IN *F. VESCA*

Several lines of data support the presence of *FvFT1*-*FvSOC1*-*FvTFL1* regulatory pathway in the SD *F. vesca*. *FvFT1* up-regulates *FvSOC1* in the shoot apex at least in H4, and *FvSOC1* activates the expression of *FvTFL1* that encodes a strong floral repressor. Therefore, the photoperiodic flowering response is reversed in the SD *F. vesca* compared to H4 (Koskela et al., 2012; Mouhu et al., 2013). We found that in the SD *F. vesca*, similarly to H4, *FvFT1* and *FvSOC1* gene expression levels were higher under the end-of-day FR and B light treatments compared to R and SD treatments. However, in contrast to H4, SD *F. vesca* was induced to flower under R light and SD treatments while FR and B light inhibited flowering. Taken together, the expression of *FvFT1* and *FvSOC1* correlated negatively with the flower induction in the SD *F. vesca* in all light quality treatments tested in this study as well as in the photoperiodic treatments in previous works (Figure 5; Koskela et al., 2012; Mouhu et al., 2013). We also found that the expression of *FvTFL1* closely followed that of *FvSOC1* in all light quality treatments indicating that the presence of the functional *FvTFL1* not only reverses photoperiodic flowering response (Koskela et al., 2012), but also the effect of the end-of-day B light and the phytochrome-mediated R/FR light on flowering. The ortholog of *FvTFL1* may control light responses also in the cultivated strawberry, since the SD cultivar of the cultivated strawberry responds to R/FR treatments similarly to the SD *F. vesca* (Vince-Prue and Guttridge, 1973) and the *F. vesca* is one of its ancestors (Hirakawa et al., 2013). Although significant amount of data support the presence of *FvFT1*-*FvSOC1*-*FvTFL1* pathway in the SD *F. vesca*, functional analysis is needed to

confirm whether FvFT1 acts as an anti-florigen in the presence of FvTFL1. Antiflorogens have recently been reported in sugar beet and chrysanthemum (Pin et al., 2010; Higuchi et al., 2013).

FvCO GENE EXPRESSION DO NOT COINCIDE WITH FvFT1 mRNA PEAK

In the *F. vesca*, *FvFT1* mRNA expression peaks in the late evening under LD (Koskela et al., 2012), similarly to *FT* homologs in several other species (Cremer and Coupland, 2003; Böhnenius et al., 2006; Pin et al., 2010). In Arabidopsis, *FT* is induced by CO when CO mRNA expression peak coincide with light in the evening under LD (Suarez-Lopez et al., 2001). Our data do not support similar regulation in the *F. vesca*. The Arabidopsis coincidence model would require *FvCO* to peak in the afternoon before *FvFT1*. However, *FvFT1* is highly up-regulated 16 h after dawn, when the *FvCO* expression level is low, and *FvCO* mRNA level peaks later toward dawn similarly to Arabidopsis *COL2* and *Populus deltoides CO1* and *CO2*, which have little or no effect on the onset of flowering (Ledger et al., 2001; Hsu et al., 2012).

Both FR and B light increase the expression of *CO* and *FT* in Arabidopsis, whereas lower mRNA levels are observed in R rich light (Imaiizumi et al., 2003; Valverde et al., 2004; Kim et al., 2008; Wollenberg et al., 2008). Although light quality treatments strongly affected *FvFT1* mRNA levels in *F. vesca* and caused an additional *FvFT1* expression peak 4 h after dawn, the end-of-day light quality treatments had no effect on *FvCO* daytime expression. Since light quality affects the stability of CO protein in Arabidopsis (Valverde et al., 2004), one possible scenario is that the stabilization of *FvCO* in the morning up-regulates *FvFT1*. However, further studies are needed to reveal whether *FvCO* has a role in the regulation of *FvFT1* in different phases of the diurnal cycle in the *F. vesca*.

CONCLUDING REMARKS

We have shown that both B and R/FR light signals contribute to the regulation of flowering and flowering time genes in *F. vesca*, although phytochrome mediated R/FR signals have stronger effect at least in the end-of-day treatments (Figure 6). In the perpetual flowering LD accession H4, the floral promoter *FvFT1* has a central role in flowering responses to different light qualities, whereas *FvSOC1* seems to be specifically required for B light mediated activation of flowering. In the SD *F. vesca*, however, the flowering response to different light spectra is reversed, because of the up-regulation of the strong floral repressor *FvTFL1* by *FvFT1*/*FvSOC1*. These new insights highlight the importance of the regulation of *FvTFL1* and *FvFT1* also in the light quality responses, in addition to photoperiodic flowering, in the perennial Rosaceae model plant *F. vesca* (Koskela et al., 2012). Our result, that flowering of both perpetual (LD) and seasonal (SD) flowering strawberries can be controlled by light quality treatments, may have practical applications in the strawberry cultivation under controlled climate.

AUTHOR CONTRIBUTIONS

Marja Rantanen, Pauliina Palonen, Paula Elomaa and Timo Hytönen planned the study. Marja Rantanen, Takeshi Kurokura and Katriina Mouhu carried out the experiments and analyzed the data. Paulo Pinho, Eino Tetri and Liisa Halonen designed and

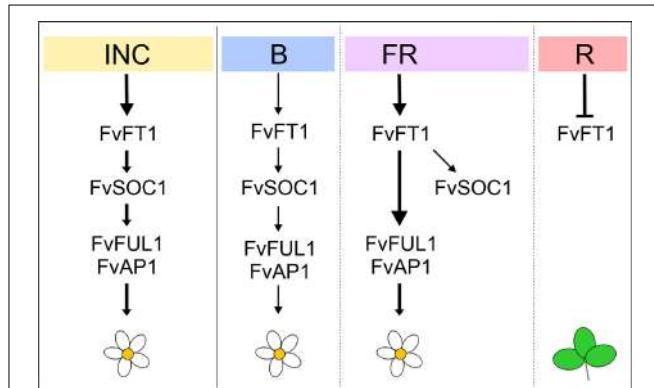


FIGURE 6 | Model showing the regulation of flowering by light quality in the *F. vesca*. Daylength extension with incandescent light (INC) promotes flowering through the up-regulation of *FvFT1* and *FvSOC1* in the perpetual flowering *F. vesca* accession Hawaii-4 (Mouhu et al., 2013). These genes are essential also for the promotion of flowering by the end-of-day blue (B) light. Under FR light, however, *FvFT1* induces flowering independently of *FvSOC1*, whereas R (red) light down-regulates *FvFT1* and prevents flower induction. In the SD *F. vesca*, *FvTFL1*, a strong floral repressor that functions downstream of *FvFT1* and *FvSOC1*, reverses the responses to different light qualities. Arrows indicate activation, and bars indicate repression. Thicker line indicates stronger response.

Paulo Pinho constructed the LED luminaires. Marja Rantanen and Timo Hytönen wrote the manuscript with input from other authors. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00271/abstract>

Supplementary Table 1 | qPCR primers used in this study.

Supplementary Figure 1 | qPCR program used in this study.

Supplementary Figure 2 | Stability of the normalization gene *FvMSI1*.

Supplementary Figure 3 | Flowering time and *FvFT1* expression in Hawaii-4 at 22°C.

Supplementary Figure 4 | The down-regulation of *FvSOC1* in the RNAi-lines.

Supplementary Figure 5 | Diurnal expression rhythm of *FvCO* in the SD *F. vesca* and Hawaii-4.

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HvFT1 polymorphism and effect—survey of barley germplasm and expression analysis

Jorge Loscos¹, Ernesto Igartua¹, Bruno Contreras-Moreira^{1,2}, M. Pilar Gracia¹ and Ana M. Casas^{1*}

¹ Department of Genetics and Plant Production, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas, Zaragoza, Spain

² Fundación ARAID, Zaragoza, Spain

Edited by:

Maria Von Korff Schmising, Max Planck Society, Germany

Reviewed by:

Ben Trevaskis, Commonwealth Scientific and Industrial Research Organisation, Australia
Muhammad Aman Mulki, Max Planck Institute for Plant Breeding Research, Germany

***Correspondence:**

Ana M. Casas, Department of Genetics and Plant Production, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas, Avda. Montañana 1005, 50059 Zaragoza, Spain
e-mail: acasas@eead.csic.es

Flowering time in plants is a tightly regulated process. In barley (*Hordeum vulgare* L.), HvFT1, ortholog of *FLOWERING LOCUS T*, is the main integrator of the photoperiod and vernalization signals leading to the transition from vegetative to reproductive state of the plant. This gene presents sequence polymorphisms affecting flowering time in the first intron and in the promoter. Recently, copy number variation (CNV) has been described for this gene. An allele with more than one copy was linked to higher gene expression, earlier flowering, and an overriding effect of the vernalization mechanism. This study aims at (1) surveying the distribution of *HvFT1* polymorphisms across barley germplasm and (2) assessing gene expression and phenotypic effects of *HvFT1* alleles. We analyzed *HvFT1* CNV in 109 winter, spring, and facultative barley lines. There was more than one copy of the gene (2–5) only in spring or facultative barleys without a functional vernalization *VrnH2* allele. CNV was investigated in several regions inside and around *HvFT1*. Two models of the gene were found: one with the same number of promoters and transcribed regions, and another with one promoter and variable number of transcribed regions. This last model was found in Nordic barleys only. Analysis of *HvFT1* expression showed that association between known polymorphisms at the *HvFT1* locus and the expression of the gene was highly dependent on the genetic background. Under long day conditions the earliest flowering lines carried a sensitive *PpdH1* allele. Among spring cultivars with different number of copies, no clear relation was found between CNV, gene expression and flowering time. This was confirmed in a set of doubled haploid lines of a population segregating for *HvFT1* CNV. Earlier flowering in the presence of several copies of *HvFT1* was only seen in cultivar Tammi, which carries one promoter, suggesting a relation of gene structure with its regulation. *HvCEN* also affected to a large extent flowering time.

Keywords: barley, flowering time, copy number variation, *HvFT1*, *HvCEN*

INTRODUCTION

In temperate cereals, like barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.), flowering is regulated by the integration of two seasonal signals (Laurie, 2009): photoperiod (day length) and vernalization (prolonged exposures to low temperatures). Flowering time is also closely linked with agronomic performance. Plants must flower at the appropriate time of the year, when conditions are most favorable for pollination, seed development and high grain yield.

The responses to day length and temperature serve to classify barley varieties according to their adaptation pattern. Based on the response to day length, varieties are divided into photoperiod-sensitive (long days accelerate flowering) or -insensitive (plants flower almost independently of the day length). Based on the response to vernalization, barley varieties are classified as winter (vernalization is required for timely flowering) or spring (flowering irrespective of vernalization), although the presence of an allelic series at *VrnH1* produces intermediate genotypes (Hemming et al., 2009). Usually, winter varieties are sown in autumn, spring varieties in winter and spring, and there is a third category known as facultative varieties, that can be sown anytime.

Several major genes are the main responsible for the responses to photoperiod and vernalization, which are described next.

Allelic differences in the photoperiod genes *PpdH1* and *PpdH2* are associated with natural variation in the response to day length. *PpdH1* (a member of the *Pseudo Response Regulator* family) is part of the circadian clock of the plant and promotes flowering under long days (Turner et al., 2005). Recessive mutations in the *PpdH1* gene result in delayed flowering under long days (Turner et al., 2005; Hemming et al., 2008). The *PpdH1* gene acts in parallel to *HvCO1* (Campoli et al., 2012), which is one of the barley homologs of the *Arabidopsis* (*Arabidopsis thaliana*) *CONSTANS* (*CO*) gene. Overexpression of *HvCO1* results in the up-regulation of *HvFT1* (the ortholog in barley of the *Arabidopsis* *FLOWERING LOCUS T*, or *FT*) and the acceleration of flowering (Campoli et al., 2012). *PpdH2* has been long acknowledged as the responsible for acceleration of flowering in response to short photoperiod, although its role is being re-defined (Casao et al., 2011). It is a paralog of *HvFT1*, (alternatively named *HvFT3* by Faure et al., 2007 and Kikuchi et al., 2009). Its effect on flowering is not as strong as *HvFT1* and it seems to be restricted to winter genotypes under short days or long days without vernalization (Casao et al., 2011).

Another paralog of *HvFT1* with a large effect on time to flowering, particularly at Mediterranean latitudes (Boyd et al., 2003; Cuesta-Marcos et al., 2008), is *HvCEN*, and its two main haplotypes are differentially distributed over spring and winter barley varieties (Comadran et al., 2012).

Natural variation in barley vernalization requirement is mainly found in the vernalization loci *VrnH1* (Trevaskis et al., 2003; Yan et al., 2003), *VrnH2* (Yan et al., 2004), and *VrnH3* (Yan et al., 2006). The *VrnH1* gene is closely related to the *Arabidopsis* gene *APETALA1* (*API*), responsible for the transition from the vegetative to the reproductive stage (Trevaskis et al., 2007). Different alleles of *VrnH1* have been identified, with insertions or deletions in the first intron of the gene (von Zitzewitz et al., 2005; Cockram et al., 2007; Hemming et al., 2009), affecting the length of the optimum vernalization period. Alleles lacking large sections of the ~11 kb intron are more active and are associated with earlier flowering without vernalization, whereas alleles lacking small segments are associated with only a moderate increase in *VrnH1* activity and weaker promotion of flowering (Szűcs et al., 2007; Hemming et al., 2009). *VrnH2* includes three closely related genes designated as *HvZCCTa-c*, which are characterized by a putative zinc finger and a CCT-domain. *VrnH2* is considered to play the role of repressor of flowering and it has been shown that deletions of all the three *HvZCCT* genes result in spring growth habit (Karsai et al., 2005; Trevaskis et al., 2006). Finally, *VrnH3* was shown to correspond to *HvFT1*, the ortholog of the *Arabidopsis FT* gene (Yan et al., 2006; Faure et al., 2007).

FT is considered as the main flowering integrator of the photoperiod and vernalization pathways in both monocot and dicot species (Turck et al., 2008). In barley, the most accepted hypothesis for the regulation of *HvFT1* establishes that, during the fall, when temperate cereals germinate, *HvFT1* is repressed by *VrnH2* (Yan et al., 2006; Hemming et al., 2008; Distelfeld et al., 2009). During winter, vernalization up-regulates *VrnH1* (Trevaskis et al., 2006; Oliver et al., 2009), which results in the repression of *VrnH2* in the leaves and, consequently, the activation of *HvFT1* transcription in the spring (Loukoianov et al., 2005; Trevaskis et al., 2006; Hemming et al., 2008; Chen and Dubcovsky, 2012). The precise molecular mechanisms of action of these genes are still the object of numerous studies in barley and other cereals. *FT* transcription is induced in the leaves and it has been demonstrated in different species that the encoded protein travels through the phloem to the stem apical meristem, where it plays a central role in triggering flowering (Corbesier et al., 2007; Tamaki et al., 2007). In *Arabidopsis*, *FT* interacts with the bZIP transcription factor FD and up-regulates the expression of the meristem identity gene *API* at the shoot apex (Abe et al., 2005; Wigge et al., 2005). A similar interaction has been reported in wheat, where the orthologous *FT* protein interacts with an FD-like protein and has the ability to bind *in vitro* the promoter of *VrnH1*, the wheat homolog of *API* (Li and Dubcovsky, 2008).

Ample natural variation in the *HvFT1* gene has been found, with polymorphisms reported in the promoter and in the first intron. This variation has been linked to differences in flowering phenotypes in a number of studies (Yan et al., 2006; Hemming et al., 2008; Casas et al., 2011). It seems clear now that, in *Arabidopsis*, the *FT* promoter and first intron contain

cis-regulatory sites that are important for its transcriptional regulation (Tiwari et al., 2010). However, the *FT1* regulatory regions of barley and wheat are not as well characterized. Yan et al. (2006) found an association between growth habit and mutations in the first intron, but the sequencing of the *HvFT1* alleles from populations previously used to map QTL for flowering time (Hemming et al., 2008) failed to reveal any significant association between the two linked SNPs in intron 1 and flowering time. Further results reported in other surveys of *HvFT1* allelic variation (Cuesta-Marcos et al., 2010; Casas et al., 2011) were also in disagreement with Yan et al. (2006) regarding the direction of the effect assigned to the functional polymorphism in the first intron. Yan et al. (2006) also identified two promoter haplotypes, characterized by seven linked SNPs and two indels in the first 550 bp upstream of the start codon. Using primers specific to differentiate those indels, Casas et al. (2011) analyzed natural variation for the promoter haplotypes (135–146 vs. 139–142 bp) and the intron 1 haplotypes (AG/TC) in a collection of barley landraces. In that study the intron AG haplotype was clearly associated with later flowering than the TC haplotype. The results for the promoter haplotypes hinted at a role of these polymorphisms on flowering time, but of lesser magnitude than intron polymorphism. The combination of the 135–146 promoter with the TC intron was associated with earliest flowering (Casas et al., 2011; Ponce-Molina et al., 2012). Further evidence from other populations (Nitcher et al., 2013) confirmed the description of promoter haplotypes as “early” (135–146) and “late” (139–142). Another SNP in the promoter of the *HvFT1* gene, upstream of the studied region was also suggested to have an additional role on flowering time (Cuesta-Marcos et al., 2010; Casas et al., 2011).

The scope of *HvFT1* polymorphism has been recently widened even further by including copy number variation (CNV), first described by Nitcher et al. (2013). Recently, this type of polymorphism has been proposed as a key contributor to intra-species genetic variation, along with SNPs and indel polymorphisms. Some data suggest that CNV mainly affects the members of large families of functionally redundant genes and that the effects of individual CNV events on phenotype are usually modest (Zmieńko et al., 2014). Nevertheless, there are many cases in which CNVs for specific genes have been linked to important traits such as flowering time and plant height and resistance (Zmieńko et al., 2014). Regarding *HvFT1*, it has been reported recently that a genotype with high gene copy number (BGS213, derived from cultivar Tammi) was responsible for early flowering and an epistatic override of winter growth habit, caused by the combination of *vrnH1* and *VrnH2* alleles (Nitcher et al., 2013). This study pursues to carry out a comprehensive survey of *HvFT1* polymorphisms, including CNV, in barley accessions of different origins and germplasm groups. Also, we aim to provide further new information on gene expression and phenotypic effects of contrasting genotypes at *HvFT1*.

MATERIALS AND METHODS

PLANT MATERIALS

HvFT1 polymorphism

A set of 109 genotypes was used to survey the polymorphisms present at *HvFT1*, 89 cultivars, mainly European, and 20 inbred

lines derived from Spanish landraces (Igartua et al., 1998). They were classified into 60 winter and 49 spring types, according to their genetic constitution at vernalization and photoperiod genes (**Table S1**). These genotypes constitute a representative sample of barley germplasm available to European breeders.

HvFT1 phenotypic effect

Several biparental populations used in past studies were reassessed to account for possible phenotypic effects of the polymorphisms at *HvFT1* (**Table 1**). In some cases, these effects were already described in the references cited. In others, further genotyping allowed a better resolution of the QTLs or the discovery of previously unknown polymorphism. QTL x QTL interaction analyses were done using the *unbalanced analysis of variance* option implemented in Genstat 14 (Payne et al., 2009), following a factorial model with the markers closest to the QTL peaks and “environment” as factors. The field experiments of the population Beka x Mogador are explained in the publication by Cuesta-Marcos et al. (2008).

Gene expression analysis

Seven spring cultivars and two landrace-derived inbred lines, selected to represent *HvFT1* CNV types, were used for gene expression analysis. Also, eight doubled haploid (DH) lines of the population Beka x Mogador (**Table 2**) were used for this purpose. Several major flowering time genes were segregating in this population (Cuesta-Marcos et al., 2008). To focus only on variation at *HvFT1*, DH lines were selected with spring (Beka) alleles at *VrnH1*, *VrnH2*, and *PpdH2*. Variation in *HvCEN*, the most important factor determining flowering time in this population, was also considered. We aimed at having two plants per *HvCEN-HvFT1* haplotype, but found only one for the Mogador-Mogador class (an extra plant was allocated to the Beka-Mogador class). *PpdH1* was not segregating in this cross. Both parents carry the recessive, long-day insensitive allele.

SEQUENCE POLYMORPHISMS AT MAJOR GENES

DNA sequence polymorphisms for the 109 accessions were screened with allele-specific primers of candidate genes. *VrnH1* was scored based on the size of the first intron of its candidate *HvBM5A* (Yan et al., 2003; von Zitzewitz et al., 2005). Alleles were classified according to Hemming et al. (2009); *VrnH2* was evaluated as presence of *HvZCCT-Ha* and *HvZCCT-Hb* (Karsai et al., 2005). *PpdH1* was genotyped using SNP22 in the *CCT* (*Constans*, *Constans-like*, *TOC1*) domain of its candidate gene *HvPRR7*, after digestion with *BstU I* (Turner et al., 2005). *PpdH2* was scored as presence of the *HvFT3* gene as reported by Casao et al. (2011). Regarding *VrnH3*, two indels in the promoter and two SNPs in

the first intron of the *HvFT1* gene were assessed (Casas et al., 2011). *HvCEN*, candidate gene for *Mat-c* or *Eam6* was partially sequenced in 24 genotypes. The haplotypes are identified as reported by Comadran et al. (2012).

It was not possible to assess *HvFT1* polymorphism directly at the Beka x Mogador DH population (Cuesta-Marcos et al., 2008) because the sequences of the parental alleles were conserved and they differed only in copy number (2 Beka, 1 Mogador). The population was reanalyzed based on the new information found (parents polymorphic at *HvFT1* for CNV). Two microsatellite markers, in the *HvFT1* region, were mapped (EBmac0603 and AF022725A), to provide better resolution of the flowering time QTL found in this region.

HvFT1 CNV ANALYSIS BY qPCR

Genomic DNA was isolated from frozen barley leaves using the NucleoSpin Plant II kit (Macherey-Nagel, Germany) and used as template for CNV analysis by qPCR in an ABI 7500, essentially as described by Nitcher et al. (2013) with some modifications. Briefly, 100 ng genomic DNA were mixed with 2 μM of each primer and 10 μl of 2X Power SYBR Green Supermix (Thermo Fisher Scientific, Waltham, MA). The PCR program comprised 10 min at 95°C, 40 cycles of 10 s 95°C and 50 s 60°C, and a

Table 2 | Haplotypes for major flowering time genes and markers closest to QTLs (see Cuesta-Marcos et al., 2008) for selected doubled haploid (DH) lines of the population Beka × Mogador.

| DH | <i>VrnH2</i> | <i>VrnH1</i> | <i>PpdH2</i> | <i>HvCEN</i> | <i>HvFT1</i> | FLE [†] | AE [‡] |
|------|---------------|--------------|--------------|----------------|-----------------|------------------|-----------------|
| line | <i>HvZCCT</i> | <i>HvBM5</i> | <i>HvFT3</i> | <i>Bmac132</i> | <i>E41M47_e</i> | | |
| 1840 | B | B | B | B | B | 42 | * |
| 1892 | B | B | B | B | B | 41 | * |
| 1927 | B | B | B | B | M | 43 | * |
| 1933 | B | B | B | B | M | 44 | * |
| 2011 | B | B | B | B | M | 44 | * |
| 1873 | B | B | B | M | M | 30 | 36 |
| 1837 | B | B | B | M | B | 31 | 37 |
| 2009 | B | B | B | M | B | 40 | 46 |

Alleles conferring lateness are highlighted in gray. Dates for developmental stages in the pot experiment used for gene expression analysis are also included.

B, Beka; M, Mogador.

[†]FLE: days from sowing to 50% of plants with flag leaf expanded.

[‡]AE: days from sowing to 50% of plants with emerged awns.

*more than 48 days, as the experiment was terminated at that date and the plants had not reached awn emergence yet.

Table 1 | Biparental populations analyzed in this study.

| Population | Type | Number of lines | <i>HvFT1</i> polymorphism | References | Present study |
|-------------------|------|-----------------|---------------------------|-----------------------------|-------------------------------------------------------------|
| Henni x Meltan | DH | 118 | Promoter | Borràs-Gelöñch et al., 2010 | Genotyping of <i>HvFT1</i> promoter |
| SBCC154 x Beatrix | DH | 168 | Intron and CNV | Unpublished | QTL analysis, genotyping of <i>HvFT1</i> |
| Beka x Mogador | DH | 120 | CNV | Cuesta-Marcos et al., 2008 | New markers in <i>HvFT1</i> region and CNV. QTL interaction |

melting curve stage. Number of copies of the first exon of *HvFT1* was tested in all 109 genotypes. Morex was selected as the calibrator genotype and *SNF2* as the housekeeping gene (Yan et al., 2002). Then, in a subset of lines, two other *HvFT1* regions (promoter and exon 3) and three other genes close to *HvFT1* (*UCW118*, *UCW123*, and *UCW120*) were tested as reported by Nitcher et al. (2013). Efficiency for each primer pair was obtained by serial dilutions of barley genomic DNA and it was taken into account for CNV calculation (Weaver et al., 2010). Efficiencies for *SNF2*, *HvFT1*-promoter, *HvFT1*-exon1, *HvFT1*-exon3, *UCW118*, *UCW120*, and *UCW123* were 0.95, 0.96, 0.92, 0.95, 0.96, 1.03, and 0.85, respectively.

GROWTH CONDITIONS FOR EXPRESSION STUDIES

Barley plants used for expression analysis were grown in pots of 11.5 (diameter) × 16.0 (height) cm with a mix of peat, sand, and perlite. Six seeds of one genotype were sown per pot. After emergence, they were thinned to three seedlings per pot. The plants were grown in a growth chamber, under long-day conditions (16 h light, 250 μE m⁻² s⁻¹, 20°C, 60% relative humidity/8 h dark, 16°C, 65% relative humidity) for 7 weeks. There were three pots per genotype, which were used as replicates.

Two experiments were carried out with samplings at two different times. In the first experiment, leaf tissue was harvested in the middle of the light period, after 8 h light, as reported by Kikuchi et al. (2009). In the second experiment, to maximize circadian expression of *HvFT1* (Turner et al., 2005) harvesting took place 2 h before dark, after 14 h light. In both cases, leaf tissue (last expanded or flag leaf) was harvested and frozen immediately in liquid nitrogen before tissue homogenization (Mixer Mill model MM 400, Retsch, Germany). At each sampling time, three samples were analyzed per treatment and genotype. Each sample came from a different plant and pot.

mRNA EXTRACTION, cDNA SYNTHESIS, AND GENE EXPRESSION ANALYSIS

For qPCR analysis of *HvFT1* expression levels, 1 μg of total RNA (purified using the NucleoSpin RNA Plant kit, Macherey-Nagel) was transcribed to cDNA by using the SuperScript III reverse transcriptase and 2.5 μM poly(dT)₂₀ primer according to the manufacturer's instructions (Invitrogen). The reaction mixture for qPCR and the PCR program have been previously described. cDNA was quantified using a Nanodrop system (Thermo Fisher Scientific) and equal amounts were used for all samples. *Actin* was selected as the housekeeping gene (Trevaskis et al., 2006), and expression of *HvFT1* and *HvCEN* was analyzed using the same primers as in Yan et al. (2006) and Comadran et al. (2012), respectively. Efficiencies for *Actin*, *HvFT1*, and *HvCEN*, were 0.97, 0.86, and 0.96, respectively.

RESULTS

SURVEY OF *HvFT1* CNV

Winter and spring genotypes were classified as follows: winter genotypes carry a functional *VrnH2* allele and a winter or intermediate allele in *VrnH1* (wild type *vrnH1*, *VrnH1-6*, or *VrnH1-4*). Spring or facultative lines have been classified as those with a spring allele in *VrnH1* or lines in which *VrnH2* is absent. With

regard to *HvFT1*, there were accessions representing all possible combinations of intron and promoter polymorphisms, both within the winter and spring groups (Table 3). The combination of the “late” promoter with the “early” intron, however, was the most frequent. *HvFT1* exon 1 copy number ranged from 0.35 ± 0.13 in the winter cultivar Igri to 5.12 ± 0.28 in the spring cultivar Zaida (Figure 1). The results for several genotypes were intermediate between two classes, and were assigned to classes based on pedigree information when possible. Genotypes with several copies of *HvFT1* presented allele combinations typical of both winter and spring cultivars at all the *Vrn* and *Ppd* genes, with one exception, *VrnH2*. No genotype with the *VrnH2* gene present had more than one *HvFT1* copy.

It is remarkable that all of the winter barley cultivars, i.e., with a dominant *VrnH2* allele, had only one copy of the first exon. Barberousse, the last winter genotype in Figure 1, had 1.50 ± 0.01 copies, which we considered as a single copy. On the other hand, 28 out of the 49 spring cultivars analyzed contained more than 1.70 copies of exon 1 of *HvFT1*, which we have considered as multiple copies.

The study was extended to analyze CNV in other areas within or near the *HvFT1* gene, as in Nitcher et al. (2013). Thus, as well as qPCR primers for *HvFT1* exon 1 (amplifying the region of +200 to +293 bp downstream from the ATG start codon), we used primers for *UCW118* (nearest known gene in the flanking Morex BAC 455J22 upstream from *HvFT1*), *HvFT1* promoter (-727 to -656 bp upstream from the ATG), *HvFT1* exon 3 (+778 to +902 bp downstream from the ATG), *UCW123* (+6.6 kbp downstream from *HvFT1*), and *UCW120* (nearest known gene in the flanking Morex BAC 761F04 downstream from *UCW123*). This analysis was carried out in 10 barley varieties from Northern Europe (Figure 2A), 18 Spanish landraces (Figure 2B), and in another nine varieties from diverse origins (Figure 2C). Although all genotypes were not analyzed for all the genes, several results merit further attention. Two main patterns of *HvFT1* promoter/exon 1/exon 3 copy number were found in Northern European barleys: Asplund, Olli, Herse, Stella, Tammi, and Maskin contained one single copy of the promoter and multiple copies of exon 1 and exon 3, whereas Henni, Meltan, Pallas,

Table 3 | Number of spring and winter accessions classified according to *HvFT1* haplotypes defined by polymorphisms at the promoter (indel 1–indel 2), first intron and number of copies of exon 1.

| Promoter | Intron | Number of copies | |
|--------------------------|--------|------------------|----|
| | | 1 | >1 |
| SPRING ACCESSIONS | | | |
| 135–146 | AG | 3 | 14 |
| 139–142 | AG | 1 | 1 |
| 135–146 | TC | 3 | 2 |
| 139–142 | TC | 14 | 11 |
| WINTER ACCESSIONS | | | |
| 135–146 | AG | 9 | |
| 139–142 | AG | 7 | |
| 135–146 | TC | 6 | |
| 139–142 | TC | 38 | |

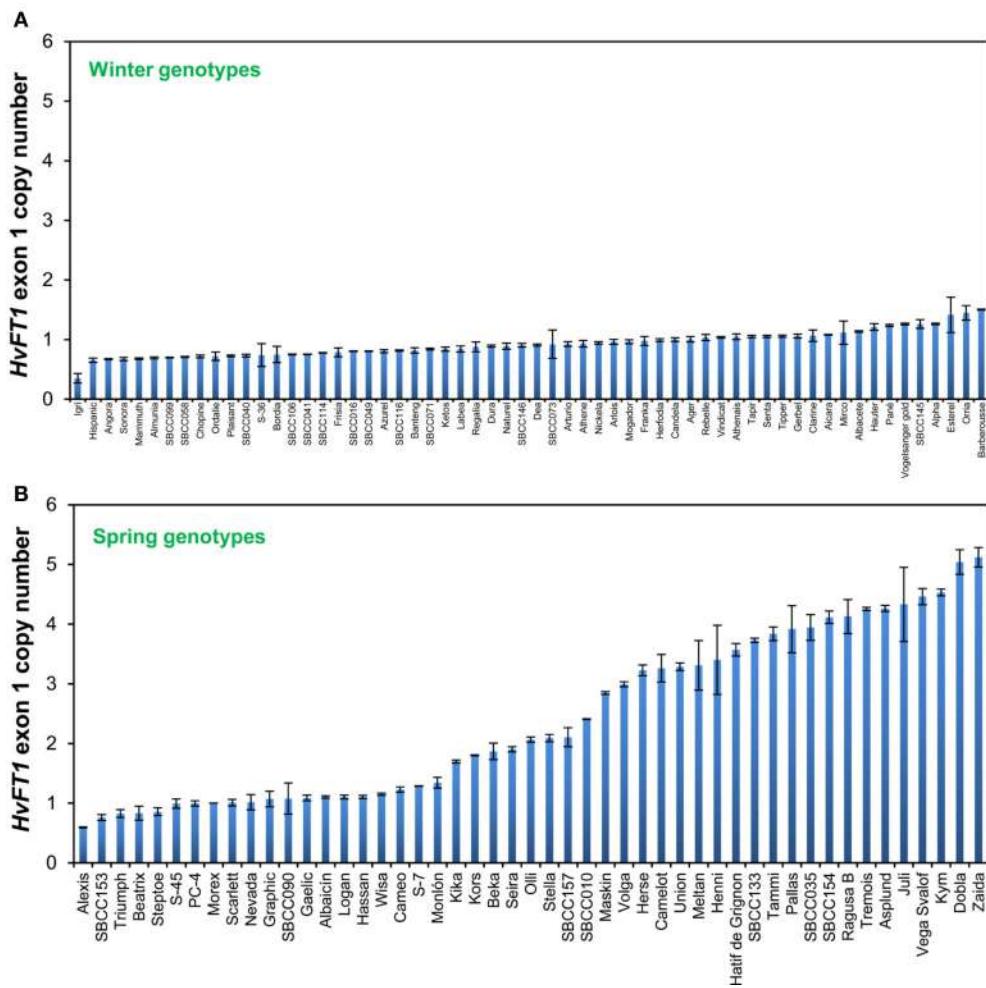


FIGURE 1 | Determination of copy number variation for the first exon of *HvFT1* in winter (A) and spring or facultative (B) barleys. Bars represent means \pm s.e.m. Morex was used as the calibrator genotype (copy number = 1). SBCC, Spanish Barley Core Collection.

and Juli contained multiple copies of promoter, exon 1, and exon 3. These different gene structures may affect gene functionality, as we will discuss later. CNV in other genes around *HvFT1* was only analyzed in 11 accessions. *UCW118* (next to the promoter), and *UCW123* and *UCW120* (next to the exon 3) were similar to the corresponding CNV in the adjacent regions, with the exception of Tammi, which contained multiple copies of *UCW118* but only one promoter. We do not provide results for *UCW120* in Meltan since the primers gave no amplification. Regarding the Spanish lines, only 4 out of 18 genotypes analyzed (SBCC157, SBCC154, SBCC133, and SBCC035) contained multiple copies of the promoter, exon 1 and exon 3. For the other genotypes analyzed, only Beka and Dobla presented several gene copies in the *HvFT1* promoter, exon 1, and exon 3.

EFFECT OF *HvFT1* POLYMORPHISM IN POPULATIONS

We have reassessed several populations to illustrate the effect of sequence and CNV polymorphism at *HvFT1*.

The population Beka x Mogador (Cuesta-Marcos et al., 2008) is a spring x winter population with very large flowering time

variation. It has been reassessed because we have found that, although the sequence of *HvFT1* is conserved in both genotypes (1 distal mismatch in 2547 bp sequenced), it still presents CNV polymorphism: Beka has two copies of the gene, whereas Mogador has one. A small flowering time QTL in the vicinity of *HvFT1* was already reported, although the region was not well covered with markers in the original study. Close markers were now identified and genotyped in the population to increase coverage. The QTL already detected on 7HS in this cross was made more conspicuous with the markers introduced for this study (Figure 3), with the peak hinting at the *HvFT1* position, and a high significance [-log₁₀(P) above 11]. In this case, Beka contributed the early allele, about 2.2 days earlier than the Mogador allele. If this QTL is truly due to the effect of *HvFT1*, it must be caused by differential effect of the number of copies. The possible effect of *HvFT1* CNV on the vernalization mechanism, as described by Nitcher et al. (2013), should have been evident in this population as an interaction of the QTL found at the *VrnH1*, *VrnH2* regions with the QTL at the *HvFT1* region, in trials without enough vernalization. This could have occurred in the three

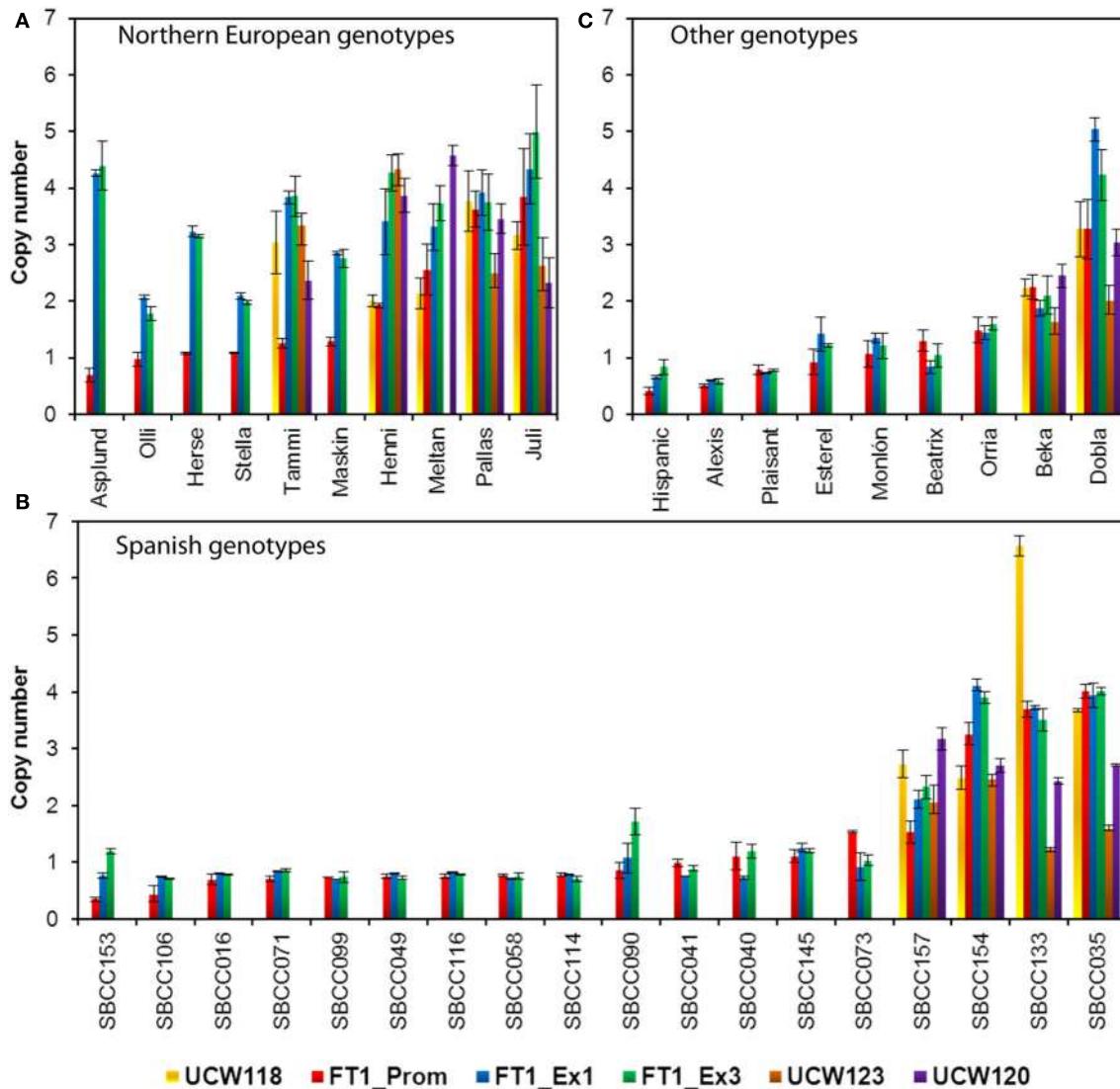


FIGURE 2 | Analysis of copy number variation for *HvFT1*(promoter, exon 1, and exon 3), and for regions upstream (UCW118) or downstream (UCW123 and UCW120) of *HvFT1* on barley genotypes of Northern

European (A), Spanish (B), and other origins (C). Bars represent means \pm s.e.m. Morex was used as the calibrator genotype (copy number = 1). SBCC, Spanish Barley Core Collection.

late sowings, in which the number of cooling degree days (a measure of vernalizing potential) was much lower than at the fall sowings (Table S1 in Cuesta-Marcos et al., 2008). The six field trials were reanalyzed for interactions between the five major QTL (*VrnH1*, *VrnH2*, *HvCEN*, *PpdH2*, *HvFT1*), using the closest marker to each peak, and dividing the six trials into fall sowings (November) and winter-spring sowings (late February to late March). The effect of *HvFT1* was clear across all trials, but did not interact much with other genes. There was just one possible interaction of *HvFT1* with *VrnH1* but in the fall-sown trials and not in the late sown trials. It was caused by a significant difference for the *HvFT1* alleles (with Beka, the one with two copies, inducing earliness) only in the presence of the winter (Mogador) allele at *VrnH1* (Table 4). The expected interaction between vernalization and *HvFT1* at the late-sown trials, however, was not detected at

all. Therefore, we can conclude from this result that the possible promoting effect of the double *HvFT1* gene of parent Beka was not strong enough to affect the vernalization requirement of the winter lines of this population.

SBCC154 x Beatrix is a cross of two spring genotypes. The population was described by Hofmann et al. (2013), but the flowering time data have not been reported yet. SBCC154 has four copies of *HvFT1*, whereas Beatrix has only one. Both have the putatively late (139–142) promoter (Nitcher et al., 2013), whereas Beatrix has the early intron (TC) and SBCC154 the late one (AG). The *HvFT1* marker detected a significant QTL with an effect of 2.5 days, with Beatrix as the early allele (Figure 4).

The population Henni x Meltan (Borràs-Gelonch et al., 2010), a cross of two spring cultivars, was an example of polymorphism just at the promoter. Each genotype has four copies of *HvFT1*

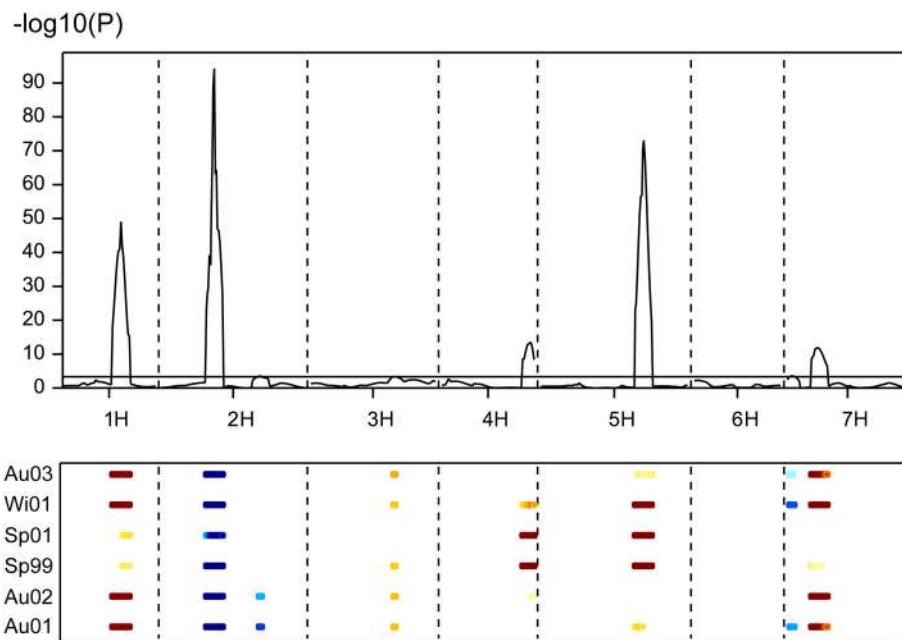


FIGURE 3 | Multi-environment QTL analysis for days to flowering from January 1st for six field experiments carried out with 120 doubled haploid lines and the parents of the population Beka x Mogador. The peaks above the threshold (dashed line) indicate presence of QTL significantly affecting the trait. Data taken from Cuesta-Marcos et al. (2008), enriched with new markers

on the 7HS chromosome. In the lower part of the figure, field trials are coded with Au (autumn sowing), Wi (winter sowing), or Sp (spring sowing) and two digits for the year; the colored dashes indicate the extent of the QTL and its direction: blue means that the early allele came from Mogador, yellow-brown from Beka, with intensity proportional to the size of the effect.

and, although they may have just two copies of the promoter, the parents present the same number of copies across the whole gene. The only polymorphism found was at the promoter, with Meltan carrying the early promoter (135–146) and Henni the late one (139–142). Confirming this expectation, the early allele of the QTL in Borràs-Gelonch et al. (2010) was contributed by Meltan, the difference being 23°C d (around 1–2 days).

EXPRESSION ANALYSIS OF *HvFT1* IN SELECTED SPRING BARLEYS

After finding CNV variation for *HvFT1*, the next step was to evaluate the effect of CNV variation on gene expression. If the effect of several copies of the gene was always as large as reported by Nitcher et al. (2013), then it should be detectable as a large increase of gene expression and a very early phenotype, overriding the effect of any other polymorphisms at *HvFT1*. This hypothesis was tested by evaluating gene expression on a set of spring and facultative cultivars representative of different *HvFT1* copy number alleles: Morex (reference genotype for one copy of *HvFT1*) and Beatrix as single *HvFT1* copy number, SBCC154, SBCC157, Dobla, Beka, Pallas, and Juli as examples of multiple promoter and exon 1 copies, and Tammi as representative of the genotypes with one promoter but multiple exon 1 copies (Table S1 and Figure 5A). *HvFT1* expression was found, in general, to increase according the developmental stage of the plants, although the expression levels differed widely among genotypes. At the second sampling date all genotypes, except Dobla, had not reached awn emergence yet. At this point, SBCC154, SBCC157, and Dobla, the earliest genotypes, displayed the highest *HvFT1* mRNA levels (awn emergence dates are included in Figure 5A).

Apart from this observation, we did not observe much correlation between *HvFT1* expression and time to awn appearance. Interestingly, these three varieties had the dominant allele for *PpdH1*, whereas the rest had the recessive allele at this gene. Also, we could not find a clear correlation between number of *HvFT1* copies or sequence polymorphism and *HvFT1* mRNA expression. For example, Juli and Tammi contained multiple copies of *HvFT1* (four promoters and four genes in Juli, one promoter and four genes in Tammi), but both of them showed lower *HvFT1* expression than SBCC154, SBCC157, or Dobla, at the same sampling date. The influence of the difference in *HvFT1* promoter copies on *HvFT1* expression observed in Tammi with respect to Juli and Pallas will be discussed later. Thus, we can conclude that CNV has not a prevailing effect on *HvFT1* expression, over other types of polymorphisms at the same gene. It is not the main factor controlling *HvFT1* expression and flowering time (measured as awn appearance), and it depends largely on the genetic background, and polymorphisms at other genes, especially *PpdH1*, as has been reported previously in the literature in studies done before taking into account CNV (Turner et al., 2005; Hemming et al., 2008).

EXPRESSION ANALYSIS OF *HvFT1* IN SELECTED LINES OF THE BEKA x MOGADOR POPULATION

In a second gene expression experiment, eight DH lines of the Beka x Mogador population were analyzed, together with some genotypes in common with the first experiment (Figures 5B,C). This population was found to contain a QTL for flowering time in *HvFT1* gene, as shown above. Individuals were selected according to CNV in *HvFT1*: Beka contained two copies while Mogador

contained only one. qPCR analysis were performed as before, but this time the material was harvested 2 h before dark. Sampling time actually had a major effect on the detection of *HvFT1* expression: under these conditions, *HvFT1* mRNA levels were, in

Table 4 | Probabilities of significance from three analyses of variance of flowering date recorded at six field trials, three fall-sown and three winter- or spring-sown in the Beka x Mogador population.

| Source | All trials | Fall-sown | Winter- spring-sown |
|---------------------|--------------|--------------|---------------------|
| | CPROB* | | |
| Trial | 0.000 | 0.000 | 0.000 |
| <i>VrnH1</i> | 0.000 | 0.280 | 0.000 |
| <i>VrnH2</i> | 0.000 | 0.000 | 0.000 |
| <i>HvFT3</i> | 0.000 | 0.000 | 0.000 |
| <i>HvCEN</i> | 0.000 | 0.000 | 0.000 |
| <i>HvFT1</i> | 0.000 | 0.000 | 0.003 |
| Trial. <i>VrnH1</i> | 0.000 | 0.237 | 0.000 |
| Trial. <i>VrnH2</i> | 0.000 | 0.511 | 0.000 |
| Trial. <i>HvFT3</i> | 0.092 | 0.006 | 0.632 |
| Trial. <i>HvCEN</i> | 0.000 | 0.612 | 0.001 |
| Trial. <i>HvFT1</i> | 0.766 | 0.248 | 0.698 |
| <i>VrnH1.VrnH2</i> | 0.000 | 0.424 | 0.000 |
| <i>VrnH1.HvFT3</i> | 0.489 | 0.527 | 0.644 |
| <i>VrnH2.HvFT3</i> | 0.947 | 0.934 | 0.811 |
| <i>VrnH1.HvCEN</i> | 0.000 | 0.008 | 0.000 |
| <i>VrnH2.HvCEN</i> | 0.786 | 0.072 | 0.074 |
| <i>HvFT3.HvCEN</i> | 0.817 | 0.305 | 0.276 |
| <i>VrnH1.HvFT1</i> | 0.097 | 0.011 | 0.699 |
| <i>VrnH2.HvFT1</i> | 0.564 | 0.760 | 0.652 |
| <i>HvFT3.HvFT1</i> | 0.330 | 0.509 | 0.477 |
| <i>HvCEN.HvFT1</i> | 0.109 | 0.078 | 0.548 |

The sources of variance are the trials plus five markers close to QTL peaks representing five major flowering time genes: *VrnH1* (*HvBM5*), *VrnH2* (*HvZCCT*), *HvFT3* (*Bmag382*), *HvCEN* (*Bmac132*), and AFLP E41M47_e (*HvFT1*). Three way interactions are not shown, as none was significant. The analyses of variance were done on genotype means, taking as error the residual genotypic variance.

*Conditional probability of significance for each term, when added to a full model with the rest of terms already included. P-values below 0.05 highlighted in bold type.

general, clearly higher than at the first experiment (Figure 5A), in which samples were harvested 8 h after the commencement of the light period, instead of 14 h. The varieties used in the first experiment, which had a recessive *PpdH1* allele, were also included for comparison. Apart from the clear induction of expression from a dominant *PpdH1* allele, we were not able to establish a clear relationship between *HvFT1* copy number, mRNA levels and flowering time for these plants (Figure 5B). *HvFT1* expression in the DHs was similar and stable during the experiment after 19 and 33 days. No significant differences were observed in *HvFT1* mRNA levels between the two alleles as main effects, although there was a significant interaction ($P = 0.022$) between the alleles at *HvCEN* and *HvFT1*. At the second sampling date, the plants with the Beka allele in *HvFT1* showed significantly higher expression than plants with Mogador allele, only if the allele at *HvCEN* also came from Beka. The Mogador allele at *HvCEN* had a major effect on earliness, and these lines (1837, 1873, and 2009) flowered markedly earlier than the others (flag leaf unfolding dates are included in Figure 5C).

Another interesting result of the second experiment was that Tammi showed the largest *HvFT1* expression after 33 days, and also flowered the earliest. It seems that Tammi has a more effective *HvFT1* gene to promote flowering than the others. This could be related to the fact that Tammi, as indicated above, presented four copies of the *HvFT1* gene but only one copy of the promoter, whereas Juli and Pallas, for example, have four copies of both promoter and exon 1.

EXPRESSION ANALYSIS OF *HvCEN* IN THE BEKA x MOGADOR POPULATION AND IN SELECTED SPRING BARLEY GENOTYPES

As we have shown previously, CNV and different haplotypes for *HvFT1* are not sufficient to establish a clear relationship between them, *HvFT1* mRNA levels and awn appearance. For example, the presence of a dominant *PpdH1* allele was found to enhance *HvFT1* expression in different spring genotypes (Figure 5A). Additionally, CNV in *HvFT1* did not have a clear role to determine flag leaf unfolding in the Beka x Mogador DHs (Figure 5C). For these reasons, we decided to analyze the mRNA levels of another gene involved in the establishment

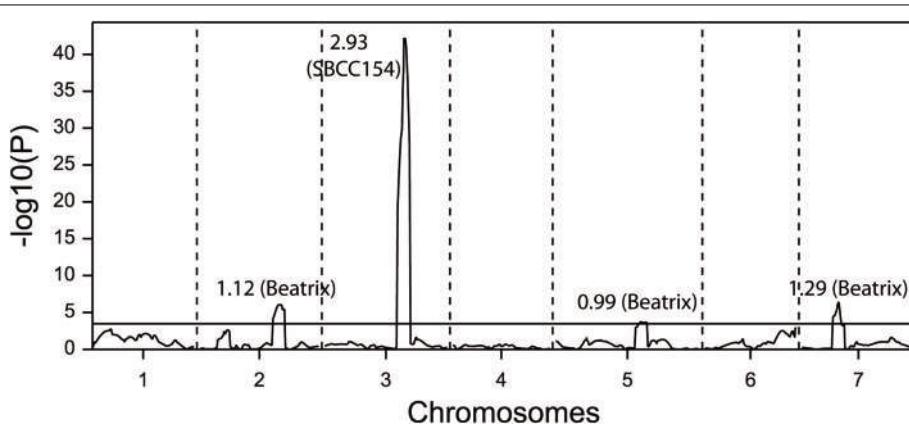


FIGURE 4 | QTL scan for flowering time at a field trial for a population of 168 doubled haploid lines from the cross SBCC154 x Beatrix. The figures besides the QTL peaks indicate the size of the effect, with the early allele indicated in parentheses.

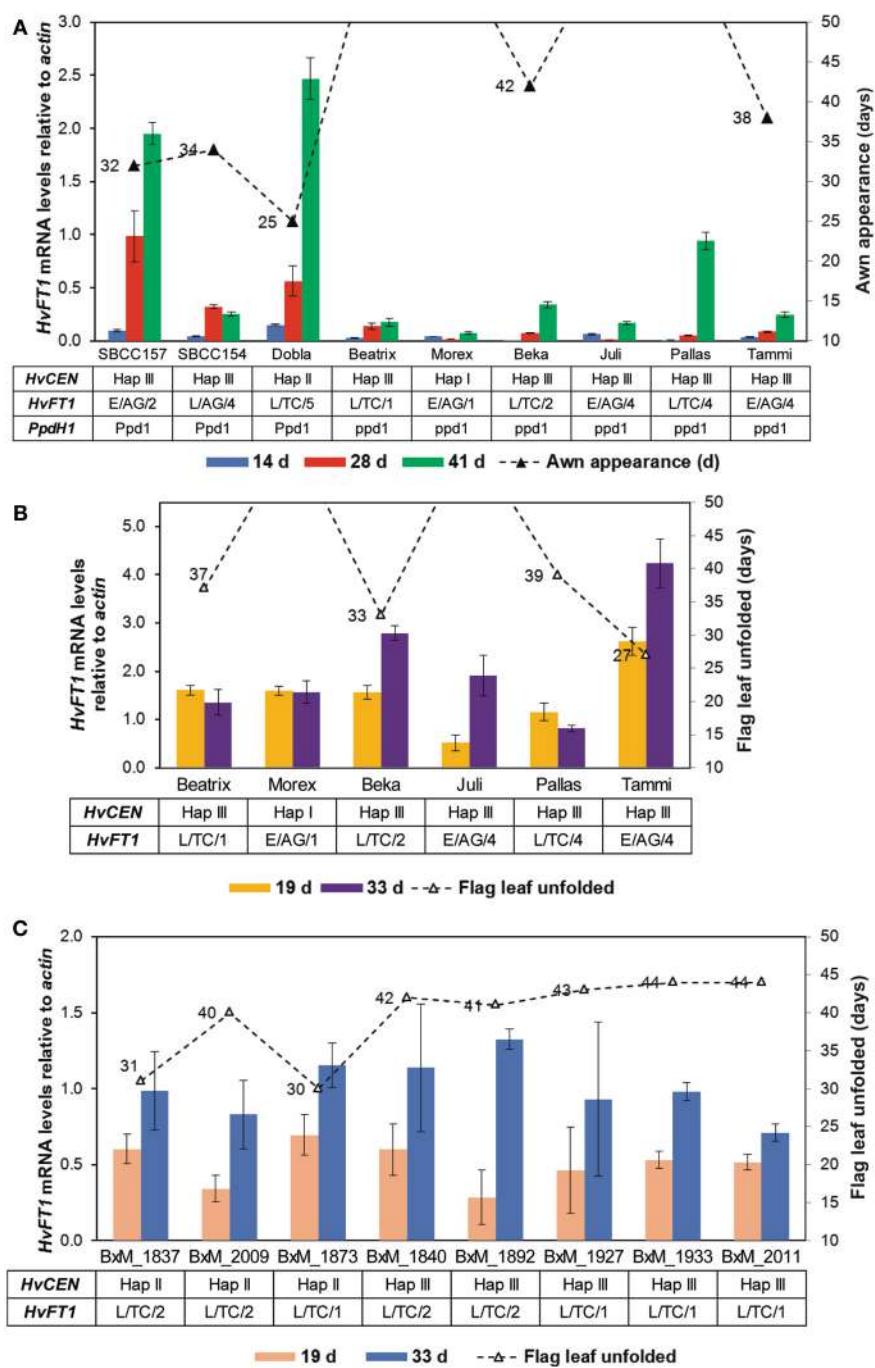


FIGURE 5 | *HvFT1* expression analysis by qPCR. In a first experiment (**A**), leaf tissue was harvested in the middle of the light period (after 8 h light) 14, 28, or 41 days after sowing. Days until awn appearance (triangles) are shown for the whole duration of the experiment (50 days). *Hvcen*, *HvFT1*, and *Ppdh1* haplotypes for each plant are also indicated. In the case of *HvFT1*, polymorphisms for promoter (E, “early”; L, “late”), intron 1 (AG or TC) and copy number variation (for

exon 1) are shown. In the second experiment (**B,C**), harvesting took place 2 h before dark (after 14 h light), 19 or 33 days after sowing. Days until full unfolding of the flag leaf (triangles) are shown for the whole duration of the experiment (50 days). As previously, *Hvcen* and *HvFT1* haplotypes for each plant are included. (**C**) Expression in selected doubled haploid lines of the Beka x Mogador population (BxM, see Table 2). Bars represent means \pm s.e.m.

of flowering time, *Hvcen*, a paralog of *FT* and *TFL1* in *Arabidopsis* (Kobayashi et al., 1999). We used the same cDNAs as for the previous expression studies (Figure 6). We included haplotype information for some genotypes about *Hvcen* in Table S1 for comparison. As observed for *HvFT1*, our results

suggest that *Hvcen* mRNA levels are not the main responsible to explain awn appearance (Figure 6). *Hvcen* expression dramatically increased when samples were harvested 14 h after dawn instead of 8 h (Figures 6A–C), as we observed for *HvFT1*. Another remarkable observation is that *Hvcen*

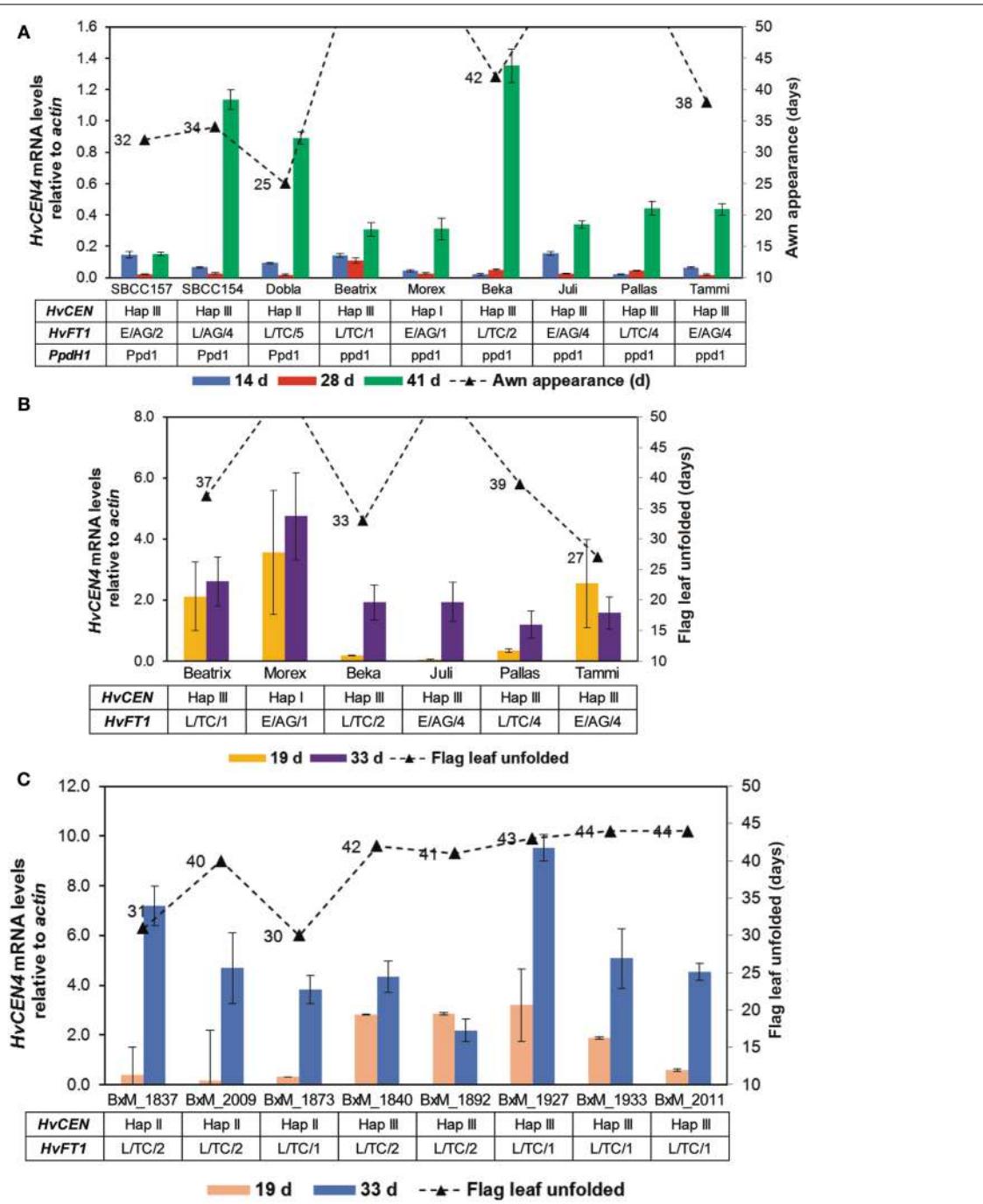


FIGURE 6 | *HvCEN* expression analysis by qPCR. *HvCEN* mRNA levels were quantified using the same conditions as for Figure 5. **(A)** leaf tissue was harvested in the middle of the light period (after 8 h light) 14, 28, or 41

days after sowing. **(B,C)**, harvesting took place after 14 h light, 19 or 33 days after sowing. **(C)** Expression in selected doubled haploid lines of the Beka x Mogador population (BxM, see Table 2). Bars represent means \pm s.e.m.

expression also changed during the development, in disagreement with previously observed results for *HvCEN* expression (Comadran et al., 2012). For example, Morex contained significantly higher *HvCEN* mRNA levels than Tammi, but Morex did not flower after 50 days and awns in Tammi appeared after 33 days.

DISCUSSION

DISTRIBUTION AND PHENOTYPIC EFFECT OF *HvFT1* POLYMORPHISMS OVER BARLEY GERMPLASM

In this paper, we analyzed the extent of *HvFT1* CNV and its effect on *HvFT1* expression in more than 100 different spring and winter barley genotypes, mainly European, including some landraces

from the Spanish Barley Core Collection (SBCC) (Igartua et al., 1998). Recently, Nitcher et al. (2013) demonstrated that the *FT1* allele present in the barley genetic stock BGS213, which carried several copies of *HvFT1*, showed earlier transcriptional up-regulation of *FT1*, and was associated with a dominant spring growth habit. Cultivar Tammi, from Finland, was the donor of the mutation present in that genetic stock (Franckowiak and Konishi, 1997). This allele is not frequent and was reportedly found only in spring cultivars originating from regions of extremely high latitude or high altitude (Takahashi and Yasuda, 1971). We confirmed the finding by Nitcher et al. (2013) that the Tammi/BGS213 allele was characterized by having a single copy of the promoter and several copies of the transcribed region. This kind of allele was present in a group of 6 six row Northern European cultivars, some of them related by pedigree (Baumer and Cais, 2000; Chiapparino et al., 2006). The origin of this allele could be traced back to primitive cultivar Asplund, which is in the parentage of Tammi and Herse, both showing alleles with multiple exon copies and one promoter (Figure 2). This allele seems to be particularly beneficial at high latitudes because both parents of Tammi (Asplund and Olli) come from the Northern limits of the barley cultivation range (Manninen and Nissila, 1997).

We found several copies of *HvFT1* in accessions from apparently disconnected germplasm groups, like Scandinavian and Spanish landraces. Although the sample examined is not enough to derive definitive conclusions, the distribution of CNV alleles over type of growth habit haplotypes is intriguing. Multiple copies at *HvFT1* occurs only in genotypes that do not have winter growth habit. This suggests a possible disadvantageous agronomic effect of the presence of several copies of the gene in interaction with *VrnH2*. A possible mechanism that provides a plausible explanation for this is that the increased copy number of *HvFT1* is epistatic to winter alleles for *VrnH1* and *VrnH2* (as reported by Nitcher et al., 2013). Muñoz-Amatriaín et al. (2013) have recently shown that high levels of CNVs are found in the barley genome, around 9.5% in coding genes, at increasing frequencies as distance to the centromere increases. This is a widespread phenomenon, contributing to phenotypic variation in barley. *HvFT1* CNV may be neutral in spring genotypes, but breeders could have selected against this duplication when breeding winter barley to avoid early transition to reproductive growth and exposure of reproductive tissues to damaging low temperatures. However, we have shown that this epistatic mechanism is not universal, as it does not occur in the Beka x Mogador population.

The reassessment of three biparental populations representing polymorphisms of different kind identified QTL in the region of *HvFT1*. Previous studies also shed some light on the possible phenotypic effect of *HvFT1*. The QTL found in the cross SBCC145 x Beatrix (Ponce-Molina et al., 2012), whose peak coincides with the gene, must be due to polymorphisms at the promoter region, as both parents have one copy of the gene and same first intron sequence. The early allele was contributed by SBCC145, a Spanish landrace from the Canary Islands, which carries the “early” promoter. It must be pointed out, however, that SBCC145 has a distinct promoter, with additional polymorphisms compared to others, similar to cultivars Dairokkaku (Casas et al., 2011) and Meltan. The cross SBCC154 x Beatrix offered the opportunity to

contrast two different polymorphisms (promoter for SBCC145 x Beatrix, first intron, and CNV for SBCC154 x Beatrix) against a common parent (Beatrix). This population was tested at a single trial sown in autumn, in the same field as the population SBCC145 x Beatrix, and the result was the presence of a QTL, exactly at the marker used to genotype *HvFT1*, with SBCC154 as the late allele. In this case, high copy number (SBCC154) was late compared to one copy, but this could be due to an effect of SBCC154 carrying the “late” intron. Therefore, any acceleration of flowering that might be produced as a consequence of high copy number was secondary to the lateness associated to the sequence polymorphism. It must be noted that *PpdH1* was segregating in this population. However, flowering occurred too early to allow for a significant effect of *PpdH1*, which was not detected as QTL.

In the cross SBCC016 x Esterel (Casas et al., 2011), each genotype had one copy of *HvFT1*. Esterel has the “early” intron (TC) and SBCC016 the “late” one (AG). Accordingly, the early allele of the QTL, which also peaked at the gene itself, was contributed by Esterel. Hayes et al. (1993) and Borràs-Gelonch et al. (2012) reported a QTL in this region for the population Steptoe x Morex. Although the size of the effect was not large, in both studies the Morex allele was significantly later. Both parents have just one copy of the gene, and a mixture of late and early polymorphisms at the promoter and the intron. It seems that the intron effect is stronger, as Steptoe carries the early (TC) allele at this position.

A QTL in the region of *HvFT1* was also detected in the classical studies carried out in the Igri x Triumph population (Laurie et al., 1995), with the Triumph allele conferring earlier flowering, although the nucleotide sequences were identical (Yan et al., 2006). CNV has been recently identified in this population, with two copies of the *HvFT1* gene in Triumph (R. Nitcher, personal communication). This result would match our findings in the Beka x Mogador population. Nevertheless, we could not reproduce this result, since the Triumph seed we analyzed had only one copy of the gene. We cannot discard that the samples analyzed are different, because this cultivar is known under two different names, Trumpf in Germany and Triumph in the UK, where it was reselected from somewhat heterogeneous seed (van Harten, 1998).

In summary, these findings reported in the literature, combined with the results presented in this study, reveal that detection of flowering time QTL in the region of *HvFT1* in biparental populations representing all kinds of polymorphisms at *HvFT1* (promoter, first intron, and CNV) is common. There is no functional proof that this gene is responsible for all the QTL, but it is a good candidate. An alternative explanation could be the presence, at least in some cases, of an additional flowering time gene closely linked to *HvFT1*. In any case, we have to wait until there are either functional proofs or increasing evidence from other populations to declare that *HvFT1* is the responsible for the variation observed. We expect that the catalog of polymorphisms presented in this study will help other researchers to contribute information to clarify the issue.

The survey of *HvFT1* polymorphisms allows us to conclude that *HvFT1* (*VrnH3*) is far from being effectively fixed in cultivated barley, as stated up to now (Stracke et al., 2009; Comadran

et al., 2012). This statement is probably true if one only considers the allele responsible for the huge phenotypic effect observed by Nitcher et al. (2013) on a winter barley, which confirmed observations of the seminal work by Takahashi and Yasuda (1971). The effects that we have found in different populations (this study), association studies (Casas et al., 2011) and also reported in the literature (previous paragraphs) point at smaller phenotypic effects that are linked to all types of polymorphisms in this gene (promoter, first intron, CNV). These effects, however, cannot be easily extrapolated to different genetic backgrounds.

CNV AND GENE EXPRESSION

The commencement of the reproductive stage in barley and the duration of the time period until flowering are controlled by a variety of factors that act interactively. In temperate cereals like wheat and barley, flowering is promoted by long days. In barley, the up-regulation of *VRN3/FT1* appears to be the main trigger for the initiation of flowering (Faure et al., 2007), although an alternative view is that its role may be to accelerate inflorescence development and reduce the time taken from double-ridge to head emergence (Sasani et al., 2009). Its expression is tightly regulated, repressed by *VRN2* under long days (Hemming et al., 2008), which actually competes with CO to bind to protein complexes that activate *FT* (Li et al., 2011). Also in response to long days, *FT1* expression is promoted by CO-like proteins (Li et al., 2011; Campoli et al., 2012) and the pseudo response regulator *PpdH1* (Turner et al., 2005; Faure et al., 2007). This promotion may occur by interaction of CO with the promoter of *FT*, as happens in *Arabidopsis* (Tiwari et al., 2010; Li et al., 2011; Andrés and Coupland, 2012).

The findings of this study add to mounting evidence pointing at a complex control of the timing of head emergence that cannot be easily reduced to a simple scheme. On one hand, *HvFT1* displays a variety of polymorphisms in regions that are compatible with a regulatory role. On the other hand, its expression pattern is compounded by its nodal position, downstream of the vernalization and long day pathways, whose genes also have large influence on duration of plant development.

The first gene expression experiment results confirmed the induction of *HvFT1* expression by the long day pathway, irrespective of the polymorphisms present at *HvFT1*. The genotypes that reached first awn appearance in that experiment were all those having a sensitive/dominant *PpdH1* allele, particularly at the second sampling date. This date is probably the most meaningful because all *PpdH1* genotypes reached awn appearance just a few days later. This result agrees with other reports where *HvFT1* expression in *ppdH1* background was markedly lower (Turner et al., 2005; Hemming et al., 2008; Kikuchi et al., 2009). Therefore, we can conclude that gene duplication of *HvFT1* does not always ensure higher expression and, in any case, the scale of its effect is minor compared to the induction by *PpdH1*. The case of high *HvFT1* expression in presence of *ppdH1* described in Nitcher et al. (2013) seems an exception, probably due to a unique genetic background.

Our results confirm that the triggering of events at the meristem leading to flowering is not determined just by *HvFT1* expression. There must be additional factors that probably need the

presence of *HvFT1* product to interact with. We cannot rule out, however, that copy number is related to a dosage effect, precisely by the different genetic background in each genotype, which may lead to differences in the induction of *HvFT1*. A beneficial increase in dosage is actually one of the evolutionary forces explaining the conservation of gene duplications, particularly for genes that mediate the interaction between the organism and the environment, as reported for *Ppd-B1* and *Vrn-A1* in wheat by Díaz et al. (2012), or for genes with dosage-sensitive functions, owing to protein-protein interaction (Innan and Kondrashov, 2010). *HvFT1* corresponds to the first class, and may as well be part of the second. CNV is widespread in plants (Zmíšek et al., 2014), and certainly in barley (Muñoz-Amatriaín et al., 2013), but its effects vary in each case. For instance, in barley, the powdery mildew resistance allele *mlo-11* acts by disrupting its expression through accumulation of non-functional copies of the gene upstream of the wild-type copy (Piffanelli et al., 2004). On the other hand, the effect of the freezing tolerance locus *FrH2* in barley depends on the number of *CBF* genes transcripts produced, that are proportional to the number of copies present in the gene cluster identified as responsible for this QTL (Pasquarello et al., 2014). The plant immunity locus *GER4*, also in barley, is also a cluster of tandemly duplicated genes. In this case, the enhanced transcript dosage was proposed as the evolutionary driving force for the local expansion and functional redundancy of the locus (Himmelbach et al., 2010).

The results of Nitcher et al. (2013) and the earliness induced by the double copy “Beka” allele in the Beka x Mogador population point in the direction of a dosage effect. However, the direct comparison of *HvFT1* expression figures for different alleles is hampered by the large influence of the alleles at *PpdH1* on *HvFT1* and at *HvCEN* on flowering time. The analysis of the eight sister lines of the Beka x Mogador population indicates a slightly earlier flowering and higher *HvFT1* expression of lines with the Beka allele only in presence of the Beka allele at *HvCEN* (spring, Hap III), but the evidence is too small to declare that we have found a clear dosage effect.

These results led us to speculate that the most efficient version of the barley gene could be one with a single conserved promoter, and variable number of transcribed regions, as seen in cultivar Tammi, or one promoter and one copy of the gene. Other versions of the gene, with several promoters and transcribed regions may take longer to be induced. Indeed, we do not know whether the multiple copies of *HvFT1* are functional or not. They seem to be expressed in Tammi/BGS213, but there is no evidence for other alleles. If they are, the presence of several full and functional copies of the gene may affect differently the expression of the gene, depending on the concentration of the promoting signal. If it is low, its dilution over several copies may delay transcription, whereas an abundant promoting signal would enhance transcription proportionally in alleles with several copies. The special case of Tammi/BGS213 indicates either that the transcription of several copies is triggered by a single promoter, or that the additional copies of the gene are placed under the control of other promoters and thus escape the regulatory control of the wild-type gene. For other multiple copy alleles, with equal number of promoters and transcribed regions, we can

speculate that the copies are full and perfect replicates of the original gene, and that the expression of them all is affected by the same processes.

The difference in overall gene expression between the two experiments agrees with the reports on pattern of diurnal oscillation of *FT1* expression during the day (Turner et al., 2005; Kikuchi et al., 2009). Actually, the same observation can be made for *HvCEN* (**Figure 6**). This information, combined with the sequence analogy between the two genes, suggests also that *HvCEN* could be subject to circadian rhythm.

INTERACTIONS OF *HvFT1*

The DH lines (**Figures 5C, 6C**) presented very different dates to reach a developmental stage (flag leaf unfolding in this case) in the presence of rather similar levels of *HvFT1* expression. As pointed out before, in this case the cause for differences in development seems to lie on the allele present at *HvCEN*. It is clear that accelerated development in the presence of the Mogador *HvCEN* allele (Hap II) did not depend on higher expression of *HvFT1*. Therefore, early development in these lines must be caused by some mechanism acting in parallel to *HvFT1*, either through protein-protein interaction with *HvFT1* or by a combined effect with *HvFT1* by focusing on the same targets.

Actually, the interaction between *HvFT1* and *HvCEN* is by no means unexpected. *HvCEN* and *HvFT1* are probably paralogs as suggested by their high sequence identity (59%), and indeed have been annotated as members of closely related protein families before (Higgins et al., 2010; Andrés and Coupland, 2012; Comadran et al., 2012). Therefore, it is likely that their products display similar interactive patterns with other proteins. There are many evidences in the literature pointing at the interaction of FT proteins with other proteins in the flowering promotion pathway. Ahn et al. (2006) reported that the family of small proteins coded by *FT* and homologous genes act as “either scaffolds or regulators of signaling complexes” in *Arabidopsis*. The bZIP transcription factor FD also seems to play a central role in flowering (Wigge et al., 2005). More recently, Jaeger et al. (2013) put forward a model to explain flowering control in *Arabidopsis* in which TFL1 (the product of another paralog gene) competed with FT to form the complex with the FD gene product, needed to trigger floral meristem specification. Some evidence for this kind of process in cereals was found by Li and Dubcovsky (2008), who detected interaction between the proteins of *TaFT* (the *FT* homolog of bread wheat) and *FD-like2*, and also in rice (Taoka et al., 2011), although in this case the interaction needed the intermediation of a third protein. In barley, however, *FD* orthologs can only be predicted in terms of sequence similarity.

A search in the Protein Data Bank revealed that, besides the FT-FD interaction, *Arabidopsis* homodimers of FT and also TFL1 have been reported. Thus, it is also conceivable that proteins coming from genes as similar as *HvFT1* and *HvCEN* may form heterodimers as well, making the speculation about the interaction between the products of these two genes more plausible. The *HvCEN* polymorphism, which translates to a non-synonymous mutation (Pro135Ala), is located in a solvent-exposed protein

loop (Comadran et al., 2012). However, with the structural evidence at hand, this loop does not directly participate in any dimeric interface and therefore nothing can be concluded about its molecular role in the interface.

The role of *HvFT1* may actually go beyond the duration of developmental phases. There is recent evidence about dramatic agronomic effects of the ortholog of *FT* in tomato, *SFT* in interaction with *SP* (*SELF PRUNING*, itself an ortholog of *HvCEN* and *HvTFL1*). *SFT* is in a “dose-dependent epistatic interaction” with *SP* which results in a modification of plant architecture that can be optimized to produce higher yields (Jiang et al., 2013). This dose-dependent action of *SFT* also opens the ground for speculation about possible dose-dependent action of multiple copies of *HvFT1* and its agronomic outcome.

CONCLUSIONS AND FURTHER WORK

The main conclusions of this study are:

- A wide survey of barley germplasm revealed that *HvFT1* duplication was only observed in spring and facultative barleys that do not possess a functional *VrnH2* allele.
- Two models of *HvFT1* duplication were observed, one that includes the promoter and the gene, the other only the transcribed region. Higher gene expression seems associated only to the second one.
- There are flowering time QTL on the region of *HvFT1* in different populations, representing all types of polymorphism at *HvFT1*, promoter, first intron and CNV.
- Analysis of *HvFT1* expression and phenotypic effects showed that they depend on gene polymorphisms but also on genetic background.

Plant breeders must be able to fully harness the development of cereal plants to be able to respond to the challenges of climate change. In this study, we present the case to state that *HvFT1* has been a hot spot to fine tune barley adaptation to environmental conditions, and will have to be given due consideration by breeders to create future cultivars.

The role of other genes in triggering flowering initiation, possibly in interaction with *HvFT1*, seems a very rich area which deserves more research. In particular, further avenues to attempt to resolve the precise sequence or structural variation in *HvFT1* which causes early or late flowering, particularly for the Tammi allele will require crossing different *HvFT1* alleles into a common genetic background, maintaining fixed *PpdH1* and *HvCEN*. This could be combined with BAC sequencing for the entire *HvFT1* region, for key alleles.

AUTHOR CONTRIBUTIONS

Ernesto Igartua, Ana M. Casas and M. P. Gracia conceived the experiments. Jorge Loscos performed qPCR analysis. Ana M. Casas and Jorge Loscos characterized the flowering time genes. Ernesto Igartua and Jorge Loscos performed computational analysis. Bruno Contreras-Moreira performed the bioinformatics searches and helped with the interpretation of the data. All authors wrote and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00251/abstract>

Table S1 | Barley accessions surveyed in this study classified according to the genetic constitution at vernalization and photoperiod genes.

Countries of origin and row number are also indicated.

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Putative sugarcane *FT/TFL1* genes delay flowering time and alter reproductive architecture in Arabidopsis

Carla P. Coelho^{1,2}, Mark A. A. Minow², Antonio Chalfun-Júnior¹ and Joseph Colasanti^{2*}

¹ Setor de Fisiologia Vegetal, Departamento de Biologia, Universidade Federal de Lavras, Lavras, Brazil

² Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Edited by:

Maria Von Korff Schmising, Max Planck Society, Germany

Reviewed by:

Yao-Guang Liu, South China Agricultural University, China
Wilma Van Esse, Max Planck Institute for Plant Breeding Research, Germany

***Correspondence:**

Joseph Colasanti, Department of Molecular and Cellular Biology, University of Guelph, 50 Stone Road East, Guelph, ON, N1G 2W1, Canada
e-mail: jcolasan@uoguelph.ca

Agriculturally important grasses such as rice, maize, and sugarcane are evolutionarily distant from *Arabidopsis*, yet some components of the floral induction process are highly conserved. Flowering in sugarcane is an important factor that negatively affects cane yield and reduces sugar/ethanol production from this important perennial bioenergy crop. Comparative studies have facilitated the identification and characterization of putative orthologs of key flowering time genes in sugarcane, a complex polyploid plant whose genome has yet to be sequenced completely. Using this approach we identified phosphatidylethanolamine-binding protein (PEBP) gene family members in sugarcane that are similar to the archetypical *FT* and *TFL1* genes of *Arabidopsis* that play an essential role in controlling the transition from vegetative to reproductive growth. Expression analysis of *ScTFL1*, which falls into the *TFL1*-clade of floral repressors, showed transcripts in developing leaves surrounding the shoot apex but not at the apex itself. *ScFT1* was detected in immature leaves and apical regions of vegetatively growing plants and, after the floral transition, expression also occurred in mature leaves. Ectopic over-expression of *ScTFL1* in *Arabidopsis* caused delayed flowering in *Arabidopsis*, as might be expected for a gene related to *TFL1*. In addition, lines with the latest flowering phenotype exhibited aerial rosette formation. Unexpectedly, over-expression of *ScFT1*, which has greatest similarity to the florigen-encoding *FT*, also caused a delay in flowering. This preliminary analysis of divergent sugarcane *FT* and *TFL1* gene family members from *Saccharum spp.* suggests that their expression patterns and roles in the floral transition has diverged from the predicted role of similar PEBP family members.

Keywords: *Saccharum spp.*, bioenergy crop, floral induction, *FT*-like genes, florigen orthologs, PEBP family

INTRODUCTION

Flowering time is a crucial and highly controlled mechanism in plants that has a direct impact on reproductive success and survival (Imaiizumi and Kay, 2006). Moreover, the floral transition in crop plants is directly related to crop yield. In order to survive imminent seasonal changes, plants have developed core signaling pathways that integrate day-length perception with developmental reprogramming. Signals are initiated outside of the shoot apical meristem (SAM) and a response cascade is triggered, ultimately reaching the SAM where cellular changes occur, leading to the formation of reproductive structures instead of leaves. The study of flowering time mutants in *Arabidopsis* has been instrumental in defining six signaling pathways: photoperiodic, autonomous, vernalization, gibberellin, ambient temperature and age-dependent control (Fornara et al., 2010). FLOWERING LOCUS T (FT)/TERMINAL FLOWER 1 (TFL1) are phosphatidylethanolamine-binding protein (PEBP) family members that are similar to mammalian PEBPs (Banfield et al., 1998; Ahn et al., 2006). In *Arabidopsis*, TFL1 is responsible for maintaining the inflorescence in an indeterminate state, with loss of TFL1 function resulting in the production of terminal flowers (Bradley et al., 1997). Although the *TFL1* gene sequence is highly similar to *FT*, *TFL1* acts antagonistically by delaying floral

commitment (Hanzawa et al., 2005; Ahn et al., 2006). Whereas the FT protein interacts with the FLOWERING LOCUS D (FD) bZIP transcription factor at the SAM to promote flowering (Abe et al., 2005; Wigge et al., 2005), TFL1 protein similarly binds to FD to repress downstream genes such as *APETALA 1* (*AP1*) and *LEAFY* (*LFY*) in the central zone of the meristem (Ratcliffe et al., 1999; Hanano and Goto, 2011). Opposite functions of *TFL1* and floral meristem genes reflect their specific expression in separate domains. *TFL1* is expressed in central cells of the SAM whereas the floral meristem genes are concentrated in the peripheral cells (Mandel et al., 1992; Kempin et al., 1995; Bradley et al., 1997). When floral meristem identity gene expression is reduced, flowers have shoot-like characteristics (Irish and Sussex, 1990; Schultz and Haughn, 1991, 1993; Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Shannon and Meekswagner, 1993). Upon floral transition, *TFL1* is up-regulated to maintain indeterminate inflorescence meristem and to counterbalance *FT* activity (Shannon and Meekswagner, 1991; Bradley et al., 1997; Ratcliffe et al., 1999; Conti and Bradley, 2007; Hanano and Goto, 2011; Jaeger et al., 2013).

Several structural and biochemical features of FT protein support the hypothesis that FT is a major component of the florigen that triggers floral evocation at the SAM (Taoka et al., 2013).

FT is expressed in phloem-specific tissues under floral inductive long-day conditions (Takada and Goto, 2003; An et al., 2004) and is able to traffic long distances intercellularly from companion cells to the SAM (Jaeger and Wigge, 2007; Mathieu et al., 2007). Characterization of *FT* homologs that induce flowering in diverse species suggests that *FT* is a highly conserved florigen (Kojima et al., 2002; Lifschitz et al., 2006; Corbesier et al., 2007; Lin et al., 2007; Tamaki et al., 2007; Lazakis et al., 2011; Meng et al., 2011). For example, the rice *FT* ortholog, Heading date3 (*Hd3a*), is a mobile signal synthesized in leaves that is capable of reaching the SAM (Kojima et al., 2002; Tamaki et al., 2007); Zea mays *CENTRORADIALIS8* (*ZCN8*) gene is expressed in the leaf and is able to induce flowering in *Arabidopsis ft* mutants when expressed under the control of a phloem-specific promoter (Lazakis et al., 2011; Meng et al., 2011); the tomato *SINGLE FLOWER TRUSS* (*SFT*) dependent graft-transmissible elements complement developmental defects in *sft* mutants and substitute long-day conditions in *Arabidopsis* (Lifschitz et al., 2006). In addition, the *Beta vulgaris* floral inducer *FT2* (*BvFT2*) is needed for normal flower initiation in sugar beet (Pin et al., 2010).

Plants typically have more than one *FT* related homolog, and domain analysis suggests that variation in specific regions of the *FT* protein are responsible for alternative functions, such as floral repression (Hanzawa et al., 2005; Ahn et al., 2006; Pin et al., 2010; Blackman et al., 2011; Harig et al., 2012). These observations, and comparison of *FT* function in various plant species suggests that the ancestor of *FT* is a floral repressor (Karlgren et al., 2011; Harig et al., 2012). Augmenting the acknowledged role of *FT* in flowering time, recent discoveries associate *FT* function with other meristem-related mechanisms (Bohlenius et al., 2006; Shalit et al., 2009; Navarro et al., 2011), consolidating *FT* as a key mobile signal that is not only related to floral transition but also related to diverse developmental events in plants.

Perennial plants depend on the maintenance of growth through several seasons, balancing nutritional status, biomass accumulation, and alternating vegetative and reproductive growth over the years. Flowering time genes are largely conserved between annual and perennial plants (Albani and Coupland, 2010), however perennial plants also must account for plant age to coordinate competence to flower. In the perennial *Arabis alpina*, sensitivity to vernalization depends on plant age; a condition which the PEBP member gene, *AaTFL1*, sets as a threshold to control the age-dependent pathway to flowering (Wang et al., 2011; Bergonzi et al., 2013). In perennial sugarcane, a qualitative short-day plant, little is known about the genetic control of floral induction. The transition to reproductive growth is undesirable in commercial sugarcane cultivars because the production of floral structures redirects carbon assimilates from stalks to inflorescences and results in loss of accumulated sucrose (Berding and Hurney, 2005). Therefore understanding the genetic underpinnings of flowering time in sugarcane provides a basis for the development of new strategies to improve agronomic traits such as increased biomass and sugar production in this important food and bioenergy crop. Here we isolate and characterize two novel sugarcane PEBP members and show that they alter flowering time and floral architecture in *Arabidopsis*.

MATERIALS AND METHODS

PLANT GROWTH CONDITIONS AND GENOTYPING

Sugarcane plants, variety RB72 454, were grown in a greenhouse under either 14-h long-day conditions at 27°C with 10-h nights at 22°C, or 12-h short-day inductive conditions representing field conditions with 20-20-20 NPK fertilizer supplemented with micronutrients added as required. *Arabidopsis* plants, ecotype *Columbia* (*Col-0*), were cultivated in Conviron growth chambers under conditions of 16-h days at 23°C with 8-h nights at 21°C, with a light intensity of 120 $\mu\text{molm}^{-2}\text{s}^{-1}$ and 60% humidity. *Arabidopsis* plants with segregating transgenes were genotyped using the Sigma REDExtract-N-Amp Plant PCR Kit (Sigma Biosciences) following manufacturer's instructions, and PCR was performed using kanamycin primers - KanrF: 5'-ATAC TTTCTGGCAGGAGCA-3' and KanrR: 5'-ACAAGCCGTTTT ACGTTTGG-3'.

ISOLATION AND CLONING OF *FT/TFL1* HOMOLOGS FROM SUGARCANE LEAVES

Mature and immature leaf tissues from sugarcane plants under inductive and non-inductive conditions were collected for total RNA extraction (TRIzol Reagent) and genomic DNA as previously described (Colasanti et al., 1998). For RNA assays, complementary cDNA was synthesized using the qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's instructions. Sequences were amplified using specific primers designed at the UTR region of the genes: *ScTFL1F*: 5'-GTCCGATTAGCTTGCTGCAT-3'; *ScTFL1R*: 5'-GGCCATG CTCATAACTTGG-3'; *ScFT1F*: 5'-ATATGGCTAATGACTCCC TGACG-3'; *ScFT1R*: 5'-CTGGACATGAGGGTAGGTAAAT-3'. Genomic and complementary *ScTFL1/ScFT1* sequences were cloned to the CloneJET PCR Cloning Gene (Thermo Scientific) and sequenced.

PHYLOGENETIC ANALYSIS OF THE *ScTFL1* AND *ScFT1* CANDIDATES WITH ORTHOLOGS OF RELATED SPECIES

Deduced amino acid sequences of sugarcane *ScTFL1/ScFT1* compared to homologs from other species were aligned with translated sequences for *Arabidopsis TFL1* and *FT*; *ZCN1*, *ZCN2*, and *ZCN8* (maize); *RCN1* and *Hd3a* (rice); *NtFT1* to *NtFT4* (tobacco); and *BvFT1* and *BvFT2* (sugar beet), using the software BioEdit 7.1.3.0 (Hall, 1999). Phylogenetic trees were constructed by MEGA software, version 4.0 (Tamura et al., 2007), with the neighbor-joining comparison model (Saitou and Nei, 1987), *p*-distance method and pair-wise deletion. Bootstrap values from 1000 replicates were used to assess the robustness of the trees (Felsenstein, 1985). Phylogenetic analysis that included the deduced amino acid sequences of incomplete sugarcane genes was corrected by deleting positions with gaps from the alignment. Gene structure information for homologs was accessed at the Phytozome 9.1 genome database available online (www.phytozome.net).

CONSTRUCTION OF OVEREXPRESSION VECTOR AND ARABIDOPSIS TRANSFORMATION

Candidate genes for *TFL1* and *FT* amplified from sugarcane leaf RNA were cloned into Gateway entry vector pDONR-221 using

the BP recombination reaction and the subsequent products were recombined with the destination vector pK2GW7 by a LR clonase originating from the expression vector 35S::*ScTFL1* and 35S::*ScFT1*. Gateway sites were added to the sequencing primers for cloning purposes as follows:

*ScTFL1*gatF: 5'-GGGGACAAGTTGTACAAAAAGCAGGC
TGTCCGATTAGCTTG-CTGCAT-3' and *ScTFL1*gatR: 5'-GGGG
ACCACTTGTACAAGAAAGCTGGTGGCCATG-CTCATAAC
TTTGG-3' and *ScFT1*gatF: 5'-GGGGACAAGTTGTACAAAA
AAGCAGGC-TATATGGCTAATGACTCCCTGACG-3' and *ScFT1*gatR: 5'-GGGGACCACTTGTACAA-GAAAGCTGGTCTGG
ACATGAGGGTAGGTAAAT-3'. *Agrobacterium tumefaciens* strain GV3101::pMP90 containing the over-expression constructs were introduced into Arabidopsis plants by floral dip (Clough and Bent, 1998). *Agrobacterium* containing *ScTFL1* and *ScFT1* over-expression constructs were introduced to the Columbia (*Col-0*) ecotype. Fifty T1 individuals overexpressing *ScTFL1* and 18 T1 lines carrying the 35S::*ScFT1* construct germinated on MS plates supplemented with 50 µg/ml kanamycin and resistant seedlings were transplanted to soil to obtain T2 seeds. Segregation analysis showed that 20 and 14 T2 lines, respectively, had single insertions of the 35S::*ScTFL1* and 35S::*ScFT1* transgenes (X2 test, $p < 0.05$). All individuals with the transgene showed the late flowering phenotype whereas segregants without the transgene flowered normally. Ten individuals from four independent single insertion lines each that were homozygous for the transgene were selected for phenotypic analyses of *ScTFL1* and *ScFT1* overexpression. Flowering time was scored by counting the number of rosette leaves at the appearance of the first floral bud in primary inflorescences.

GENE EXPRESSION ANALYSIS USING SEMI-QUANTITATIVE AND QUANTITATIVE RT-PCR

Gene and transgene expression analysis was carried out using semi-quantitative RT-PCR and real time RT-PCR. For *ScTFL1* and *ScFT1* expression analysis, RNA was extracted as described above from sugarcane mature leaves, immature leaves and the SAM enriched region. Complementary DNA (cDNA) was prepared using qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's instructions. *ScTFL1* primers were designed to assess transgene expression in the *ScTFL1* transgenic lines: *ScTFL1*qRTF: 5'- GACTTGCCTGCTTC
TCACA -3'; *ScTFL1*qRTR: 5'- AGGCATCTGTTGTCAGGT -3'. Expression of the *ScFT1* gene in transgenic plants was assessed by qRT-PCR analysis with the primers *ScFT1*qPCR-F (GGC
TAATGACTCCCTGACGA) and *ScFT1*qPCR-R (CCATCCCTT
CAAACACTGGT). PerfeCTa SYBR Green SuperMix (Quanta Biosciences) and an Applied Biosystems 7300 Real Time PCR instrument were used, and data was analyzed by the Pfaffl method with efficiency correction to obtain fold difference in expression (Pfaffl, 2001). Three biological replicates of three technical replicates were analyzed. *Actin8* (*ActinrF*: 5'-GCCGATGCT
GATGACATTCA-3' and *ActinrR*: 5'-CTCCAGCGAATCCAGCC
TTA-3') and *ScGAPDH* (*ScGAPDH*F: 5'-CACGGCCACTGG
AAGCA-3' and *ScGAPDHR*: 5'-TCCTCAGGGTTCCTGATGCC-3') were used for normalization and the calibrator was the average ΔCt for the independent line with lower expression level.

Statistical significance is reported by the Student's *t-test* with $P < 0.05$.

SCANNING ELECTRON MICROSCOPY (SEM)

At least three inflorescences per plant were harvested from *ScTFL1* over-expressed plants and images were captured with a Hitachi Tabletop TM-1000 Scanning Electron Microscope. Dimension bars were added using the ImageJ software (Abràmoff et al., 2004).

RESULTS

ISOLATION OF A *TFL1* HOMOLOG FROM *SACCHARUM* SPP. AND EXPRESSION ANALYSIS IN DIFFERENT TISSUES

Candidates for *FT/TFL1* gene family members were identified in the sugarcane EST database, SUCEST (Vettore et al., 2001; Coelho et al., 2013). A complete sequence for a *TFL1-like* subfamily candidate was identified and termed *ScTFL1*. The deduced *ScTFL1* protein had highest similarity to maize genes ZCN1 and ZCN2 proteins (93 and 84% amino acid identity, respectively), 92% identity to rice RCN1, and 70% identity to *Arabidopsis TFL1* (Figure 1A). *ScTFL1* sequence is more similar to the rice and maize homologs compared to *Arabidopsis* (Figure 1D). The founding member of this family, *Arabidopsis TFL1*, is highly conserved between species, and homologs have been reported in several different species (Hecht et al., 2005; Danilevskaya et al., 2010; Taylor et al., 2010; Mauro-Herrera et al., 2013).

Plant PEBP proteins are general regulators of signaling complexes, as shown by the tomato SELF PRUNNING (SFP) protein, a *TFL1* homolog that acts through interaction with different proteins (Pnueli et al., 2001). The PEBP family consists of three gene subfamilies named *MOTHER-of-FT* (*MFT*)-like, *TFL1*-like and *FT*-like (Chardon and Damerval, 2005). In sugarcane, eight candidate PEBP gene family members were identified by *in silico* analysis in the sugarcane database and found to belong to several different subfamilies; one *MFT-like* gene, one *TFL1*-like and six *FT*-like candidates (Coelho et al., 2013). Six members of the *TFL1*-like subfamily were reported in maize, ZCN1 to ZCN6 (Danilevskaya et al., 2010), and four members were identified in rice: Ocen1 to Ocen4 (Nakagawa et al., 2002). Completion of the sugarcane genome sequence likely will reveal more PEBP members in this species.

Comparing the deduced *ScTFL1* amino acid sequence to known *TFL1*-like homologs from other plant species shows that they all share a histidine residue at position 89 (H89) (Figure 1A); in *Arabidopsis* this residue is at a key position that determines whether *TFL1* or *FT* act as a floral repressor or promoter, respectively (Hanzawa et al., 2005). The gene structure of *ScTFL1* is similar to the related *TFL1* orthologs, consisting of three introns and four exons of similar sizes. (Figure 1B). The fourth exon contains a specific region, segment B, that is critical for *FT* to function as a floral promoter or *TFL1* as a floral repressor (Ahn et al., 2006). Residues Gln140, Asp144 and Glu141 (found in *FT*, *TFL1*, and *BFT*, respectively) of segment B may have an important function in determining *FT*-like or *TFL1*-like activity (Ahn et al., 2006; Yoo et al., 2010). This segment forms an external loop that varies in *TFL1* but not in *FT*, and it seems that the opposite activity in flower induction is derived from hydrogen bond formation near

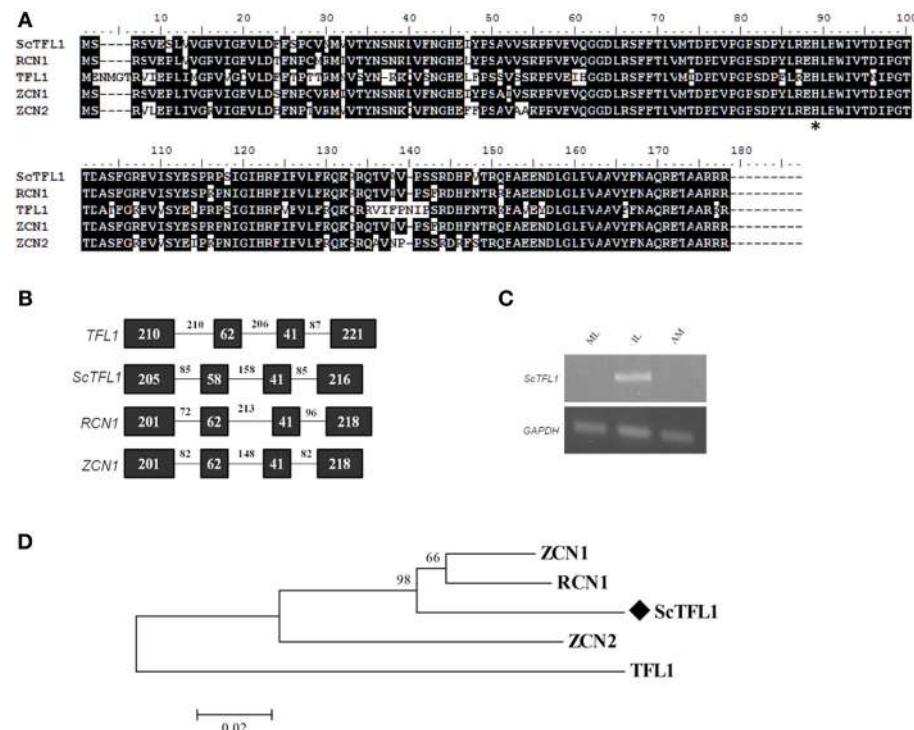


FIGURE 1 | Sequence conservation among TFL-like genes. (A) Alignment of the *ScTFL1* candidate with homologs from different species: *Arabidopsis TFL1*; rice *RCN1*; and maize *ZCN1*. Asterisk (*) highlights the amino acid residue conserved in all TFL1 homologs. **(B)** *TFL1* gene structure conservation among *TFL1* homologs, consisting of four exons and three introns. Boxes represent exons and lines, introns. Numbers indicate size of each exon and intron. **(C)** Expression pattern of *ScTFL1* in different tissues by semi-quantitative PCR; sugarcane *GAPDH* endogenous control was used as control. IL: apex-surrounding immature leaves; ML, mature leaves; AM, apical

meristem. **(D)** Evolutionary relationship of *TFL1* homologs. Amino acid sequences from different species were aligned using ClustalW, the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap values from 1000 replications were used to assess the robustness of the trees. Sugarcane *TFL1* candidate gene is highlighted by a diamond symbol (◆) and *TFL1* homologs from related species are deposited at the Genbank database. Accession numbers: *ZCN1* (ABX11003.1), *ZCN2* (ABX11004.1), *RCN1* (ABA95827.1), and *TFL1* (AED90661.1), *ScTFL1* (KJ496328).

the binding pocket in *TFL1* but not in *FT*, suggesting that this segment is crucial for the co-activation of specific, yet-to-be identified *FT/TFL1* interactors (Ahn et al., 2006; Pin et al., 2010; Taoka et al., 2011; Harig et al., 2012; Taoka et al., 2013). Consistent with this, *FT* has a tyrosine residue at position 85 (Y85), a key difference that specifies *FT* function as a floral promoter in *Arabidopsis* (Hanzawa et al., 2005), although in some species it has been reported that the *FT*-like containing the Y85 residue may act as a floral repressor if there is variation in segment B.

We determined the expression pattern of *ScTFL1* at the vegetative apical meristem region, mature leaves and the immature leaves surrounding the meristem in 7-month old sugarcane plants. *ScTFL1* is expressed in the young leaves that enfold the meristem, however no transcript was detected in the shoot apical region. (Figure 1C). In *Arabidopsis*, *TFL1* is expressed in young axillary meristems and is later confined to the central core of the meristem (Conti and Bradley, 2007). The *ScTFL1* expression pattern suggests that this gene acts in regions adjacent to the meristem in vegetative sugarcane plants. Similarly, in maize, which is an annual plant, *ZCN1* and *ZCN2* are expressed in both vegetative and reproductive phases, with *ZCN1* mRNA detected in vascular bundles of leaf primordia and *ZCN2* in leaf axils of shoot apices (Danilevskaya et al., 2010).

ECTOPIC EXPRESSION OF *ScTFL1* ALTERS FLOWERING TIME AND MAINTAINS INDETERMINATE FATE OF INFLORESCENCE MERISTEMS IN TRANSGENIC ARABIDOPSIS PLANTS

To understand the role played by this sugarcane *TFL1* homolog, we examined transgenic *Arabidopsis* plants over-expressing the *ScTFL1* driven by the constitutive 35S CaMV promoter. More than 40 transgenic lines were isolated and found to flower later than wild-type; four independent T2 lines homozygous for the transgene (*ScTFL1-5*; *ScTFL1-6*; *ScTFL1-11*, and *ScTFL1-41*) were selected for further analysis. The prolonged vegetative phase was manifested as an increase in the number of rosette leaves in all transgenic lines, ranging from 15.4 to 17.7 leaves on average, compared to the 11.4 leaves in Col-0 plants (Table 1). All four lines had ectopic expression of the *ScTFL1* transgene, however the differences in flowering time did not correlate with the level of exogenous transcript (Supplemental Figure 1A). Otherwise there were no morphologic differences in vegetative structures, such as the serrated leaves that were reported from over-expression of the *BROTHER of FT and TFL1* (*BFT*) gene in *Arabidopsis* (Yoo et al., 2010).

With regard to reproductive development, ectopic expression of *ScTFL1* altered flowering time (Table 1; Supplemental Figure 2) but also affected the formation of the inflorescence

structures, as typified in the most severe line, ScTFL1-41 (**Figure 2A**). The other late flowering lines examined, ScTFL1-5, -6 and -11, showed similar defects in reproductive architecture, although to a lesser extent than the most severe line ScTFL1-41 which was also exhibited the latest flowering. In addition ScTFL1-41 plants had a highly branched phenotype (**Figures 2B,C**), shoot-like inflorescences, aerial rosettes, abnormal flower formation (**Figure 3**), and prolonged life cycle (>64 days). A similar phenotype was reported in Arabidopsis over-expressing *TFL1* and *TFL1-like BFT*, and their respective rice and maize homologs; i.e., in developmental phases were delayed and similar effects on plant architecture were observed (Ratcliffe et al., 1998, 1999; Jensen et al., 2001; Nakagawa et al., 2002; Danilevskaya et al., 2010; Yoo et al., 2010).

ScFT1 IS A PUTATIVE FT ORTHOLOG THAT DELAYS FLOWERING IN ARABIDOPSIS

In parallel with the characterization of *ScTFL1*, we isolated *ScFT1* from mature sugarcane plants and compared the sequence and expression pattern to Arabidopsis *FT* and other homologs. Comparison of the *ScFT1* deduced protein with *FT* homologs from different species (**Figure 4A**) showed it to be 59% identical to Arabidopsis *FT*, 59% to rice *Hd3a*; 57% to maize *ZCN8*; 62% to sugar beet *BvFT2* and 61% to tobacco *NtFT4*. Sugar beet and tobacco candidates that act antagonistically to flowering had less similarity to *ScFT1*: *NtFT1*, -2, -3 were 57, 54, and 54%, respectively, and the sugar beet *BvFT1*, 59%.

Table 1 | Flowering characteristics of four 35S::*ScTFL1* independent transgenic lines.

| Plant genotype | Days to flowering | Number of leaves | Number of plants |
|-----------------|-------------------|--------------------------|------------------|
| Col-0 wild-type | 32 | 11.4 ± 0.54 | 5 |
| ScTFL1-5 | 41 | 15.4 ± 1.35 ^a | 10 |
| ScTFL1-6 | 41 | 14.3 ± 1.34 ^a | 10 |
| ScTFL1-11 | 41 | 15.2 ± 1.73 ^a | 10 |
| ScTFL1-41 | 47 | 17.7 ± 1.60 ^a | 10 |

^aIndicates statistically different from wild-type with $p > 0.05$ by student *t*-test.

Phylogenetic analysis of FT-like proteins showed that the FT-like floral repressors from tobacco clade together and the floral promoter *NtFT4* clades with the FT-like floral promoters, *FT* and *Hd3a* (**Figure 4D**). As sugarcane and maize are more closely related to each other than to other species examined it is not unexpected that *ScFT1* clades with maize *ZCN8*, but not to other FT-like proteins, such as *FT* and *Hd3a*. Despite the finding that *ZCN8* does not clade with FT-like floral promoters, the strong association of *ZCN8* with a maize flowering time QTL and its ability to complement an Arabidopsis *ft* mutant suggests that it acts as a floral promoter in maize (Lazakis et al., 2011; Meng et al., 2011). Similar to all PEBP family members, the *ScFT1* gene consists of four exons and three introns, with similar exon sizes but largely varying the number of nucleotides in the introns (**Figure 4B**).

Expression analysis in sugarcane showed that *ScFT1* transcript is present in mature leaves of vegetative phase plants, although it was expressed in immature leaves and SAM of the same plants. Interestingly, *ScFT1* transcript was detected in mature leaves of flowering plants (**Figure 4C**), suggesting a possible role in post-floral transition plants.

TRANSGENIC PLANTS OVEREXPRESSING *ScFT1* DELAYED FLOWERING AND CAUSED ABNORMAL SILIQUE DEVELOPMENT

Sugarcane *ScFT1* was over-expressed in Arabidopsis to test whether this *FT-like* candidate is involved in controlling flowering time. Four independent transgenic lines were selected for flowering time analysis and shown to over-express the transgene (**Supplemental Figure 1B**). Unexpectedly, in all cases *ScFT1* over-expression resulted in late flowering plants, with an average range of 16.1–24.5 rosette leaves, compared to the 11.4 rosette leaves of Col-0 wild-type (**Table 2**). The most severe effect on flowering time was observed in *ScFT1-3*, which had a significantly higher transgene expression than the other three lines (**Supplemental Figure 1B**).

In addition to later flowering, all *ScFT1* over-expressing lines often exhibited defects in floral organ formation. Unlike *ScTFL1* lines, where the latest flowering phenotype was associated with defects in reproductive structures, the *ScFT1* over-expressing line with the most severe reproductive abnormalities was *ScFT1-1*, which consistently had a high number of sterile flowers and

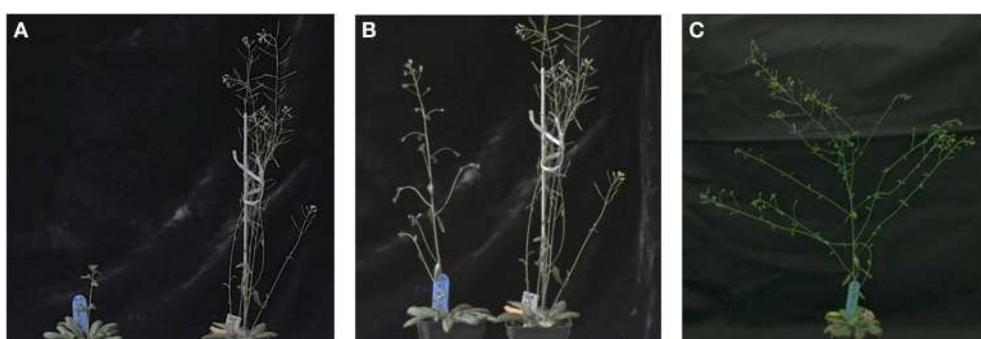
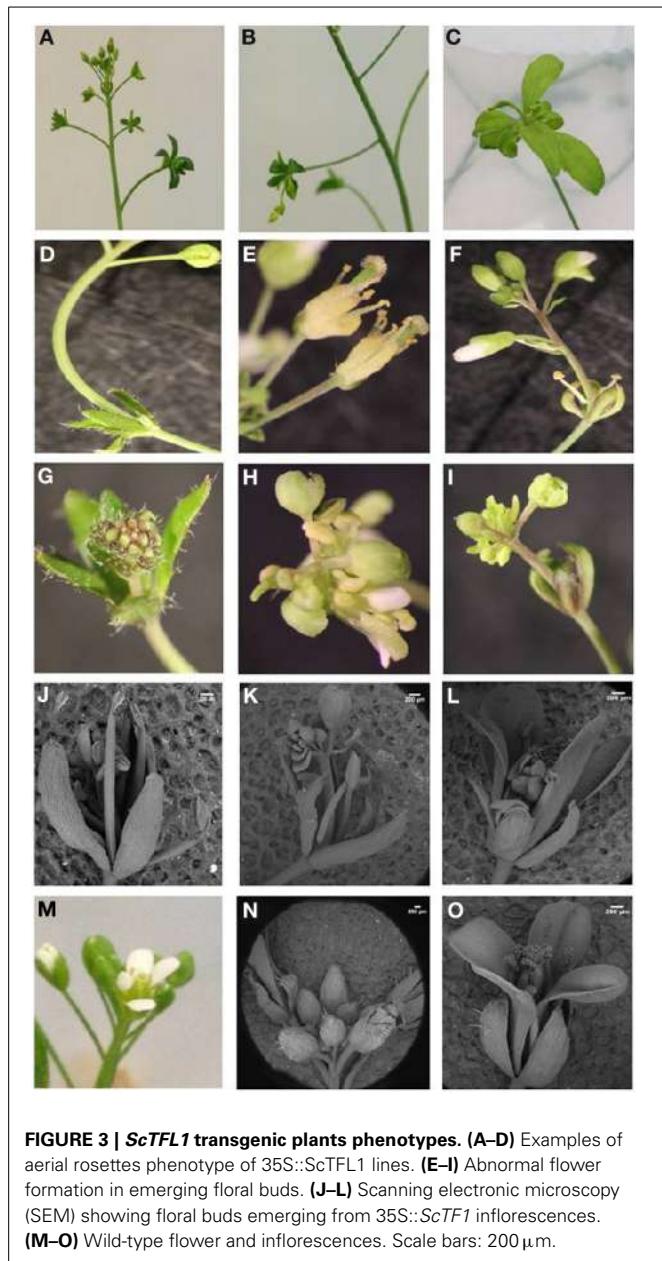


FIGURE 2 | Ectopic *ScTFL1* expression affects inflorescence architecture in transgenic Arabidopsis. (A) Growth of 35S::*ScTFL1-41* transgenic plants (left) and Col-0 (right) under long-day conditions after 50

days; and **(B)** 55 days; and **(C)** 64 days of germination, at this point Col-0 wild-type plants have completed the life cycle. All plants are in the Col-0 background.



formed abnormally shorter siliques (**Figure 5A**). Furthermore, most siliques had abnormal development, leading to poor seed set and mostly sterile plants. In *ScFT1* transgenic lines, open flowers did not self-fertilize and siliques did not develop from fertilized carpels, which may explain the shorter siliques (**Figure 5B**).

These results suggest that *ScFT1* may be involved in meristem activities that control flowering time and production of fertile organs, although further analysis is required to understand the effects of *ScFT1* overexpression in meristem development. Other studies have reported that loss of *FT-like* function caused meristem-associated abnormalities (Bohlenius et al., 2006; Shalit et al., 2009; Krieger et al., 2010; Danilevskaya et al., 2011; Navarro et al., 2011).

YET TO BE CHARACTERIZED ScFT-LIKE GENES MAY BE INVOLVED IN SUGARCANE FLORAL INDUCTION

ScFT1 is the only full-length *FT-like* candidate we were able to isolate from the sugarcane genome. Four other incomplete sequences were identified in the sugarcane EST database (SUCEST), which we designate *ScFT2*, *ScFT3*, *ScFT4*, and *ScFT5*. Of the candidates that were analyzed, *ScFT2* is most closely related to *FT-like* candidates maize *ZCN8* and *ScFT1*. The *ScFT3* and *ScFT4* putative homologs clade with all floral promoter *FT-like* genes, *Hd3a*, *FT*, and *BvFT1* (**Figure 6A**), indicating that we cannot rule out the possibility that one or both of them may act as florigen in sugarcane. Functional characterization of these candidates will enlighten this hypothesis. Phylogenetic analysis indicates that *ScFT3* and *ScFT4* clade with floral promoters, given the high degree of similarity of segment B compared to other FT floral promoting proteins (**Figure 6B**).

DISCUSSION

ScTFL1 MAINTAINS MERISTEM INDETERMINACY IN ARABIDOPSIS, SUGGESTING A SIMILAR ROLE IN SUGARCANE

Although extreme late flowering is a negative agronomic trait in many crops, it is of great advantage in commercial sugarcane plants, where a non-flowering phenotype is a highly desirable trait that is the objective of many sugarcane-breeding programs (Berding and Hurney, 2005; Van Heerden et al., 2010). Maintaining sugarcane plants in a vegetative state prevents the loss of sugar accumulation in the stalks that would result from precocious flowering, especially in the tropics where day-length is inductive for floral transition throughout the year.

As a first step in elucidating the molecular mechanisms that control flowering in sugarcane we isolated *FT/TFL1* gene family homologs of key flowering time genes first characterized in Arabidopsis. Two of these genes, *ScTFL1* and *ScFT1*, were analyzed for their role in flowering by ectopic expression in Arabidopsis. We have validated this technique previously by showing that over-expression of another monocot flowering gene, the maize *FT-like* gene *ZCN8*, had a dramatic effect on Arabidopsis flowering (Lazakis et al., 2011). Similarly ectopic expression of *FT/TFL* related genes have been demonstrated for diverse plant species (Jensen et al., 2001; Nakagawa et al., 2002; Mimida et al., 2009; Pin et al., 2010; Karlsgren et al., 2011; Klintenaas et al., 2012).

Late flowering was observed in Arabidopsis plants over-expressing *ScTFL1*. This suggests that *ScTFL1* acts by extending the duration of growth phases and maintenance of the inflorescence meristem in sugarcane. The latest flowering *ScTFL1* overexpressing plants also had abnormal floral organ structures, which may be due to an imbalance between *TFL1* and *AP1* expression. This leads to floral reversion to vegetative structures, triggering the appearance of floral buds inside the aerial rosettes, resulting in enhanced indeterminate growth. In *wild-type* plants, *AP1* down-regulates *TFL1* in floral meristems and, in turn, *TFL1* maintains indeterminate growth of the vegetative center (Ratcliffe et al., 1999). Although complete inhibition of flowering has not been observed in any single mutants in Arabidopsis, this is not the case for double mutants. Floral transition is never completed in *pernywise* and *pound-foolish* (*pnw pnf*) double mutants (Smith et al.,

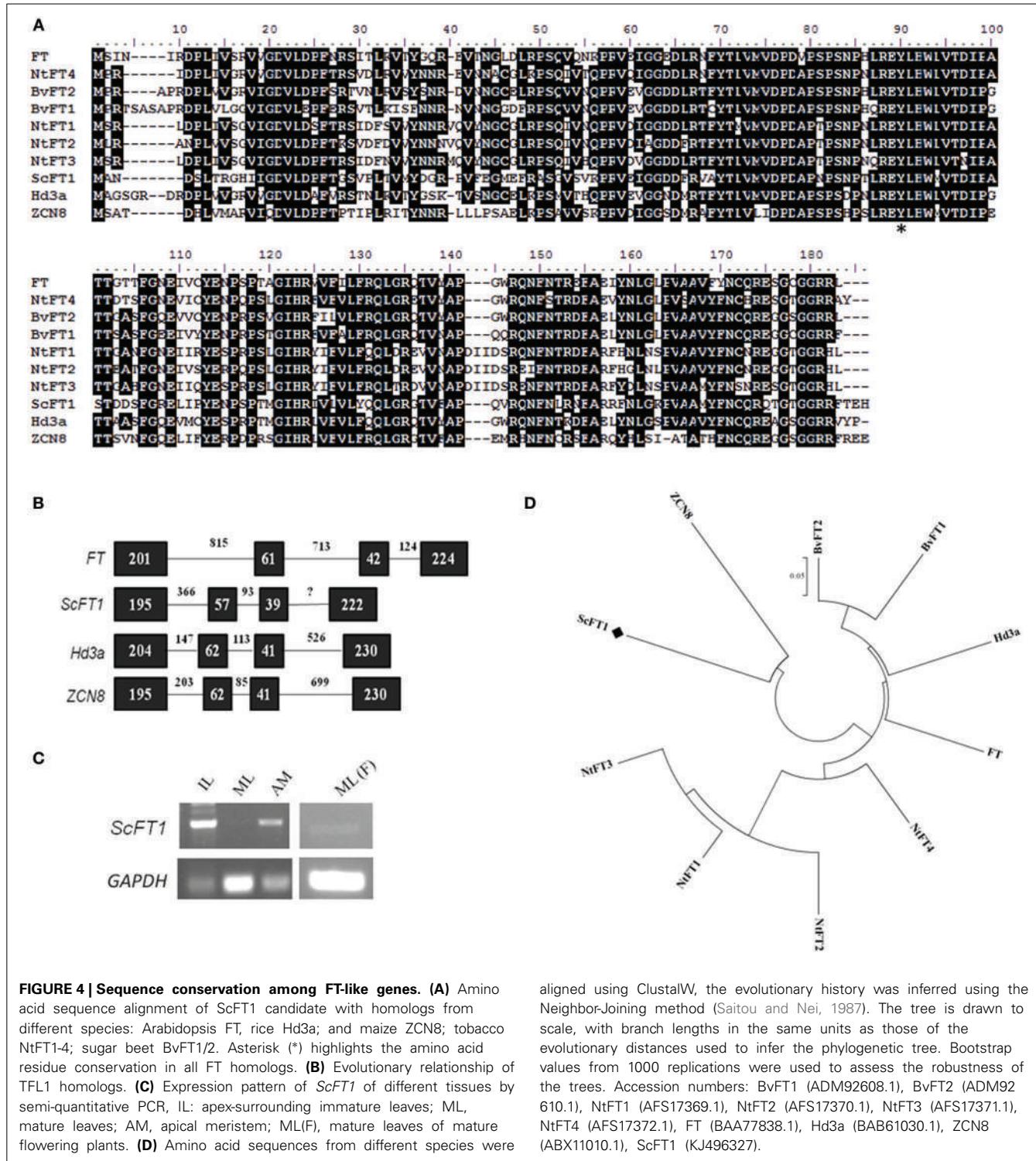


FIGURE 4 | Sequence conservation among FT-like genes. (A) Amino acid sequence alignment of ScFT1 candidate with homologs from different species: Arabidopsis FT, rice Hd3a; and maize ZCN8; tobacco NtFT1-4; sugar beet BvFT1/2. Asterisk (*) highlights the amino acid residue conservation in all FT homologs. **(B)** Evolutionary relationship of TFL1 homologs. **(C)** Expression pattern of ScFT1 of different tissues by semi-quantitative PCR, IL: apex-surrounding immature leaves; ML, mature leaves; AM, apical meristem; ML(F), mature leaves of mature flowering plants. **(D)** Amino acid sequences from different species were aligned using ClustalW, the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bootstrap values from 1000 replications were used to assess the robustness of the trees. Accession numbers: BvFT1 (ADM92608.1), BvFT2 (ADM92610.1), NtFT1 (AFS17369.1), NtFT2 (AFS17370.1), NtFT3 (AFS17371.1), NtFT4 (AFS17372.1), FT (BAA77838.1), Hd3a (BAB61030.1), ZCN8 (ABX11010.1), ScFT1 (KJ496327).

2004). Loss of both of these duplicate BELL homeobox genes results in ectopic *TFL1* expression at high levels in the vasculature, the same site of *FT* expression. This indicates that ectopic overexpression of *TFL1* can result in a non-flowering phenotype.

The altered architecture of the ScTFL1-41 is similar to that observed in the Arabidopsis late-flowering ecotype, Sy-0, which

also has aerial rosettes formation in flowering stems (Grbic and Bleeker, 1996). Aerial rosette formation is often related to loss of floral meristem identity genes responsible for signal transduction of the *AP1* gene, an integrator of all flowering pathways that determines floral organ formation. By the time *AP1* is expressed, floral determination is initiated and plants continue to flower

independent of environmental signals (Hempel et al., 1997). Grbic and Bleeker (1996) suggested that this phenotype is a result of the interaction of two main dominant genes, *AERIAL ROSETTE* (*ART*) and *ENHANCER OF AERIAL ROSETTE* (*EAR*). Mutant phenotypes of *aerial rosette 1* (*art1*) are a consequence of

Table 2 | Flowering characteristics of four 35S::ScFT1 independent transgenic lines.

| Plant genotype | Days to flowering | Number of leaves | Number of plants |
|-----------------|-------------------|--------------------------|------------------|
| Col-0 wild-type | 32 | 11.4 ± 0.54 | 5 |
| ScFT1-1 | 37 | 16.1 ± 0.87 ^a | 10 |
| ScFT1-2 | 37 | 17.3 ± 2.16 ^a | 10 |
| ScFT1-3 | 46 | 24.5 ± 1.84 ^a | 9 |
| ScFT1-4 | 37 | 19.6 ± 1.41 ^a | 10 |

^aIndicates statistically different from wild-type with $p > 0.05$ by student *t*-test.

a delay from the vegetative (V) to reproductive (R) phase transitions, resulting in the formation of a new type of metamer consisting of V1 → V2* → R* → R, in which aerial rosettes are formed by the V2* stage (Poduska et al., 2003). Similar to this, 35S::TFL1 plants also have a prolonged vegetative phase and produce aerial rosettes (Ratcliffe et al., 1998, 1999). Aerial rosette formation also was reported when another *BELL* gene, *ATH1*, was over-expressed in Arabidopsis (Proveniers et al., 2007).

Increased axillary branching of the ScTFL1 transgenic plants is similar to the effects observed when other *TFL1* homologs are over-expressed in Arabidopsis. It was suggested that this phenotype may be a consequence of interaction of TFL1 with hormones, since plant hormones such as auxin, cytokinin and strigolactone play a role in the branching and outgrowth of plants (McSteen, 2009; Danilevskaya et al., 2010). The TFL1 protein complex was reported previously, and the external loop seems to be the site for co-repressors/co-activators to bind and trigger developmental responses. Nevertheless, these co-repressors/co-activators have



FIGURE 5 | Ectopic expression of ScFT1-1 affects flowering time and siliques development. (A) Comparison of development timing of ScFT1-1 (right) with Col-0 wild-type plant (left). (B) Close-up at the siliques from Col-0 (left), ScFT1-1 flowers (middle), and abnormal siliques (right).

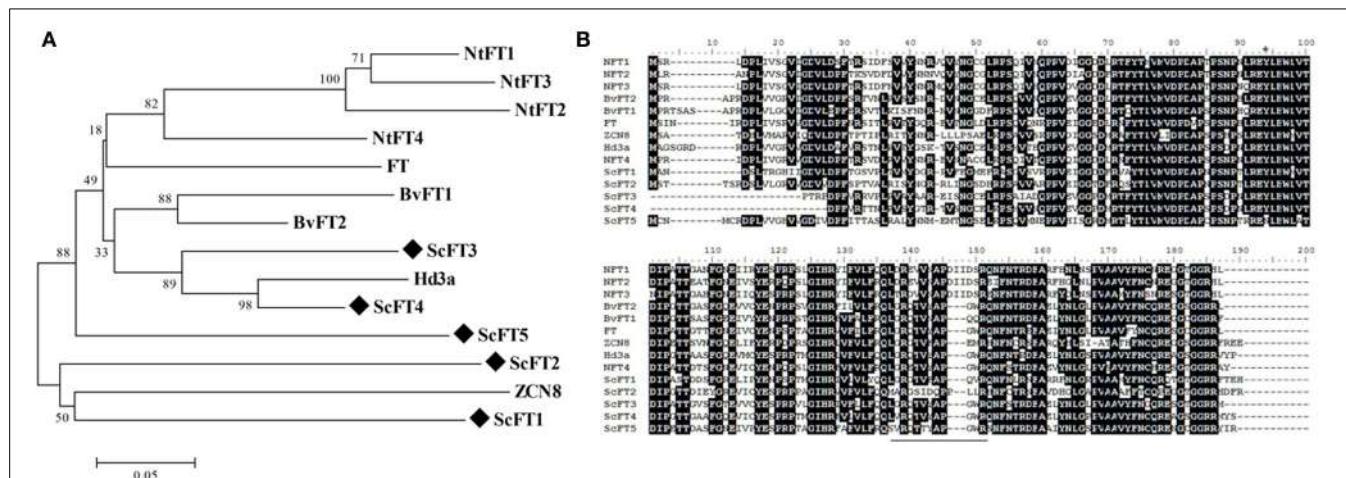


FIGURE 6 | Sequence analysis of ScFT protein candidates with other FT homologs. (A) Amino acid sequences from different species were aligned to five ScFT-like candidates using ClustalW. (B) Phylogenetic tree of FT homologs. Bootstrap values from 1000 replications were used to assess the robustness of the tree. Sugarcane ScFT-like candidate genes are highlighted

by a diamond symbol (♦) and FT homolog from related species are deposited at the Genbank database. A black line highlights segment (B). Accession numbers: BvFT1 (ADM92608.1), BvFT2 (ADM92610.1), NtFT1 (AFS17369.1), NtFT2 (AFS17370.1), NtFT3 (AFS17371.1), NtFT4 (AFS17372.1), FT (BAA77838.1), Hd3a (BAB61030.1), ZCN8 (ABX11010.1).

not yet been identified (Taoka et al., 2013), raising the possibility that plant hormone activity could be connected to the mechanisms by which *ScTFL1* results in the observed phenotypes.

Mechanisms of *TFL1* function are less clear than that of *FT*; moreover *TFL1* function may vary among annual and perennial plants. *TFL1* was reported to act in an age-dependent flowering pathway in the perennial *Arabis alpina*. In these plants, *AaTFL1* is responsible for the maintenance of vegetative growth of young plants, even under inductive conditions, preventing all axillary meristems from becoming determined. As the shoot ages, *AaTFL1* sets an increasing flowering threshold and the plant is able to develop perennial traits (Wang et al., 2011). In perennial ryegrass, the *TFL1* homolog *LpTFL1* is up-regulated in the apex once the temperature and day-length increases, allowing for lateral branching, and consequently, the promotion of tillering (Jensen et al., 2001). It is possible that *ScTFL1* acts in a similar manner in perennial sugarcane, perhaps explaining the expression of this gene in leaves surrounding the peripheral regions of the meristem of vegetatively growing plants. Further studies of the expression pattern of *ScTFL1* at the shoot apex and surrounding developing leaves of mature flowering plants may provide insights about this possibility.

***ScFT1* MAY CONTROL FLOWERING TIME AND INFLORESCENCE FORMATION IN SUGARCANE**

Evolutionary analysis of the PEBP family suggests that *FT*-like and *TFL1*-like subfamilies arose from a common *TFL1*-like ancestor, and that the *FT*-like floral promoter evolved within the angiosperm clade (Karlgren et al., 2011; Klinten et al., 2012). Therefore it is possible that floral repressor activity of *FT*-like genes persists among angiosperms, as has been reported for tobacco (Harig et al., 2012) and sugar beet (Pin et al., 2010).

The present work suggests that *ScFT1* functions as a floral repressor in sugarcane. Strikingly, expression of *ScFT1* varies under non-inductive and inductive conditions. Under non-inductive long-day conditions, *ScFT1* is expressed in both immature leaves and the apical meristem region, but is not detected in mature leaves. Under inductive short day conditions, however, *ScFT1* is expressed in mature leaves, which are the source of the florigen signal. Together with the late flowering phenotype observed in the overexpressing *Arabidopsis* lines, this could indicate that *ScFT1* is associated with an anti-florigen signal that originates in mature leaves under floral inductive conditions to counter-balance the florigen signal.

The effect of *ScFT1* overexpression on siliques development is similar to that observed in *Arabidopsis* *dyyt1* mutants; *DYSFUNCTIONAL TAPETUM1* (*DYT1*) is involved in tapetum differentiation and function and, without functional *DYT1*, normal anther development is interrupted, generating plants with very small siliques (Zhang et al., 2006). *BEL1* and *SHORT INTEGUMENT* (*SIN1*) control ovule development in *Arabidopsis* as *bel1* mutants transform ovule integuments into carpels due to ectopic expression of *AGAMOUS* (*AG*) in these tissues (Ray et al., 1994). Alterations of *bel1* mutants include increased axillary buds, delayed senescence and short abnormal

siliques formation, similar to what we observe in *ScFT1* plants (Robinson-Beers et al., 1992). Loss of *SIN1* also affects flowering time, resulting in an increased number of rosette leaves and cophlorescence branches. *SIN1* is epistatic to *TFL1* and may act in an independent pathway to suppress, at least in part, the *tfl1* phenotype (Ray et al., 1996).

All *FT*-like proteins involved in floral promotion have a conserved segment B region encoded in the fourth exon that is essential for these homologs to act as florigens in diverse plant species (Ahn et al., 2006; Pin et al., 2010; Harig et al., 2012). Segment B of *ScFT1* varies in three amino acid residues compared to the *FT* and *Hd3a* floral proteins. In sugar beet variation of three amino acids in segment B of two *FT*-like proteins is sufficient for them to act antagonistically (Pin et al., 2010). Comparison of partial sequences of several *ScFT*-like genes from the sugarcane EST database (SUCEST) indicates that *ScFT3* and *ScFT4* candidates may be involved in the floral promotion, considering the sequence conservation and phylogenetic relationship to *FT*-like homolog subfamilies. Full-length transcripts need to be characterized to evaluate the effect of sequence plasticity and divergence of functions of sugarcane *FT*-like genes.

Recent discoveries suggest that *FT*-like proteins act not only as floral repressors but also in diverse developmental events, such as potato tuberization (Navarro et al., 2011), seasonal control of growth cessation in poplar trees (Bohlenius et al., 2006), termination of meristem growth and fruit yield in tomato (Shalit et al., 2009; Krieger et al., 2010), plant architecture in maize (Danilevskaya et al., 2011), and stomatal control in *Arabidopsis* (Kinoshita et al., 2011). Together these different activities raise a fundamental question about whether *FT*-like proteins function as versatile mobile signals orchestrating diverse processes in plant development rather than solely acting as a florigen (Taoka et al., 2013).

AUTHOR CONTRIBUTIONS

Carla P. Coelho and Joseph Colasanti designed the experiments and organized the manuscript. Carla P. Coelho and Mark A. A. Minow performed the experiments. Antonio Chalfun-Júnior and Joseph Colasanti edited the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00221/abstract>

Supplemental Figure 1 | Expression analysis of the transgenes in Arabidopsis independent lines. (A) *ScTFL1* expression relative to *Arabidopsis ACTIN8* expression in the lines ScTFL1-5, ScTFL1-6, ScTFL1-11, and ScTFL1-41. (B) *ScFT1* expression relative to *ACTIN8* expression in the lines ScFT1-1, ScFT1-2, ScFT3, and ScFT4. Error bars denote relative quantity maximum and minimum values from triplicate biological samples, with each sample a pool of five plantlets.

Supplemental Figure 2 | Ectopic *ScTFL1* expression affects flowering in four independent lines of transgenic *Arabidopsis*, under long-day conditions after 43 days. (A) 35S::ScTFL1-5; (B) 35S::ScTFL1-6; (C) 35S::ScTFL1-11; (D) 35S::ScTFL1-41. Transgenic plants are on the right plants and Col-0 wild-type plants are on the left in all panels.

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Post-fertilization expression of *FLOWERING LOCUS T* suppresses reproductive reversion

Liangyu Liu^{1,2,3}, Sara Farrona^{1†}, Sonja Klemme^{1†} and Franziska K. Turck^{1*}

¹ Max Planck Institute for Plant Breeding Research, Carl von Linné Weg 10, Cologne, Germany

² Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan, China

³ University of Chinese Academy of Sciences, Beijing, China

Edited by:

Christian Jung, Christian Albrechts University of Kiel, Germany

Reviewed by:

Stefan De Folter, CINVESTAV-IPN, Mexico

Takato Imaizumi, University of Washington, USA

***Correspondence:**

Franziska K. Turck, Max Planck Institute for Plant Breeding Research, Carl von Linné Weg 10, Köln, NRW, Germany
e-mail: turck@mpipz.mpg.de

†Present address:

Sara Farrona, Department of Biology, Heinrich Heine University of Düsseldorf, Düsseldorf, Germany; Sonja Klemme, Bayer CropScience NV – INNOVATION CENTER, Belgium

FLOWERING LOCUS T (FT) encodes a systemic signal communicating the perception of long day photoperiod from leaves to the shoot apex to induce the floral transition. Transient expression of *FT* in the phloem companion cells of rosette leaves for one to several days was previously shown to be sufficient to commit plants to flowering. Here we show that partial commitment results in pleiotropic inflorescence meristem reversion phenotypes. *FT* expression is much stronger in organs formed after the floral transition such as cauline leaves, sepals, and developing siliques. We show that expression of *FT* and its paralog *TWIN SISTER OF FT (TSF)* after the floral transition plays a role in inflorescence meristem stabilization even if plants flower very late in development. *CONSTANS (CO)*, the major activator of *FT*, is not required to prevent late reproductive reversion. The requirement for *FT* is temporal since reproductive reversion to a vegetative state occurs only in recently formed inflorescence meristems. Unlike for the expression of *FT* in leaves, neither the distal putative *FT* enhancer nor long-day photoperiod is required for *FT* expression in developing siliques. Expression of *FT* in developing siliques and their supporting stems is sufficient to stabilize flowering during the sensitive developmental window indicating that fruit generated *FT* participates in inflorescence stabilization.

Keywords: flowering, *FLOWERING LOCUS T*, floral reversion

INTRODUCTION

In many plant species, *FLOWERING LOCUS T (FT)*-like genes play a critical role in the photoperiod dependent timing of the transition from the vegetative to the reproductive stage (Ballerini and Kramer, 2011). In the model plant *Arabidopsis thaliana*, *FT* integrates environmental and developmental variables at the level of its transcriptional regulation (Andres and Coupland, 2012). As part of florigen, *FT* protein produced in the leaves migrates to the shoot apical meristem where it triggers the reprogramming of regulatory networks resulting in a change from vegetative to reproductive growth (Corbesier et al., 2007). Prior to the floral transition, *FT* expression is restricted to the leaves and only occurs if days are longer than the critical day length (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Adrian et al., 2010). Photoperiod control of *FT* is dependent on *CONSTANS (CO)*, which acts as direct transcriptional activator presumably in a complex involving NF-Y transcription factors (Wenkel et al., 2006; Kumimoto et al., 2008, 2010; Tiwari et al., 2010). If days are longer than the critical day length, *CO* protein is stabilized by light and thus capable of promoting *FT* expression (Valverde et al., 2004). In the absence of light, *CO* is rapidly degraded in the dark and unable to promote *FT* expression. Apart from photoperiod, other external and internal cues participate in *FT* regulation. For example, an increase in ambient temperature overrules *FT*'s dependency on photoperiod and *CO* by affecting chromatin accessibility (Kumar et al., 2012). In biennial *Arabidopsis* plants, high levels of the transcription factor *FLOWERING LOCUS C (FLC)* prior vernalization prevent the activation of *FT* in long-day photoperiod

(Hepworth et al., 2002; Michaels et al., 2005). *FLC* directly binds to putative regulatory regions in the first intron of *FT* (Searle et al., 2006). The effect of *FLC* is partially dependent on the presence of *SHORT VEGETATIVE PHASE (SVP)*, with which it may form a complex (Hartmann et al., 2000; Li et al., 2008). *SVP* preferentially binds to regions in the *FT* promoter containing several putative CArG boxes. The effect of *SVP* repression on *FT* is particularly visible in young plants as well as in cold ambient temperature (Lee et al., 2007). Plant age participates in *FT* regulation via the *microRNA 156 (miR156)* pathway (Mathieu et al., 2009).

Recently, more pleiotropic roles of *FT* and *FT*-like genes have been reported (Pin and Nilsson, 2012). During the analysis of multi-parent recombinant inbred lines in *Arabidopsis*, *FT* was identified as quantitative trait locus (QTL) implicated in the formation of axillary meristems after, but not before, the floral transition (Huang et al., 2013). *FT* and its closest paralog *TWIN SISTER OF FT (TSF)* were shown to play a role in side shoot outgrowth with *ft* and *tsf* single mutants showing a reduced elongation rate of side shoots in long days (LD) and short days (SD), respectively (Hiraoka et al., 2013). Double *ft;tsf* mutants showed an enhanced reduction of side shoot elongation in both photoperiods. In addition, *FT* expression in stomata was shown to result in stomata opening, indicating a participation of *FT* in transpiration control (Kinoshita et al., 2011). In potato, some *FT*-like genes were shown to be involved in tuber initiation in response to long days, whereas other paralogs were regulating flowering in short days (Navarro et al., 2011). In poplar, high expression of *FT*-like genes causes early flowering and thus overcomes the

juvenility phase that usually prevents the floral transition in the first years of development (Bohlenius et al., 2006; Hsu et al., 2006, 2011). However, expressed at lower levels, functional divergence of poplar *FT1* and *FT2* paralogs becomes obvious. Poplar *FT1*, which is induced by prolonged cold in various tissues including buds and leaves, triggers the onset of reproductive bud formation, whereas the long-day induced poplar *FT2*, which expresses in the leaves, is much less effective in this process. Moderate expression of *FT2* prevents growth cessation, which is usually triggered in short days or after stress perception in natural conditions but is not sufficient to trigger reproductive bud formation. Thus the role of *FT2* is to maintain vegetative growth during the growing season (Hsu et al., 2011).

Interestingly, *FT* in Arabidopsis is expressed at much higher levels after the floral transition, notably in cauline leaves, sepals, petals and developing siliques (Schmid et al., 2005; Adrian et al., 2010), and the expression in developing siliques is independent of photoperiod (Hiraoka et al., 2013). Here we set out to elucidate if *FT* expression in developing fruits fulfills a biological function or rather reflects a non-functional relaxation in the tight repressive control of transcription that is necessary to prevent precocious flowering earlier in development. We show that photoperiod-independent expression of *FT* in developing fruits plays a role in inflorescence maintenance.

RESULTS

SEVERAL DAYS OF INDUCTION OF FT EXPRESSION ARE REQUIRED FOR FULL FLORAL COMMITMENT OF ARABIDOPSIS PLANTS TO FLOWER

Previous studies reported that 3 days in LD growth conditions during which *FT* is expressed are sufficient to induce early flowering in the Arabidopsis accession *Ler* whereas *Col* plants require 5 LDs for full induction of early flowering (Corbesier et al., 2007; Torti et al., 2012). We wanted to quantify more precisely how *FT* expression contributed to the commitment of the plants to flowering. We grew Arabidopsis plants of the accession *Col-0* in 18 SD before shifting them transiently for an increasing number of days to extended short day (ESD) conditions and back to SD. ESD growth conditions provide 8 h of full light supplemented by 8 h of low intensity light sufficient to trigger long day (LD) light responses, such as *FT* induction, while minimizing the differences in growth rate observed between plants grown in SD and LD conditions. As expected, plants showed accelerated flowering after three ESDs as compared to control plants grown in SD conditions (Figure 1A).

Plants flowering after experiencing few ESDs showed partial transition/reversion phenotypes. We grouped all shifted plants into three progressive phenotypic categories, which were “1: as wild-type with a main shoot showing clear apical dominance and cauline leaves with a different shape than rosette leaves,” “2: shortened main shoot without apical dominance and with aerial rosettes,” “3: main shoot fully arrested with less than 1 cm bloting or not detectable, frequent aerial rosettes on side shoots formed at the axils of rosette leaves” (Figures 1B–D). The severity of the phenotype was inversely correlated with the number of ESDs the plants had experienced (Figure 1E) indicating that maintained *FT* expression participates in preventing reversion of the inflorescence meristem to a more vegetative or arrested state. In addition, plants that were older at the start of the ESD

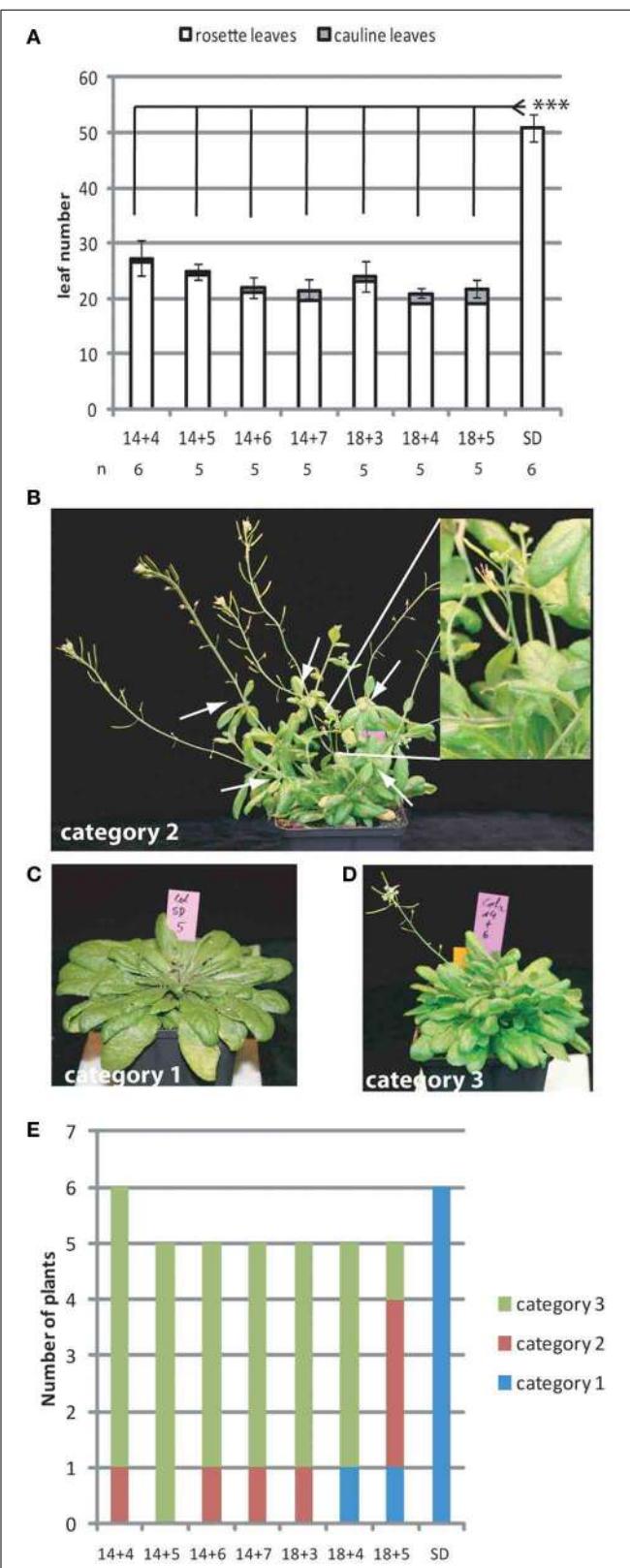


FIGURE 1 | Several ESDs are required to fully commit the shoot apical meristem to flowering. *Col* plants grown in SD for 14 or 18 days were transiently shifted to an increasing number of days in ESD as indicated. (A) (Continued)

FIGURE 1 | Continued

Flowering was determined by counting rosette and cauline leaves of the main inflorescence. In the case of SD grown plants only rosette leaves were counted. Numbers of total leaves are shown as the mean \pm SE. Statistically significant differences between treatments was assessed by One-Way ANOVA followed by Tukey Honest Significant Differences ($***p < 0.01$). (B–D) Plants typical for phenotypic categories 1–3, respectively. Categories are: “1: as wild-type,” “1: reduced apical dominance and aerial rosettes at main shoot,” “2: main shoot arrested, aerial rosettes on side shoots at the rosette” White arrows indicate aerial rosettes, the inset shows an arrested main shoot without apical dominance. (E) Quantification of the number of plants per category observed for different photoperiod treatments as indicated.

treatment showed less severe phenotypes than plants treated at a younger age (Figure 1E).

TRANSIENT REPRODUCTIVE REVERSION IS OBSERVED IN *ft* MUTANT PLANTS

The hypothesis that maintained *FT* expression is required to prevent reversion of the inflorescence is challenged by the fact that reversion has not previously been reported for plants grown either in SD growth conditions or carrying non-functional *FT* alleles. We assessed whether reversion of flowering could have been overlooked in plants that flower late in development. Although we never observed reversion in Col-0 plants grown in SDs, partial reproductive reversion was commonly observed in *ft-10* mutants (Figures 2A,B). This reproductive reversion phenotype was different from that observed in younger plants induced by a suboptimal length of ESDs, since bolting rate or apical dominance of the main shoot were not affected (Figure 1). Instead, reversion was observed in flowering shoots early after the formation of the first true flowers. The inflorescence meristem reverted to form vegetative side branches with rosette-like cauline leaves (Figures 2A,B). Reversion was transient, affecting between 3 and 10 nodes before the formation of flowers was resumed. Furthermore, reversion was not observed in *co* mutants but was enhanced in *ft-10;tsf-1* double compared to *ft-10* single mutants, which also showed a tendency to go through repeated cycles of reversion (Figures 2A,B). Taken together, the results indicate that the reversion phenotype is independent of CO but dependent on the expression of the two FT-like genes *FT* and *TSF*.

FT EXPRESSION IN DEVELOPING FRUITS IS NOT DEPENDENT ON PHOTOPERIOD AND THE DISTAL FT ENHANCER Block C

Before the reproductive transition, *FT* is expressed in the phloem companion cells of the minor leaf veins if CO protein is stabilized in response to LD photoperiod (Valverde et al., 2004). After the transition, the expression level of *FT* dramatically increases in the cauline leaf veins in long days (Figure 3A and Supplemental Figure 1). *FT* expression is also detected along the vascular bundles of sepals, petals, the funiculi and septum of developing siliques and in the vasculature of floral stems during siliques maturation as indicated by a *GUS* reporter gene driven by the full-length 5.7 kb *FT* promoter (Figures 3B–E). Expression of *FT* in developing siliques and supporting stems under the control of this promoter is independent of LD photoperiod (Figures 3C–E). This is similar to data shown by Hiraoka

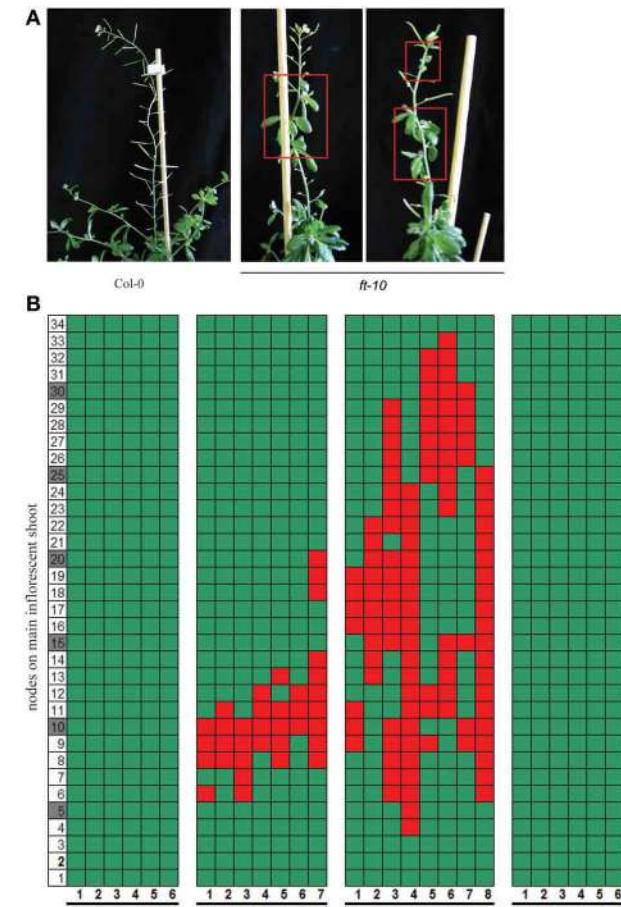
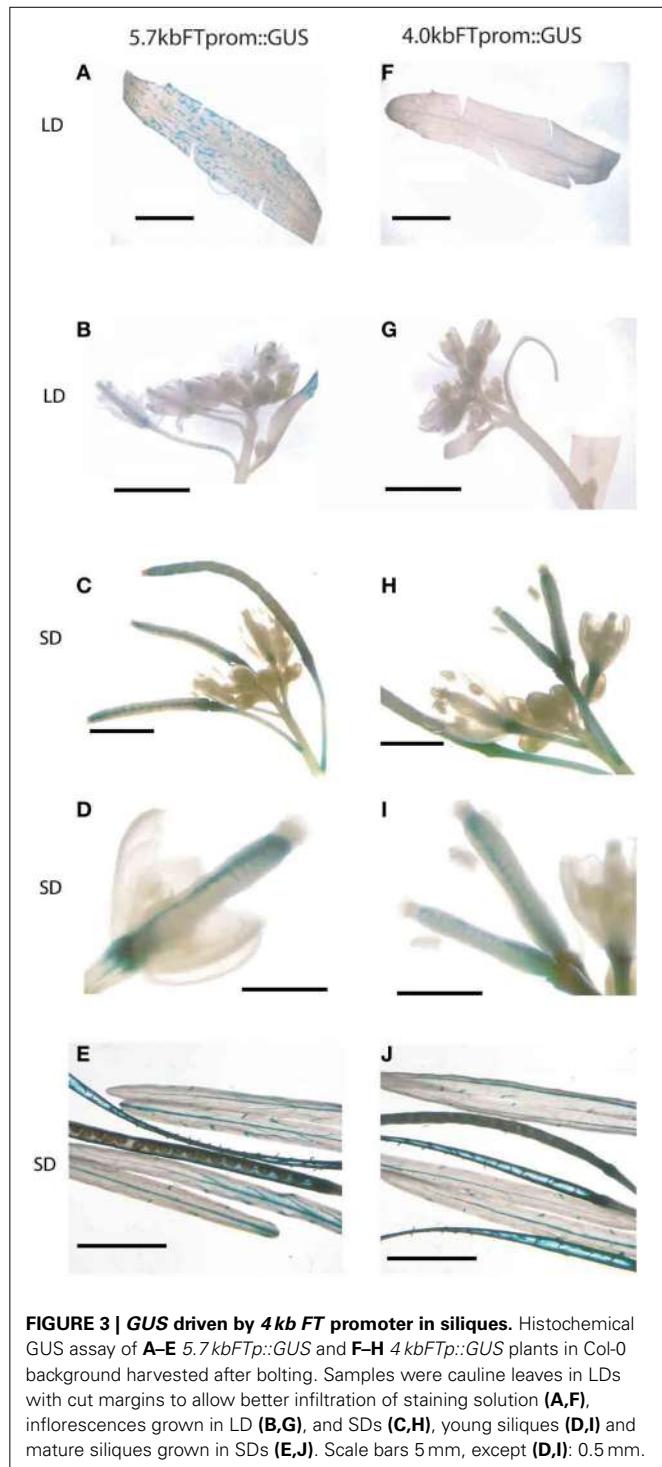


FIGURE 2 | Reversion phenotype of photoperiod pathway mutants in SDs. Plants were grown under SD conditions. (A) Plants typical for flowering phenotype of Col-0 wildtype and *ft-10* mutant (B) Side shoots on the main inflorescence were counted from the first flower (1) toward the top (until node 34). Flowers are indicated by green, cauline leaves by red squares, the number of plants assessed is indicated on the x-axis.

et al., who used a genomic *FT*:*GUS* fusion that included all intronic regions and a slightly longer *FT* promoter (Hiraoka et al., 2013). We have previously shown that induction of *FT* by LD photoperiod requires the presence of a putative distal enhancer region located between 5.7 and 4.0 kb of the transcription start site (Adrian et al., 2010). While expression of *FT* in all leaf tissues before fertilization was dependent on the presence of the distal regulatory regions (Figures 3F,G) its expression in the funiculi, the septum of developing siliques and the supporting stem did not require the presence of this putative enhancer indicated by the detection of *GUS* reporter gene expression under the control of a 4.0 kb *FT* promoter (Figures 3H–J).

Taken together, the data indicate that distal regulatory regions are important to express *FT* highly in cauline leaves whereas *FT* utilizes a different regulatory network for activation in developing siliques where the expression is also photoperiod independent.



REVERSION OF THE INFLORESCENCE MERISTEM IS PREVENTED BY EXPRESSION OF FT IN DEVELOPING SILIQUES

Reproductive reversion in *ft-10* mutants was observed in SD growth conditions when expression of *FT* is mainly restricted to the developing siliques and their stems indicating that expression in these organs after fertilization may participate in maintaining the recently committed inflorescence meristem. We made

use of the observation that the 4kb FT_{pro} ::GUS transgene was exclusively expressed in these tissues to test the functional relevance of *FT* expression in these organs. Transgenic plants that expressed *FT* cDNA under the control of the 4 kb *FT* promoter in the *ft-10* background showed no inflorescence reversion in two out of three transgenic lines whereas a third line showed a reduced reversion phenotype (Figure 4). This was not different from the rescue of the reversion phenotype observed when *FT* cDNA was expressed under the control of the full length 5.7 kb *FT* promoter (Figure 4). The floral reversion type was not correlated to slight differences in flowering time that were observed between the transgenic plants and the parental *ft-10* mutant in SD growth conditions (Supplemental Figure 2).

Taken together, the data indicate that photoperiod independent expression of *FT* in developing siliques and their supporting stem is functional and participates in maintaining the commitment of the inflorescence meristem.

DISCUSSION

We addressed the question whether expression of *FT* plays a functional role in *Arabidopsis* development after the plants have transitioned to flowering. The interest in solving this question was ignited by the previous observation that *FT* levels are much higher in organs formed after the floral transition than in rosette leaves (Supplemental Figure 1). Recently, it has been reported that *FT* and the related *TSF* play a role in controlling the elongation rate of side shoots developing from subtending caulin leaves and that *FT* protein moves from caulin leaves to axillary meristems (Hiraoka et al., 2013; Niwa et al., 2013). Our data suggest that elevated *FT* levels after the transition to flowering help stabilizing the recently formed inflorescence meristem, which seems particularly required if plants flower early in development.

Reversion of the inflorescence meristem in young plants by insufficiently persistent photoperiod signals can lead to dramatic phenotypic effects the most severe being the full arrest of the main apical meristem (Figure 1). It is currently unclear if this is an accidental consequence of the repeated reorientation of meristem identity or a deliberate developmental program. It can be argued that plants growing in natural conditions in autumn may profit from inflorescence arrest if this permits them to resume vegetative growth until the next spring instead of flowering in late autumn at an early developmental stage.

Inflorescence reversion after the formation of flowers was previously reported for plants carrying mutations in both, *SUPPRESSOR OF CONSTANS 1* (*SOC1*) and *FRUITFUL* (*FUL*) (Melzer et al., 2008). *SOC1* and *FUL* act with partial redundancy to accelerate flowering in LD (Melzer et al., 2008) and attenuate the effect of an ectopic overexpression of *FT* (Melzer et al., 2008; Torti et al., 2012). Inflorescence reversion observed in *soc1;ful* double mutants is suppressed by adding an additional mutation in the gene encoding the floral repressor SHORT VEGETATIVE PHASE (*SVP*) (Torti et al., 2012). Thus it is likely that the expression of *FT* in developing fruits and their supporting stems serves to maintain the expression of *SOC1*, *FUL*, and other unknown *SVP* target genes at the inflorescence meristem.

In wild type Col-0 plants grown in SD combined expression of *FT* and *TSF* prevents floral reversion after the formation

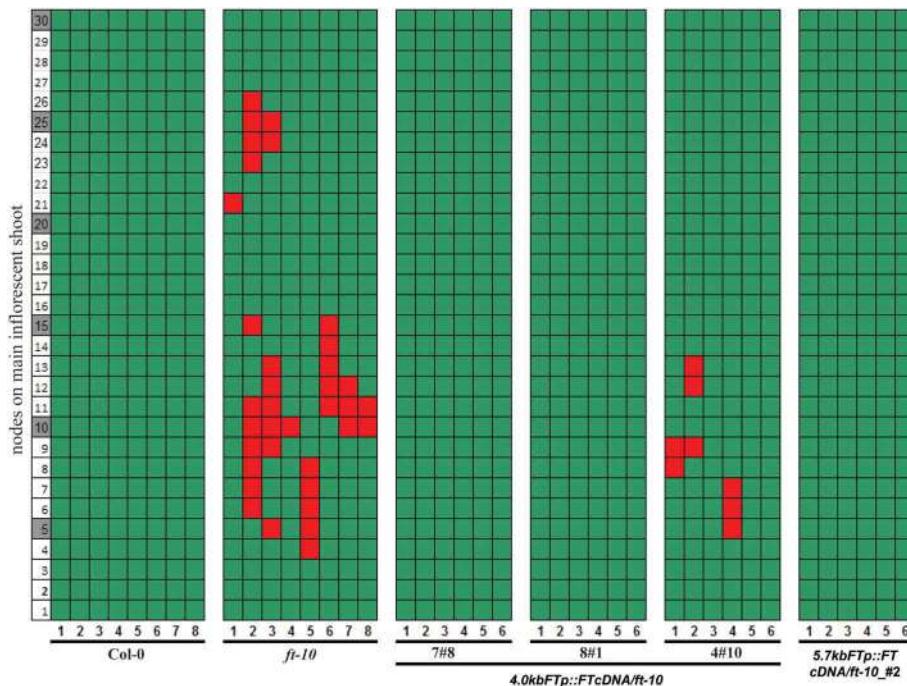


FIGURE 4 | Reversion of *ft* mutants is abolished by expressing *FT* under the control of a 4.0 kb *FT* promoter. Reversion phenotype of *ft-10* plants carrying constructs to drive *FT* cDNA by 5.7 and 4.0 kb *FTp* were measured. Three independent transgenic lines are

shown for 4.0 kb *FTp* construct. Col-0, *ft-10*, and *co-sail* were also measured. The nodes of siliques on the major influence shoot are marked as green boxes, and the reverted vegetative nodes are shown as red boxes.

of the first flowers. Expression of *FT* in the vascular bundles of developing siliques and the supporting stems is sufficient to prevent reversion of newly committed inflorescence meristems. Expression of *FT* in these tissues is independent of photoperiod and does not require the distal regulatory region that we previously showed was required for *FT* activation by CO in rosette leaves (Adrian et al., 2010). Apparently, in these particular tissues *FT* can make use of a distinct set of transcriptional activators to promote expression. These activators may not be expressed or activated in other organs. However, it is also possible that the *FT* locus itself becomes more permissive for transcription activation in developing siliques. Interestingly, the 4 kb *FT* promoter, which is sufficient for driving expression in developing siliques but not in other tissues also activates *FT* ectopically in SD in mutants affecting the chromatin-dependent Polycomb Group (PcG) pathway such as *like-heterochromatin protein 1* (*lhp1*) and *curly leaf* (*clf*) (Adrian et al., 2010; and Supplemental Figure 3). Thus, a reduction in PcG-mediated repression of *FT* could explain photoperiod independent expression in developing siliques and their supporting stems.

Feed-back from developing fruits toward programming of developing meristems is also documented in other plant species, such as fruit trees belonging to the Rosaceae family. Many apple landraces show biennial flowering (alternate bearing) because the ripening fruits inhibit the transition of newly forming meristems to an inflorescent state (Goldschmidt, 2013). However, the molecular mechanisms explaining this developmental program are not

understood and it is unclear if *FT* related genes are implicated in the process.

A delay of the reproductive phase has been associated with high performance of cereal crops grown in temperate climates that are not threatened by seasonal water shortage (Jung and Muller, 2009). Given their common potential to promote flowering in response to photoperiod, mutations in *FT*-like genes are likely to delay flowering in many species and thus may represent good targets for breeding. However, more pleiotropic roles of *FT* and related genes after the floral transition suggest that loss of *FT* function could have deleterious effects on overall seed yield. Our study shows that *FT* is important to stabilize the inflorescence meristem and recently it was reported that *FT* expression promotes side shoot formation at the axes of cauline leaves (Huang et al., 2013) as well as the rate of side shoot elongation (Hiraoka et al., 2013). Future studies should elucidate whether the pleiotropic roles *FT* plays after the floral transition are as universal as its involvement in the promotion of flowering.

METHODS

PLANT GROWTH

SD conditions included 8 h of cool-white fluorescent light (8.5 Wm^{-2}), followed by 16 h of darkness. Extended SD conditions included 8 h of cool-white fluorescent light, followed by 8 h of low-fluence rate incandescent light (0.2 Wm^{-2}), followed by 8 h of darkness. LD conditions included 16 h of cool-white fluorescent light followed by 8 h of darkness. Seeds were sowed on soil

and stratified at 4°C for 3 days. Soil trays were transferred to LD or SD growth conditions as indicated. For the shift experiment, plants grown in SDs for 14 or 18 days were transiently shifted to ESDs for 3–7 days and shifted back to SDs until setting seeds. As measure of flowering time, the total rosette, and cauline leaves on the main inflorescence shoot were counted. Plants were grown at 22°C for SD-ESD-SD shift experiments and at 20°C for inflorescence reversion experiments.

PHENOTYPING INFLORESCENCE REVERSION

For plants shifted temporarily from SD to ESD conditions, reversion was assessed by grouping plants into three phenotypic categories which were “1: as wild-type with a main shoot showing clear apical dominance and cauline leaves with a different shape than rosette leaves,” “2: shortened main shoot without apical dominance and with aerial rosettes,” “3: main shoot fully arrested with less than 1 cm blotting or not detectable, frequent aerial rosettes on side shoots formed at the axils of rosette leaves.” Reversion for plants grown in SD was assessed on the main inflorescence shoot when plants had initiated inflorescences on all side shoots.

GUS HISTOCHEMICAL STAINING

Cauline leaves, flowers, and siliques were collected from 2-month-old LD or 3-month-old SD grown plants carrying either a 5.7 or 4.0 kb *FT* promoter GUS constructs as indicated. Samples were incubated in 90% Acetone on ice for 30 min, rinsed with 50 mM sodium phosphate buffer and incubated for 24–36 h at 37°C in GUS staining solution (0.5 mg/ml X-Gluc, 50 mM sodium phosphate buffer, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X-100). To allow better penetration of the staining solution, cauline leaves were cut at the edges. After incubation, samples were washed repeatedly with 50 mM sodium phosphate buffer for 30 min and 70% ethanol until leaves turned white. GUS staining was visualized and photographed under a stereomicroscope (Leica).

TRANSGENIC PLANTS AND MUTANTS

5.7 kb-*FTpro::GUS/FTcDNA;ft-10* and 4.0 kb-*FTpro::GUS/FTcDNA;ft-10* transgenic plants have been described previously (Adrian et al., 2010). Mutants *ft-10* (GK-290E08), *tsf-1*(SALK 087522), and *co-10* (SAIL_24_H04) are caused by T-DNA insertions in the Colombia-0 ecotype and have been described previously (Michaels et al., 2005; Yoo et al., 2005; Laubinger et al., 2006).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00164/abstract>

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The relationship between flowering time and growth responses to drought in the *Arabidopsis Landsberg erecta* x Antwerp-1 population

Inga Schmalenbach¹, Lei Zhang¹, Matthieu Reymond² and José M. Jiménez-Gómez^{1,2*}

¹ Department of Plant Breeding and Genetics, Max Planck Institute for Plant Breeding Research, Cologne, Germany

² Institut Jean-Pierre Bourgin, Institut National de la Recherche Agronomique, Versailles, France

Edited by:

Maria Von Korff Schmising, Max Planck Society, Germany

Reviewed by:

Zhao Su, The Penn State University, USA

John Thomson Lovell, University of Texas, USA

***Correspondence:**

José M. Jiménez-Gómez,
Department of Plant Breeding and Genetics, Max Planck Institute for Plant Breeding Research,
Carl-von-Linné-Weg 10,
50829 Cologne, Germany
e-mail: jm.jimenez@mpipz.mpg.de

Limited water availability is one of the most prominent abiotic constraints to plant survival and reproduction. Thus, plants have evolved different strategies to cope with water deficit, including modification of their growth and timing of developmental events such as flowering. In this work, we explore the link between flowering time and growth responses to moderate drought stress in *Arabidopsis thaliana* using natural variation for these traits found in the *Landsberg erecta* x Antwerp-1 recombinant inbred line population. We developed and phenotyped near isogenic lines containing different allelic combinations at three interacting quantitative trait loci (QTL) affecting both flowering time and growth in response to water deficit. We used these lines to confirm additive and epistatic effects of the three QTL and observed a strong association between late flowering and reduced sensitivity to drought. Analyses of growth responses to drought over time revealed that late flowering plants were able to recover their growth in the second half of their vegetative development. In contrast, early flowering, a common drought escape strategy that ensures plant survival under severe water deficit, was associated with strongly impaired plant fitness. The results presented here indicate that late flowering may be advantageous under continuous mild water deficit as it allows stress acclimatization over time.

Keywords: mild drought stress, water deficit, flowering time, growth, natural variation, *Arabidopsis thaliana*, epistasis

INTRODUCTION

To ensure their survival and successful reproduction, plants need to respond appropriately to environmental changes. In the context of global climate change, limited water availability is one of the most prominent abiotic constraints to plant survival and productivity in natural environments as well as in crop production systems (Cattivelli et al., 2008). Understanding the molecular and physiological responses to drought in plants can, thus, help us to ensure food production in the future. However, our knowledge about the mechanisms involved in plant's responses to water deficit is hampered by their complexity. Depending on the specific drought scenario and the time of its occurrence during their life cycle, plants combine different strategies involving short-term and long-term responses driven by intricate regulatory networks (reviewed in Chaves et al., 2003). As demonstrated by Skirycz et al. (2011), distinct gene networks are activated depending on the severity of drought stress.

Changes in the rate of growth and/or in flowering time are two common strategies that plants use to cope with drought. Shoot growth inhibition under water deficit helps plants to reduce water loss through transpiration. Recently, Baerenfaller et al. (2012) have demonstrated that plants adapt to an early applied and continuous moderate water deficit by changes in gene expression and by reducing their growth in a constant manner. Interestingly, the

transcriptional responses observed differ significantly from modifications of gene expression in the case of a sudden drought stress (Skirycz et al., 2010; Baerenfaller et al., 2012). In terms of flowering time, an early switch from vegetative to reproductive development enables plants to reproduce before the onset of severe water deficit compromises their survival (the so called drought escape strategy; Ludlow, 1989; Sherrard and Maherli, 2006; Franks, 2011). Nevertheless, under a continuous mild water deficit, this strategy can be seen as counterproductive. Early flowering shortens the time available for carbon assimilation during vegetative development and, thus, possibly results in yield reduction. Variation in flowering time has been linked to variation in leaf growth. For instance, in the model species *Arabidopsis thaliana*, delaying the floral transition using short day photoperiods resulted in a reduced final leaf area and leaf expansion rate but an increased duration of leaf expansion (Cookson et al., 2007).

The variation found for growth, flowering time and drought responses among natural accessions in *A. thaliana* is a useful tool to understand plant water relations. Diverse studies have explored variation in physiological parameters in this species, which may play a role in its adaptation to dry environments. In particular, variation for water use efficiency (WUE), a measurement for the trade-off between CO₂ assimilation and water loss through

transpiration, has been studied extensively and used to identify quantitative trait loci (QTL; Mckay et al., 2003; Hausmann et al., 2005; Juenger et al., 2005; Mckay et al., 2008). Furthermore, few genes that underlay drought related traits and, thus, are possibly associated with environmental adaptation have been identified and characterized (Des Marais et al., 2012; Kesari et al., 2012; Lovell et al., 2013). To enable the detailed analysis of plant growth responses to drought, which result from the integration of numerous processes (reviewed in Tardieu et al., 2011), different automated phenotyping systems have been developed. Using one of these systems, Antwerp-1 (An-1) has been identified as one accession with low response of rosette growth to moderate drought stress (Aguirreza et al., 2006; Granier et al., 2006). In contrast, Landsberg *erecta* (Ler) showed a high reduction of rosette growth in response to this drought condition. QTL mapping using a recombinant inbred line (RIL) population derived from a cross between An-1 and Ler has led to the identification of loci affecting the responses of different growth related parameters to drought (Tisné et al., 2010). Highlighting their complex mode of regulation, drought responses were mainly underlined by epistasis, i.e. QTL x QTL interactions. Using near isogenic lines (NILs) developed for one specific QTL network, Tisné et al. (2010) observed an association between a delay of flowering in response to water deficit and a low response in rosette growth and epidermal cell area and number.

Some works have demonstrated a strong positive correlation between natural variation in WUE and flowering time. This association has been attributed to pleiotropic effects of the flowering time gene *FRIGIDA* (*FRI*) that is epistatic to its downstream target, the floral repressor *FLOWERING LOCUS C* (*FLC*; Johanson et al., 2000; Mckay et al., 2003; Lovell et al., 2013). Plants carrying both functional *FRI* and *FLC* alleles follow a dehydration avoidance strategy characterized by late flowering, slow growth and high WUE. In contrast, non-functional *FRI* alleles result in early flowering, fast growth and low WUE, and, thus, confer a drought escape strategy (Lovell et al., 2013). Indicating an actual relevance of this correlation for adaptation in nature, Stinchcombe et al. (2004) detected an association between the late floral transition of accessions containing a functional *FRI* allele and low January precipitation at their site of origin.

Most studies conducted so far have been focused on the adaptation of plants to severe drought stress endangering their survival and reproduction. Nevertheless, especially in temperate climate zones, moderate but continuous soil water deficit may affect plant productivity. Thus, in the present study, we intend to explore plant strategies to cope with a consistent mild drought stress during vegetative development. In this article we ask whether the timing of flowering, being central for a successful plant reproduction, is associated with growth and fitness responses to mild drought. Making use of previously described natural variation within an *A. thaliana* population (Tisné et al., 2010), we observe a strong association between flowering time and growth in response to water deficit. To further elucidate the genetic basis of this correlation, we develop and characterize a set of near isogenic lines differing in the allelic composition at several interacting QTL affecting both traits analyzed. Monitoring growth responses of the NILs over time leads to the conclusion that late flowering

may be advantageous for plant fitness under continuous mild water deficit, as a prolonged vegetative phase may enable plants to recover their growth before flowering.

MATERIAL AND METHODS

GENETIC RESOURCES

Several QTL and epistatic interactions affecting growth and flowering time have been previously detected in the Landsberg *erecta* (Ler) x Antwerp-1 (An-1) RIL population under different water regimes (Tisné et al., 2010). We developed a set of near isogenic lines (NILs) carrying all possible combinations of alleles at three of these QTL (QTL3 x QTL5.1 x QTL5.2) in a homogeneous Ler background. In order to obtain the NILs, we backcrossed RIL102 containing An-1 alleles at all three QTL to Ler. After two rounds of selfings, we obtained two homozygous NILs carrying a single An-1 introgression at QTL3 or at both QTL5.1 and QTL5.2, respectively. Both NILs were genotyped with 76 Cleaved Amplified Polymorphic Sequence markers distributed across all five chromosomes to confirm the absence of An-1 alleles outside the QTL regions (Supplemental Table 1). Finally, the two lines were crossed to each other and their F₁ selfed for two generations. In the F₃ generation, eight homozygous NILs containing the different combinations of QTL alleles were selected (Supplemental Figure 1).

DROUGHT STRESS EXPERIMENT WITH NILS

Twelve replicates per NIL and treatment were grown in an experiment that mimicked the growth conditions and water regimes of the Ler x An-1 RIL experiment conducted on the automated phenotyping platform PHENOPSIS (Tisné et al., 2010). Plants were grown in square pots (7 × 7 × 8 cm) containing a standard plant cultivation substrate (Einheitserde Spezial, Typ Mini Tray, Einheitserde- und Humuswerke Gebr. Patzer, Sinntal-Altengronau, Germany) and cultivated under controlled conditions in 12/12 h day/night regimes with temperatures of 22/18°C and an air humidity of 85/75% (day/night). Within each treatment, plants were completely randomized. Before starting the experiment, the initial weight of each pot filled with humid soil was recorded. Subsequently, some pots were removed from the experiment to quantify the initial average soil water content (SWC). To do so, these pots were dried for 3 days at 60°C, weighed afterwards and SWC calculated according to the following formula: SWC = (soil fresh weight – soil dry weight)/soil fresh weight. We assigned this initial SWC to every pot in the experiment. From sowing until the emergence of the first two leaves (growth stage 1.02, according to Boyes et al., 2001), all plants were maintained at 54–60% SWC (72–80% field capacity). From growth stage 1.02 until the end of the vegetative phase (growth stage 6.00), plants grown under well-watered (ww) condition were further maintained at 54–60% SWC, while plants under water deficit (wd) were maintained at 36–42% SWC (48–56% field capacity). The soil water content was adjusted every second day, and, thus, soil dried down over time resulting in the given ranges of SWC in each treatment. To adjust SWC, water was added manually to each pot until the respective target pot weight (corresponding to 60% and 42% SWC in ww and wd, respectively) was reached. This protocol ensured the same water availability for

all plants in the respective treatment, independently from possibly different transpiration rates. The experiment was finished for each plant at the time of flowering and for each line when less than three replicates remained without flowering.

From the start of the treatment to the respective day of flowering, we monitored rosette growth by taking photos from above of each plant individually. In these images, we separated the rosette from its background by removing the blue and red color filters and increasing contrast in the green channel in Adobe Photoshop®. Then, images were manually cropped and transformed into binary format using ImageJ. Projected rosette leaf area (RA; mm²) was calculated from binary images with the open source ImageJ Plugin Rosette Tracker (De Vylder et al., 2012).

When a plant started flowering, its final projected rosette area, flowering time (FT; in days after sowing) and total leaf number (LN; rosette plus cauline leaves) were recorded. Leaf production rate (LPR; leaves per day) was calculated as the ratio LN/FT. As a proxy for fitness, seed yield (YLD; mg/plant) was measured after complete plant ripening. To determine only the effect of water deficit that occurred during the vegetative phase on YLD, drought stressed plants were grown in ww condition from flowering until complete seed set. The relative response to drought for each parameter was calculated as follows: Response = (trait value in wd—trait value in ww)/trait value in ww.

FLC EXPRESSION ANALYSIS

For analyzing *FLC* expression we used plants grown simultaneously with the plants of the NIL drought experiment described above. All above ground tissues were harvested for each plant 21 days after sowing. Tissue from two plants was pooled for each of three biological replicates. RNA was extracted using Trizol (Ambion® TRIzol® RNA Isolation Reagent, Life Technologies) and transcribed into cDNA using Super Script® II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed on a CFX384 Touch™ Real-Time PCR Detection System (Biorad) using SYBR Green dye (iQ™ SYBR® Green Supermix, Biorad) and the following *FLC* specific primers: F-primer: 5'-CCGAACATCATGTTGAAGCTTGTGAG-3', R-primer: 5'-CGGAGATTTGCCAGCAGGTG-3'. Expression values were determined using the standard curve method and normalized to the expression of *PP2A* (F-primer: 5'-TAACGTGGCCAAATGATGC-3', R-primer: 5'-GTTCTCCACAACCGCTTGGT-3'). Normalized expression was averaged for three biological replicates each analyzed in three technical replicates.

STATISTICAL METHODS

Data for flowering time (FT), leaf number at flowering (LN) and rosette area (RA) in well watered (ww), and water deficit (wd) conditions for the *Ler* x *An-1* RIL population was obtained from the PHENOPSIS DB (<http://bioweb.supagro.inra.fr/phenopsis/>; Fabre et al., 2011). In most cases, four replicates of each RIL were present in each treatment (range from 0 to 4 replicates, mean of 3.73 and median of 4). Values for each individual plant were collected at the time of flowering. To this dataset, we added leaf production rate (LPR) as LN/FT for each individual plant.

Average values and relative responses to water deficit per genotype were calculated to study the correlation between traits and their responses to drought in the *Ler* x *An-1* RIL population. Mean values per line and trait were calculated by averaging the data from all plants grown both in ww and wd. Relative responses to drought were calculated per RIL and trait as [(mean in wd—mean in ww)/mean in ww]. *P*-values for the correlations between each trait and relative responses to drought were calculated using Pearson's correlation coefficient.

Analysis of phenotypic data from the NILs representing all possible combinations at QTL3, QTL5.1, and QTL5.2 was performed by fitting an ANOVA that included treatment (levels "ww" or "wd") and genotype at each QTL (levels "*Ler*" or "*An-1*") together with all possible interactions (phenotype ~ Q3 * Q5.1 * Q5.2 * treatm). The degrees of freedom for FT, LN, LPR, RA, and YLD were 184, 182, 182, 182, and 187 respectively. To study phenotypic differences between individual NILs we defined groups using Tukey's HSD test on the ANOVA mentioned above with a significance threshold of 0.05. Relative responses to drought were calculated per genotype and trait as [(mean in wd—mean in ww)/mean in ww].

For the analysis of *FLC* differences in expression we fitted an ANOVA with the relative expression of *FLC* with respect to *PP2A* (See Material and Methods above) including treatment and genotype at each QTL and with all possible interactions. The ANOVA had 47 degrees of freedoms. Significant classes were obtained using Tukey's HSD test with a significance threshold of 0.05.

RESULTS

IN THE *Ler* X *An-1* RIL POPULATION, FLOWERING TIME AND GROWTH RESPONSES TO WATER DEFICIT ARE POSITIVELY CORRELATED

The *Ler* x *An-1* RIL population (El-Lithy et al., 2006) has been phenotyped previously under two distinct water regimes, namely well-watered control condition (ww) and mild water deficit (wd), using an automated phenotyping platform (Tisné et al., 2010; Fabre et al., 2011). We used flowering time and growth measurements from this experiment to study the association between these traits and their response to drought stress (Fabre et al., 2011, See Material and Methods). We detected highly significant positive correlations between flowering time (FT) and responses of FT and rosette area (RA) to drought (Figure 1, Table 1). Furthermore, these three traits revealed highly significant positive correlations with leaf number (LN) and leaf production rate (LPR, Table 1). Overall, late flowering RILs exhibited a high LPR combined with a delayed floral transition and a low reduction of RA under drought. In contrast, earlier flowering RILs showed a lower LPR and a stronger decrease of RA in response to drought. In addition, early flowering RILs exhibited no FT response or flowered slightly earlier under drought.

CONFIRMATION OF AN EPISTATIC QTL NETWORK UNDERLYING A COMPLEX REGULATION OF FLOWERING TIME AND GROWTH RESPONSES TO DROUGHT USING NILs

In the *Ler* x *An-1* RIL population, a number of QTL have been previously reported for diverse growth related traits such as RA, LN, and duration of the vegetative phase and their responses to

drought (Tisné et al., 2010). In multiple cases, QTL for these traits colocalized and presented interactions with each other. For instance, Tisné et al. (2010) detected an epistatic interaction between a QTL on chromosome 3 (at 3.7 cM, in the following named QTL3) and a QTL on chromosome 5 (at 28.4 cM, in the following named QTL5.2) for RA and LN in both water regimes (ww and wd). Furthermore, for both parameters, QTL5.2 interacted with a second QTL on chromosome 5 (at 13.3 cM, in the

following named QTL5.1) in wd. In addition, QTL5.1 had an additive effect on RA and LN only in ww condition (Tisné et al., 2010).

In the present study, we developed a set of NILs each carrying one of the eight possible allelic combinations at QTL3, QTL5.1, and QTL5.2 in a homogeneous *Ler* background in order to confirm and further characterize the effects of these QTL (Supplemental Figure 1). These lines were grown under two distinct water regimes (ww and wd) mimicking the experimental set up of the RIL experiment described above (for details see Tisné et al., 2010). As in the RIL experiment, the following growth related parameters were quantified at the time of flowering: FT, LN, RA, and LPR. In addition, we measured seed yield (YLD) after complete plant ripening in order to assess fitness differences between the NILs. Finally, we calculated the relative responses to wd using the trait values from both water regimes.

An ANOVA using treatment and the genotype at the three QTL as individual factors revealed significant associations between each QTL and all traits analyzed (Figure 2, individual QTL effects). In addition, although the water regime had a strong effect in all traits, most single QTL x treatment interactions only affected individual traits (Figure 2, water regime and water regime x individual QTL effects). Two-way interactions between QTL had significant effects in all traits except YLD, where only the interaction between QTL5.1 and QTL5.2 was significant (Figure 2, two way QTL interactions). Interestingly, interactions involving QTL5.2 exhibited significant effects with treatment for most traits (Figure 2, water regime x two way QTL interactions). Finally, interactions between all three QTL were detected for FT, LN, LPR, and YLD, and between all QTL and environment for LN and RA.

In summary, we confirmed QTL previously detected in the RIL population by Tisné et al. (2010) in lines with a homogeneous genetic background. The positive correlation between FT and growth in response to drought observed in the RILs is in part based on additive and epistatic effects of three interacting QTL located on chromosomes 3 and 5.

LATE FLOWERING NILS EXHIBIT A REDUCED SENSITIVITY TO DROUGHT

The small genetic variation and large phenotypic differences present among the NILs is an ideal tool to study the

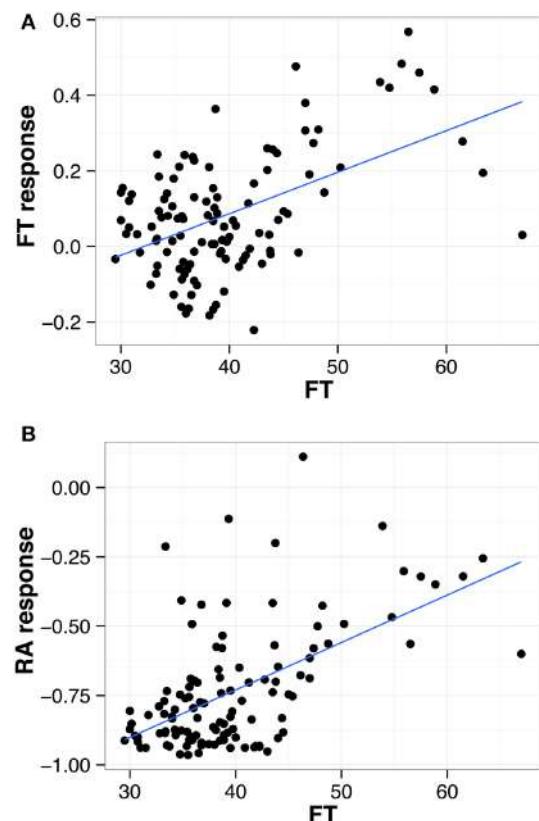
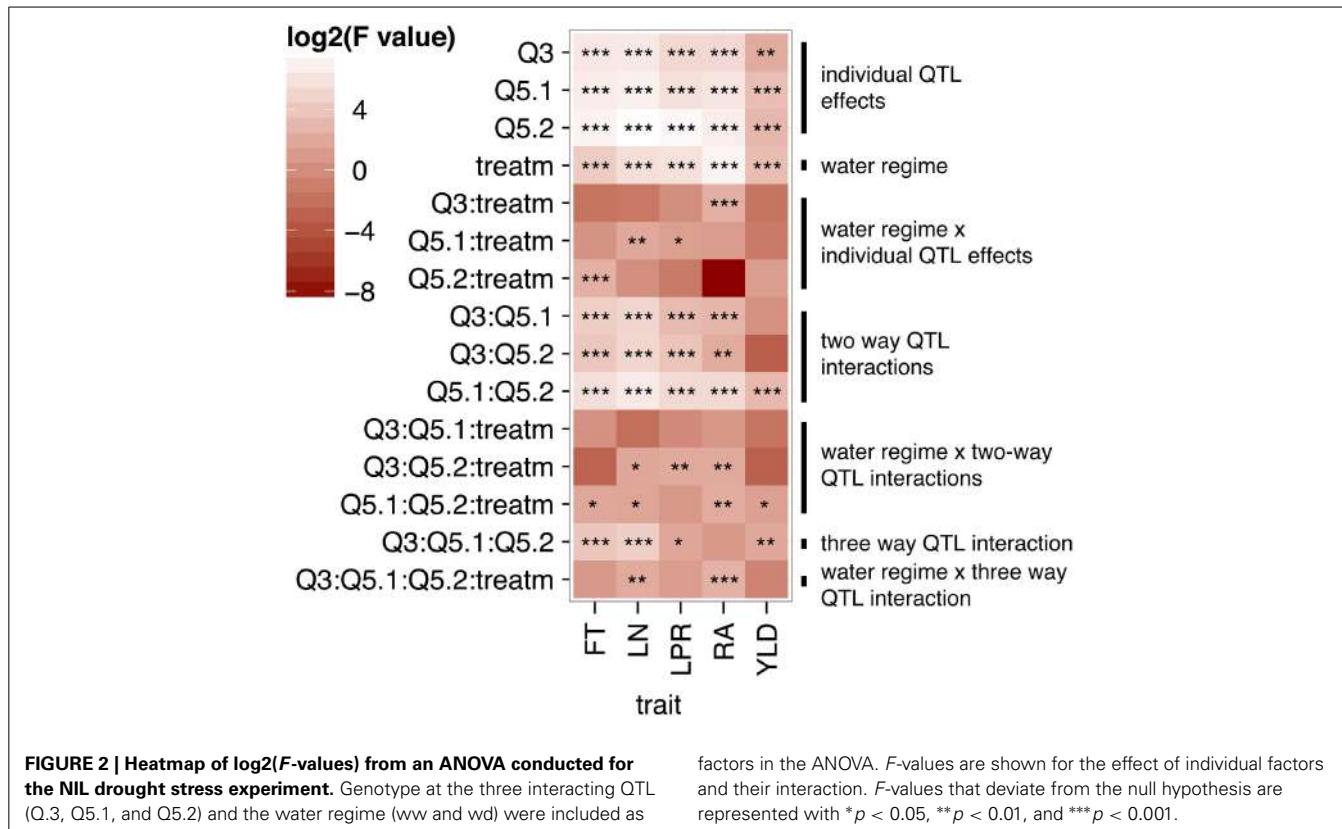


FIGURE 1 | Correlation between flowering time (FT) and (A) FT in response to water deficit and (B) rosette area (RA) in response to water deficit. Each dot represents one of the 117 *Ler* x *An-1* RILs (ww and wd; Tisné et al., 2010). The regression for all points is shown as a solid blue line.

Table 1 | Pearson's correlation coefficients (*r*) for flowering time, three growth related traits and their relative responses to water deficit in the *Ler* x *An-1* RILs.

| Traits | LN | LPR | RA | FT response | LN response | LPR response | RA response |
|--------------|---------|---------|---------|-------------|-------------|--------------|-------------|
| FT | 0.92*** | 0.83*** | 0.86*** | 0.51*** | 0.62*** | 0.32*** | 0.57*** |
| LN | | 0.97*** | 0.94*** | 0.52*** | 0.69*** | 0.40*** | 0.60*** |
| LPR | | | 0.92*** | 0.51*** | 0.66*** | 0.39*** | 0.58*** |
| RA | | | | 0.64*** | 0.68*** | 0.26** | 0.51*** |
| FT response | | | | | 0.63*** | -0.14 | 0.33*** |
| LN response | | | | | | 0.67*** | 0.75*** |
| LPR response | | | | | | | 0.68*** |

Trait abbreviations are explained in Material and Methods. For calculating correlation coefficients, the means of the performance of each RIL were averaged across the two treatments (ww and wd). The *r*-values are significant with ***P* < 0.01 or ****P* < 0.001.



relationship between flowering time and growth responses to drought. As expected from the ANOVA described above, the lines displayed significant variation for these traits in both water regimes as well as for their response to drought (**Figure 3**). With some exceptions, all lines showed a reduction of all parameters in wd condition, especially in RA (**Figure 3C**).

The line carrying *Ler* alleles at QTL3 and *An-1* alleles at both QTL5.1 and QTL5.2 (in the following called NIL LAA, for *Ler:An-1:An-1* at QTL3:QTL5.1:QTL5.2) flowered the latest and had the highest RA, LN, LPR, and YLD in both conditions (**Figure 3**). In agreement with the correlations observed in the RILs (**Figure 1, Table 1**), these phenotypes were associated with the lowest growth response to drought, i.e. the lowest reduction of RA in wd (**Figure 3C**). Moreover, line LAA did not change its LN in response to drought, whereas all other NILs had significantly less leaves in wd than in control condition (**Figure 3B**). The low growth response of NIL LAA was associated with maintained fitness under drought. As shown in **Figure 3E**, line LAA had the highest YLD under both conditions and exhibited no response to drought for this parameter. In contrast, YLD was considerably reduced in wd in all other lines except AAA that flowered the second latest and exhibited the second lowest growth reduction among all lines.

In agreement with the correlations detected in the RILs, late flowering was associated with low growth responses to drought in the NILs. As, furthermore, these phenotypes were associated with stable fitness under drought, late flowering seem to be favorable under the mild drought condition applied here.

LATE FLOWERING ALLOWS DROUGHT ACCLIMATIZATION DURING THE SECOND HALF OF VEGETATIVE DEVELOPMENT

The phenotypic differences at the time of flowering among RILs and NILs described above reflect the culmination of a drought response strategy that has occurred during the vegetative phase of the plants' life cycle. In order to describe the different strategies to cope with water deficit in detail, we monitored plant growth over time in the NILs. We quantified RA from images of plants taken approximately every 2 days in ww and wd conditions and calculated the relative RA response for each time point. Sample images from the earliest and latest flowering NIL, respectively, are shown in Supplemental Figure 2.

We observed two different phases in the drought responses of the NILs. For approximately the first 40 days of the experiment, the differences between control and drought grown plants increased steadily for all NILs, and no significant variation in this pattern was detected between the lines (**Figure 4**). All lines displayed a progressive reduction of rosette growth under wd. After this period, the early lines reached the end of their vegetative phase, i.e., they started flowering (e.g., NILs ALL and AAL). Interestingly, the late flowering NILs LAA and AAA were able to recover during the second half of their vegetative phase, as indicated by a continuous decrease in their RA response over time (**Figure 4**). During this period, the late lines grew faster in wd than in ww control condition, enabling them to compensate for previous growth deficits (Supplemental Figure 3). As NIL LAA flowered the latest, it had most time to recover, resulting in the lowest reduction of RA in response to wd at its transition to the reproductive phase (**Figure 4, Figure 3C**).

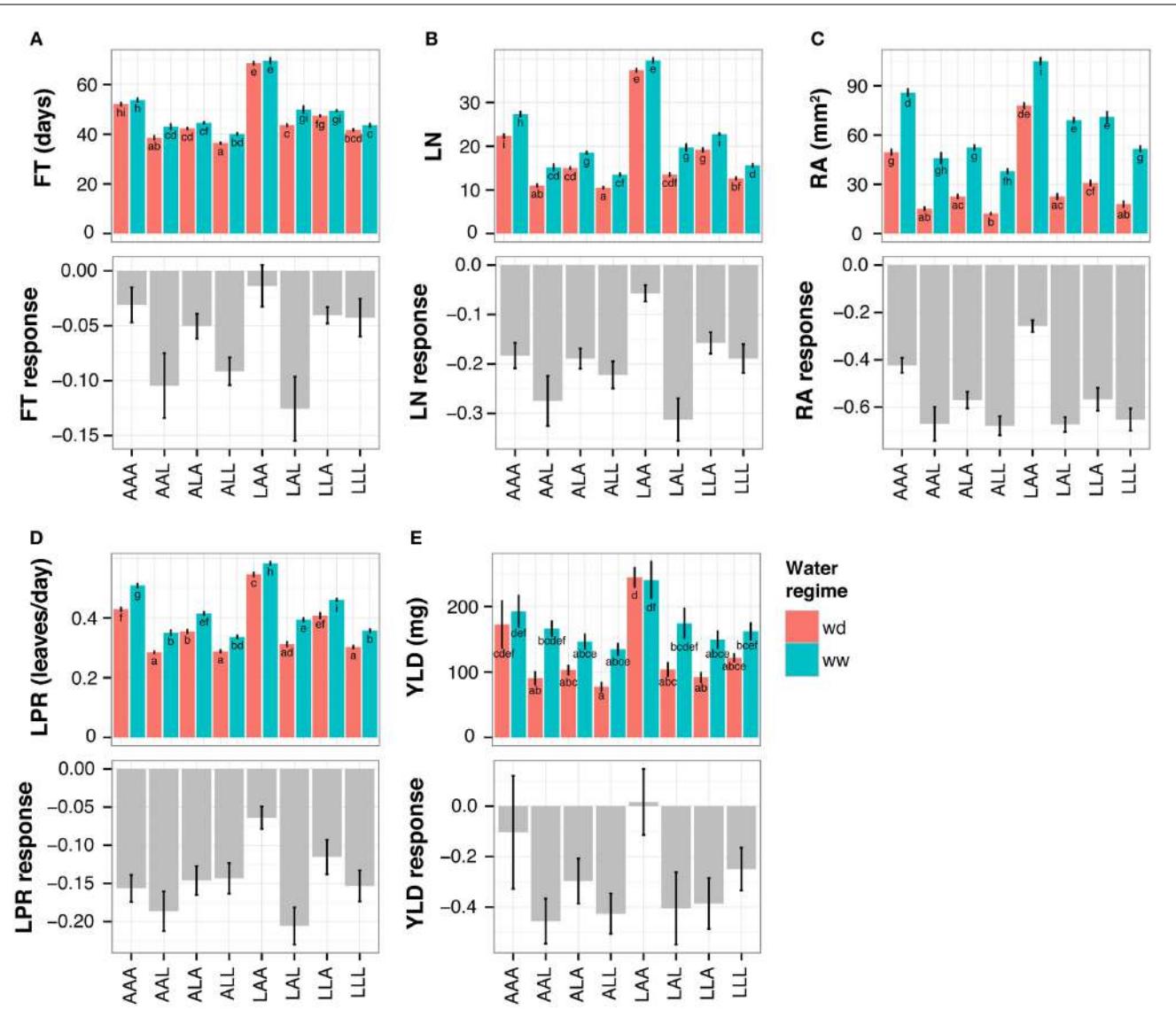


FIGURE 3 | Trait performances of NILs in both water regimes (upper panel) and in response to water deficit (lower panel, shown as relative response). At floral transition the following parameters and their responses to drought were quantified: (A) flowering time (FT; in days after sowing), (B) total leaf number (LN), (C) projected rosette area (RA) and (D) leaf production rate (LPR). Furthermore, after seed ripening (E) seed yield (YLD) and its response were quantified. The NILs are named according to their allelic combination at QTL3, QTL5.1, and QTL5.2, where the An-1 and Ler alleles are abbreviated by "A" and "L," respectively. Average trait values for 8–12 plants per NIL and treatment (mean of 11.5, median of 12). Wd, water deficit; ww, well-watered condition. Error bars indicate the standard error of the mean. Distinct letters indicate significant differences calculated with a Tukey's HSD test from an ANOVA with treatment and genotype at each QTL as factors, including all possible interactions.

of the Ler x An-1 RIL population grown under two water regimes (Tisné et al., 2010). We detected highly significant positive correlations between FT and several growth related parameters in general, i.e. averaged across the two water conditions, and in response to wd (Figure 1, Table 1). Most notably, late flowering RILs delayed their floral transition in response to wd, which was associated with low growth responses to drought. Making use of NILs, we demonstrate here that these correlations are, at least partially, controlled by an epistatic QTL network involving three QTL. In this framework of interacting loci, allelic combinations that resulted in late flowering conferred less reduced growth and

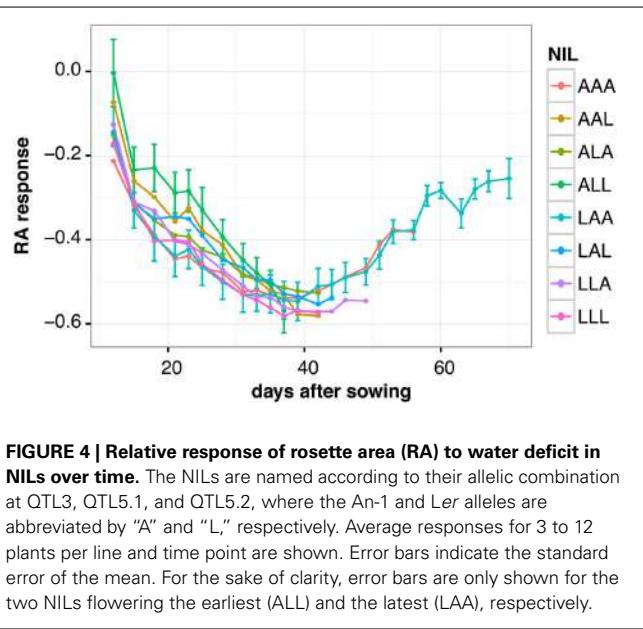
In conclusion, analysis of dynamic growth responses revealed an advantage of a late floral transition under continuous moderate drought stress as it allows plants to recover from water deficit over time before setting seeds.

DISCUSSION

AN EPISTATIC QTL NETWORK CONTROLS VARIATION IN FLOWERING TIME, GROWTH AND THEIR RESPONSES TO WATER DEFICIT IN THE Ler X An-1 POPULATION

In order to study the relationship between FT and growth in response to a continuous mild drought stress, we reanalyzed data

of the Ler x An-1 RIL population grown under two water regimes (Tisné et al., 2010). We detected highly significant positive correlations between FT and several growth related parameters in general, i.e. averaged across the two water conditions, and in response to wd (Figure 1, Table 1). Most notably, late flowering RILs delayed their floral transition in response to wd, which was associated with low growth responses to drought. Making use of NILs, we demonstrate here that these correlations are, at least partially, controlled by an epistatic QTL network involving three QTL. In this framework of interacting loci, allelic combinations that resulted in late flowering conferred less reduced growth and



stable fitness under drought, and, thus, can be seen as favorable under mild water deficit conditions (Figure 3). This association between flowering time and growth in response to drought is in agreement with the positive correlation detected in the RIL population. However, one has to be aware that in the NIL experiment, growth has been quantified as projected rosette leaf area (RA). Using this method, an overlap of leaves that appear later in development with older leaves (see Supplemental Figure 2, time points 56 and 70 d.a.s) might bias the growth measurements, and, thus, the quantification of the RA response to wd. Nevertheless, two observations support that the differences in the response to drought detected between late and early NILs are real. First, similar results as ours were obtained in the RIL experiment where RA was determined from scans of individual leaves (Tisné et al., 2010). Second, the reduced response to drought observed for the growth of late NILs was also observed for their yield, a trait that has been measured independently from the images.

Interestingly, the late flowering NILs did not delay FT in response to drought as detected for late flowering RILs (Figure 1), but displayed no change in FT under drought, whereas early flowering lines accelerated floral transition slightly (Figure 3A). A reason for this discrepancy between the NIL and the RIL experiment might be that plants in the NIL experiment were unintentionally subjected to a less severe drought stress than the RILs resulting in an overall smaller response of FT. Although we tried to mimic the experimental conditions of the RIL experiment, unavoidable differences due to for instance micro-meteorological variation might have occurred. Nevertheless, in both experiments, the late flowering lines reduced their growth less than the early flowering lines (Figures 1B, 3A,C).

Our results raise the question of whether the correlations observed are specific for the genetic material studied here or if they are a general phenomenon in *A. thaliana*. Aguirrezaibar et al. (2006) analyzed growth responses to soil water deficit of 25 natural accessions and did not detect any correlations between

leaf number (which is a common measurement of FT) and responses to drought in leaf number or RA. Furthermore, An-1, which is early flowering, was shown to maintain its growth better under drought than other accessions that are known to flower significantly later (Shindo et al., 2005; Granier et al., 2006). These results suggest the correlations we detected here are rather specific. More comprehensive studies with for instance flowering time mutants or other segregating populations would be needed to corroborate a general association between FT and growth responses to drought.

In the present study, we confirm and further characterize a complex QTL network with pleiotropic effects on two major plant traits and their responses to moderate drought stress. Multiple QTL studies in *A. thaliana* and other species have demonstrated that the analysis of epistasis (QTL × QTL interactions) is essential for describing the architecture of quantitative traits (reviewed in Mackay, 2014). Although epistasis between two QTL has been detected frequently, analyses of higher dimensional interactions like the one reported here are so far rare.

FLC AND HUA2 ARE CANDIDATE GENES FOR QTL5.1 AND 5.2, RESPECTIVELY

As described previously, the two QTL on chromosome 5 are close to genes known to affect flowering time (Tisné et al., 2010). These are *FLC* (close to QTL5.1) and the putative transcription factor *HUA2* (close to QTL5.2) that up regulates expression of *FLC* (Doyle et al., 2005). Both genes are reasonable candidates for the detected QTL as both of them exhibit functional polymorphisms for the two parental accessions. Whereas *Ler* carries a weak *FLC* allele whose expression is inhibited by an intronic transposon (Liu et al., 2004), *An-1* contains a stronger allele (El-Lithy et al., 2006). Thus, we hypothesize that the variation in flowering time we observed among NILs is associated with differences in *FLC* expression. Indeed, the two NILs flowering the latest, LAA and AAA, had the highest *FLC* expression level in both water conditions. In contrast, early flowering lines displayed low expression or no expression at all (Supplemental Figure 4). Furthermore, *Ler* contains a mutation at *HUA2* (*hua2.5*) resulting in a premature stop codon, and, thus, impairing the up regulation of *FLC* (Doyle et al., 2005). We confirmed this mutation in *Ler* and its absence in *An-1* (data not shown). The allelic nature of *FLC* and *HUA2* in the two parental accessions is in agreement with the epistatic effects observed here. The presence of strong *An-1* alleles at both *FLC* and *HUA2* (NILs LAA and AAA) is associated with high *FLC* expression levels and a late floral transition. Thus, we hypothesize that the QTL network studied here is based on a *FLC* dependent regulation involving different *FLC* regulators, such as *HUA2* and an unknown gene at QTL3. Supporting this hypothesis, Tisné et al. (2010) described a similar QTL network including QTL5.1, QTL5.2 and a QTL on chromosome 4 that also regulates FT and growth in response to drought. Here, as well, late flowering lines showed a low growth response to wd, probably based on the up-regulation of *FLC* through its main regulator *FRI* (Johanson et al., 2000), a possible candidate gene for the QTL on chromosome 4 (Tisné et al., 2010).

Several studies have linked *FLC* and plant-water relations. Different models including *FLC* as one regulator have been

proposed for the regulation of drought responsive flowering (Riboni et al., 2013; Xu et al., 2014). Furthermore, a *FLC* dependent pleiotropic effect of *FRI* on flowering time, growth rate and WUE has been reported as described below in more detail (McKay et al., 2003; Lovell et al., 2013).

Nevertheless, further analyses, such as QTL fine-mapping, cloning, and complementation, would be required to confirm the candidate genes proposed here.

LATE FLOWERING ENABLES DROUGHT ACCLIMATIZATION OVER TIME

In our lines, a late floral transition was strongly correlated with a reduced sensitivity to drought, and, thus, we assumed that late flowering plants follow a specific strategy to cope with soil water deficit. To test this, we quantified dynamic growth responses of the NILs. Although in the first half of the experiment, we could not detect any significant differences between early and late flowering genotypes, we observed a recovery of late flowering lines over time resulting in the lowest reduction of RA at floral transition. These results lead to the assumption that it is crucial for a plant at which developmental stage it is hit by drought stress. In our study, the drought treatment started for all genotypes at the same time (See Material and Methods) neglecting their different developmental stage. Thus, for early flowering plants, the wd occurred late during their vegetative phase when they already had initiated flowering. These plants had probably no time to acclimatize to the stress due to their early transition to the reproductive phase. As a consequence, growth and fitness were strongly impaired under wd and flowering slightly accelerated. In contrast, late flowering lines seemed to be able to acclimatize to the stress, possibly due to diverse physiological adjustments, as it occurred early during their life cycle. In addition, as the length of the vegetative phase was the same for late flowering lines in ww and in wd, the drought stressed plants were able to compensate previous growth deficits better than early flowering lines in wd. Based on the observations of Tisné et al. (2010) in the same population, we speculate that the late flowering NILs are able to maintain both epidermal cell area and cell number under wd better than all other lines, resulting in less reduced rosette size at floral transition. Furthermore, Juenger et al. (2005) observed a positive correlation between flowering time and WUE in the *Ler* x Cape Verde Island (Cvi) RIL population, where late flowering lines exhibited higher WUE. Similarly, Lovell et al. (2013) established a link between WUE and growth rate through variation in *FRI*. Functional *FRI* alleles confer a dehydration avoidance strategy in which plants are late flowering, have a higher WUE and decreased growth rate. This effect of *FRI* was only observed in the presence of a functional *FLC* allele (McKay et al., 2003; Lovell et al., 2013). Thus, we may hypothesize that our late flowering NILs carrying functional *FLC* alleles and showing increased *FLC* expression (Figure 4) may exhibit a higher WUE than the early flowering genotypes. In addition, we may assume that the overall physiological state of our NILs is affected by allelic variation at QTL3. Juenger et al. (2005) detected pleiotropic effects of a QTL colocalizing with our QTL3 on flowering time and WUE in the *Ler* x Cvi RILs. Analysis of a NIL revealed that presence of the *Ler* allele at this QTL does not only result in increased flowering time and WUE, but also in lower stomatal conductance and higher

transpiration efficiency. Furthermore, the *Ler* allele was associated with a decreased water loss rate measured from whole rosettes over time (Juenger et al., 2005). Whether these characteristics are advantageous under water deficit is not clear. However, to get insight into the mechanisms underlying the drought acclimatization strategy of our late flowering lines, comprehensive analyses, such as physiological studies, genome wide gene expression (Des Marais et al., 2012) or metabolite studies (reviewed in Verslues and Juenger, 2011), would be required.

In summary, our quantification of growth responses over time revealed two distinct phases of drought responses. The first phase was characterized by a strong reduction of growth due to water deficit in all lines, regardless their flowering time. Whereas most of the lines flowered after this phase, late flowering lines exhibited a second phase of growth recovery from drought. Only their late floral transition enabled them to compensate growth deficits resulting from wd through increased growth rates. In contrast, early flowering genotypes followed a drought escape strategy, which is known as a common mechanism to enable plant survival and reproduction before lethal drought conditions occur (Ludlow, 1989). Under the continuous moderate drought stress we applied here that was characterized by short cycles of drying out and re-watering (See Material and Methods), such a drought escape strategy resulted in strongly impaired growth and reduced fitness. In contrast, a late floral transition allowed plants to acclimatize to wd over time, and thus, proved to be advantageous for plant fitness under moderate drought stress.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00609/abstract>

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The role of seasonal flowering responses in adaptation of grasses to temperate climates

Siri Fjellheim¹, Scott Boden² and Ben Trevaskis^{2*}

¹ Department of Plant Sciences, Norwegian University of Life Sciences, Ås, Norway

² Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra, ACT, Australia

Edited by:

Maria Von Korff Schmising, Max Planck Society, Germany

Reviewed by:

Iain Robert Searle, The University of Adelaide, Australia

Wilma Van Esse, Max Planck Institute for Plant Breeding Research, Germany

***Correspondence:**

Ben Trevaskis, Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, GPO Box 1600, Canberra, ACT 2601, Australia

e-mail: ben.trevaskis@csiro.au

Grasses of the subfamily Pooideae, including important cereal crops and pasture grasses, are widespread in temperate zones. Seasonal regulation of developmental transitions coordinates the life cycles of Pooideae with the passing seasons so that flowering and seed production coincide with favorable conditions in spring. This review examines the molecular pathways that control the seasonal flowering responses of Pooideae and how variation in the activity of genes controlling these pathways can adapt cereals or grasses to different climates and geographical regions. The possible evolutionary origins of the seasonal flowering responses of the Pooideae are discussed and key questions for future research highlighted. These include the need to develop a better understanding of the molecular basis for seasonal flowering in perennial Pooideae and in temperate grasses outside the core Pooideae group.

Keywords: flowering, adaptation, biological, evolution, molecular, seasonality, Pooideae

OVERVIEW

Grasses from the Pooideae subfamily, family Poaceae, occur widely in temperate regions (Hartley, 1973). In addition to being important ecologically this group includes economically important pasture grasses and cereal crops, as well as invasive species and weeds. Adaptations that allow Pooid grasses to survive the seasonal extremes of temperate climates are of interest since these are central to the success of this sub-family across temperate zones. Such adaptations are also important to agriculture and are critical to the success of temperate cereal crops including wheat, barley, oats, and rye.

A key factor underlying the adaptation of grasses to temperate climates is timing. Timing biological events to occur during specific seasons is the basis for stress avoidance strategies. For example, delaying flowering until after winter allows many grasses to avoid frost damage to cold sensitive reproductive organs (see Fowler et al., 1996). Alternatively, activation of tolerance mechanisms at particular times of year can prime plant physiology for predictably harsh seasonal conditions before these occur. An example of seasonally timed tolerance is the cold acclimation response, where freezing tolerance increases during autumn to maximize chances of winter survival (Thomashow, 2010).

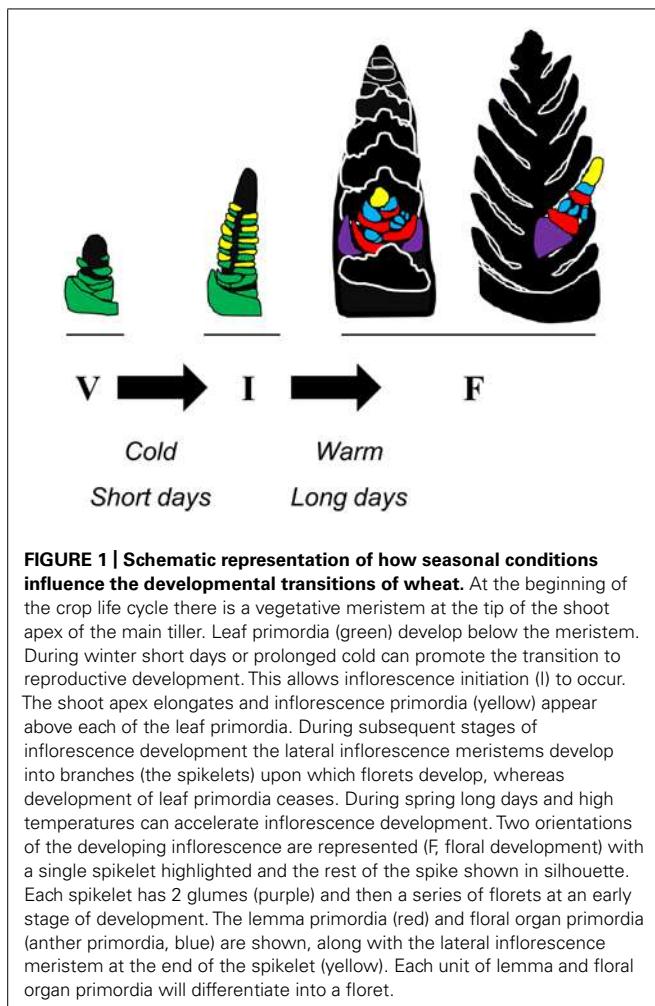
The study of the seasonal timing of biological phenomena is known as phenology. The broad topic of this review is the contribution of phenology to the success of Pooideae in temperate climates. We will highlight how different seasonal cues can adjust the developmental program of grasses to match seasonal cycles, using temperate cereals as examples, and then examine how naturally occurring variation in these responses drives adaptation to different geographical regions and climates. Then we will discuss how the molecular pathways controlling the seasonal flowering responses of temperate grasses might have evolved.

DEVELOPMENTAL PHASES OF TEMPERATE CEREALS AND GRASSES

The developmental phases of temperate cereals and related temperate grasses can be divided into two broad stages: vegetative and reproductive. During the vegetative phase the shoot apex produces only leaf primordia. Then, when the transition to reproductive growth occurs, inflorescence primordia develop above leaf primordia at the apex. This stage is referred to as inflorescence initiation (Evans, 1964). Subsequently the inflorescence primordia develop into lateral inflorescence branches, called spikelets, whereas the development of the leaf primordia ceases (Figure 1). As inflorescence development proceeds floral primordia appear on the developing spikelets and these develop into flowers, referred to as florets (see Barnard, 1964). Various arrangements of spikelet number, branch length, and floret number exist, giving rise to diverse inflorescence shapes, from compact spikes (e.g., wheat and barley) to panicles (e.g., oats). Internodes beneath the developing inflorescence elongate to form the stem, carrying the inflorescence upwards inside a sheath of leaves. The inflorescence emerges from the leaf sheath shortly before anthesis. In annual grasses the transition from vegetative to reproductive development occurs once. The situation for perennial grasses is more complex and will be discussed later.

SEASONAL REGULATION OF DEVELOPMENT PHASES IN TEMPERATE GRASSES

The different developmental phases of temperate grasses (limited to Pooid grasses in this review) are timed to optimally coordinate the plant life cycle with the changing seasons. This timing is achieved through the influence of seasonal changes in temperature and daylength on specific points in the developmental program (Figure 1). Initially there is a delay of inflorescence initiation prior to winter. This delay is removed when plants over-winter,



allowing inflorescence initiation to occur. Subsequently, increasing daylength and warmer temperatures accelerate development of the inflorescence in spring (Purvis, 1934; Evans, 1964). The overall combination of these seasonal developmental responses ensures that plants remain vegetative before winter, avoiding frost damage to developing reproductive organs, but then flower rapidly in spring, to allow flowering and seed production before the onset of heat and water limitation in summer.

THE RELATIONSHIP BETWEEN DEVELOPMENTAL PHASES AND OTHER TRAITS

Seasonal timing of developmental phases influences other traits. Sometimes this is simply a matter of timing. For example, if there is limited water at the end of the growing season the timing of flowering (heading date) will influence grain yield of cereal crops because early flowering lines can produce grain before water becomes limiting whereas late flowering lines encounter drought stress, which reduces yield. There are many field studies that demonstrate indirect effects caused by the timing of flowering (Kuchel et al., 2007, for example). In other instances there is a direct relationship between development and other traits. The best understood example is the relationship between flowering and frost tolerance in

wheat and barley. During the vegetative growth phase wheat and barley plants can acclimate to cold if exposed to non-freezing low temperatures for a period of weeks and will subsequently survive extreme winter conditions. This capacity to acclimate to cold decreases as plants develop toward flowering (Fowler et al., 2001; Limin and Fowler, 2006). Thus, seasonal regulation of development coordinates the potential for cold acclimation with seasonal conditions; vegetative plants can acclimate to cold during autumn and winter, but this capacity is lost as plants begin to flower in spring, when there is less risk of frost. The influence of developmental phases on the seasonal activation of abiotic and biotic stress tolerances is an important component of the adaptation of grasses to temperate climates.

MOLECULAR PATHWAYS CONTROLLING SEASONAL FLOWERING RESPONSES IN *Arabidopsis*

Arabidopsis (*Arabidopsis thaliana*) is adapted to temperate climates and, like temperate grasses, flowers in response to vernalization (the prolonged cold of winter) and long days (see Amasino, 2010). *Arabidopsis* provides a useful guide to the types of genes/proteins that regulate flowering and, although there are important differences, the molecular mechanisms controlling seasonal flowering responses of grasses can be viewed as variations of those that also occur in *Arabidopsis*.

Seasonal flowering of *Arabidopsis* is controlled through regulation of the floral transition, the point when the floral primordia appear at the shoot apex and flowering begins. In winter annual *Arabidopsis* ecotypes the floral transition is delayed until plants experience vernalization. This delay is caused by *FLOWERING LOCUS C* (*FLC*), which encodes a MADS box transcription factor that represses flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* is expressed at high basal levels prior to vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999). Prolonged exposure to cold represses transcription of *FLC*, through mechanisms that act at the chromatin of the *FLC* gene to limit transcription. For example, tri-methylation of histone-3-lysine 27 (H3K27Me3) in nucleosomes at the *FLC* locus is required to maintain repression of *FLC* in vernalized plants (Bastow et al., 2004; Schubert et al., 2006; Finnegan and Dennis, 2007). Stable repression of *FLC* after vernalization provides a molecular memory of winter. Genetic variation in *FLC* activity, either due to mutations in the *FLC* gene itself or in regulators of *FLC* expression, can reduce or eliminate the ancestral vernalization requirement of *Arabidopsis* and adapt ecotypes to different climates (see Amasino, 2010).

Long days accelerate the floral transition of *Arabidopsis*. The long-day flowering response depends on an internal timekeeping mechanism, the circadian clock, which activates expression of *CONSTANS* (*CO*) in the late afternoon (Suarez-Lopez et al., 2001). The *CO* protein is stable in light (Valverde et al., 2004). Thus, when days are long *CO* expression coincides with light and the protein is active. This activates transcription of *FLOWERING LOCUS T* (*FT*) in the leaves. *FT* encodes a phosphatidyl-ethanolamine binding protein (PEBP) that is translocated through the phloem from the leaves to the shoot apex (Kardailsky et al., 1999; Kobayashi et al., 1999; Corbesier et al., 2007). At the shoot apex the *FT* protein interacts with *FLOWERING LOCUS D*, a bZIP transcription

factor (Abe et al., 2005; Wigge et al., 2005). The resulting complex activates transcription of genes that promote floral development, including *AP1*, thus triggering the floral transition. Activation of *FT* by CO is likely to be mediated through interactions with nuclear factor Y proteins (NF-Y) – also known as heme activator proteins (HAP) or CCAAT binding factors (referred to hereafter as NF-Y proteins; Ben-Naim et al., 2006; Wenkel et al., 2006). These are components of a conserved eukaryotic transcriptional activation complex. Interactions between CO and NF-Y proteins are mediated by the CCT domain; a protein domain first identified in CO, CO-like and TIMING OF CAB1 EXPRESSION1 proteins (Wenkel et al., 2006). Long-day activation of *FT* is suppressed in *Arabidopsis* plants that have not been vernalized (Lee et al., 2000; Michaels et al., 2005). This is due to direct repression of *FLC*, which binds to the intron of the *FT* gene (Helliwell et al., 2006).

High temperatures ($>25^{\circ}\text{C}$) can activate expression of *FT* in short days and accelerate the floral transition (Blázquez et al., 2003; Balasubramanian et al., 2006; Turck et al., 2008). Elevated temperatures during the night activate *FT* expression. Activation of *FT* by high-temperatures is largely independent of CO and instead *PHYTOCHROME-INTERACTING FACTORS* (*PIF4* and *PIF5*) are required (Kumar et al., 2012; Thines et al., 2014). The *FLC*-like gene *FLOWERING LOCUS M1* (*FLM1*) has also been implicated in the regulation of the high-temperature flowering response of *Arabidopsis* (Balasubramanian et al., 2006). This gene is differentially spliced in a temperature dependent manner, producing a splice variant that represses flowering at lower temperatures (Lee et al., 2013; Posé et al., 2013).

As outlined in the next section, many of the classes of genes/proteins that regulate seasonal flowering responses in *Arabidopsis* also play important roles in regulating reproductive development in cereals. There are, however, few direct overlaps between *Arabidopsis* and cereals, with fundamental differences in the physiology of seasonal flowering responses and in the underlying molecular networks.

MOLECULAR NETWORKS CONTROLLING SEASONAL FLOWERING RESPONSES IN TEMPERATE CEREALS

There exists considerable diversity in seasonal flowering responses amongst temperate cereals. Some lack the requirement for vernalization, others are daylength insensitive. For the purpose of this review we will consider the presence of a vernalization requirement combined with daylength sensitivity as being the archetypal flowering behavior of temperate cereals. This view is supported by molecular evidence (see subsequent sections) that reduced vernalization requirement and reduced daylength sensitivity are acquired states that have arisen on multiple occasions through mutation.

THE VERNALIZATION REQUIREMENT DELAYS INFLORESCENCE INITIATION BEFORE WINTER

A core feature of the flowering behavior of temperate cereals and related grasses is the delay of inflorescence initiation before winter; the vernalization requirement. For example, many wheats and barleys will grow vegetatively for extended periods and will not flower without overwintering. Genetic studies of wheat and barley have identified the *VRN2* locus as a key gene controlling

vernification requirement in the cereals (Takahashi and Yasuda, 1971). Duplicated zinc finger-CCT domain proteins are found at the *VRN2* locus (Yan et al., 2004a). Some accessions of barley and diploid wheats lack a functional copy of *VRN2* due to loss-of-function mutations in the *VRN2* coding sequence or due to naturally occurring deletions of the entire *VRN2* locus (Yan et al., 2004a; Dubcovsky et al., 2005). These accessions can undergo rapid inflorescence initiation without vernalization. This only occurs in long days, however, where rapid flowering is associated with elevated expression *FT-like 1* (*FT1*), the functional equivalent of *FT* in cereals (Karsai et al., 2005; Turner et al., 2005; Hemming et al., 2008). This suggests that the normal role for *VRN2* is to block long-day induction of *FT1*. Consistent with this hypothesis, transcription of *VRN2* occurs in long days (12 h light or longer) and constitutive expression of *VRN2* down-regulates *FT1* (Trevaskis et al., 2006; Hemming et al., 2008). The *VRN2* protein is distantly related to CO and, like CO, can interact with NF-Y proteins via a CCT domain (Li et al., 2011). Binding of *VRN2* to NF-Y proteins might inhibit transcription of *FT1* by blocking binding of other CCT domain proteins that normally activate transcription of *FT1*, such as the cereal CO homologs.

SHORT DAYS ALLOW INFLORESCENCE INITIATION

Cereal varieties that are unable to flower without vernalization when grown in long days can flower in short days, suggesting that inhibition of inflorescence initiation is weaker in short days (Evans, 1987). This is also evidenced by “short-day vernalization,” where plants grown in short days for several weeks will flower when shifted to long-days (Purvis and Gregory, 1937). Low-levels of *VRN2* expression in short-days might allow inflorescence initiation in short-days (Dubcovsky et al., 2006; Turner et al., 2013). This is unlikely to occur through *FT1*, which is expressed mainly in long days. Another *FT*-like gene (*FT3*) might be important. *FT3* is normally expressed in short but not long days, and loss-of-function mutations in *FT3* delay flowering primarily in short days (Faure et al., 2007; Kikuchi et al., 2009). Interestingly, *FT3* is expressed at high levels in long days in lines that lack *VRN2*. So daylength specificity of *FT3* expression might be mediated by *VRN2* (Casao et al., 2011). It is not known whether loss-of-function mutations in *FT3* block the short-day vernalization effect. The mechanisms controlling inflorescence initiation in short-days are an interesting area for future research.

THE PROLONGED COLD OF WINTER PROMOTES INFLORESCENCE INITIATION

Prolonged exposure to low-temperatures (vernification) promotes inflorescence initiation (Chouard, 1960). Vernalization can be applied to imbibed seeds or to actively growing plants and is effective irrespective of daylength, even in total darkness (Gassner, 1918; Gott et al., 1955). The effect of vernalization on plant development can be separated from the actual low-temperature treatment. For example, vernalization of imbibed seeds promotes rapid inflorescence initiation when plants are subsequently shifted to normal glasshouse conditions (Sasani et al., 2009, for example). Thus, there is a memory of prolonged cold treatment. Typically temperatures between 0 and 10°C are effective for vernalization

and the effect of cold is quantitative, with longer cold treatments causing more rapid inflorescence initiation until a point when further cold causes no further reduction in the time taken to flower; the vernalization saturation point (Gassner, 1918; Gott et al., 1955).

The promotion of inflorescence initiation by vernalization is stronger than the effect of short days. For example, in experiments performed by Allard et al. (2012) wheat plants exposed to prolonged low-temperatures (5°C for 30 days) flowered with 8 leaves (primary tiller). Typically there are four to five leaf primordia present at the time of germination and these develop into leaves irrespective of conditions during subsequent growth, so a final leaf number of 8 is indicative of a rapid progression toward flowering. In comparison, plants grown without vernalization in long days flowered with 16 leaves. Plants grown in short days for 3–10 weeks then shifted to long days flowered with 12 leaves, showing that short days can promote inflorescence initiation, though the effect is weaker than that of prolonged cold (12 leaves following short day treatment versus 8 leaves after vernalization). At a broader level the effect of prolonged cold (*vernalization sensu stricto*) is profoundly different to that of short-day treatment because vernalization at low temperatures slows growth. This is particularly evident with vernalization of imbibed seeds where plants can emerge from prolonged cold treatment with only one or two expanded leaves. In comparison plants exposed to several weeks of short days will have many leaves on the main stem and also secondary tillers. Thus, the term “short-day vernalization” should be interpreted with caution, as noted by Evans (1987) who preferred the term “short-day induction.”

The central gene controlling vernalization in cereals is *VERNALIZATION1* (*VRN1*), a MADS box transcription factor gene related to the *API/FRUITFULL* genes of *Arabidopsis* (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Trevaskis, 2010). *VRN1* promotes inflorescence initiation but is expressed at low levels prior to vernalization and this limits the rate of progression toward inflorescence initiation (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). Exposure to low temperatures induces transcription of *VRN1* (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). This begins rapidly with the onset of cold, within 12 h, but initial expression is weak and several weeks of cold are required to elevate *VRN1* transcript levels to a level that promotes rapid inflorescence initiation (Sasani et al., 2009; Oliver et al., 2013). There is a strong relationship between the length of cold experienced, *VRN1* expression levels, and the degree to which inflorescence initiation is accelerated post-vernalization (Sasani et al., 2009). These observations are all consistent with a model where stable induction of *VRN1* provides a quantitative memory of vernalization (Trevaskis et al., 2007; Trevaskis, 2010). Indeed, transcriptome analyses show that *VRN1* is one of a limited number of genes that show lasting changes in expression levels in response to seed vernalization (Greenup et al., 2011). Low temperatures induce changes in histone modification of nucleosomes associated with *VRN1*, which might provide a mechanism for stable activation of this gene (Oliver et al., 2009, 2013).

Mutations that disrupt *VRN1* function greatly reduce the impact of vernalization, supporting a central role for *VRN1* in the vernalization response (Chen and Dubcovsky, 2012). Conversely, naturally occurring mutations in the promoter or large insertions/deletions in the first intron of *VRN1* are associated with elevated basal transcription and cause rapid flowering without vernalization (Yan et al., 2004b; Fu et al., 2005). These mutations have been used to breed and select cereal cultivars with reduced vernalization requirement, which are grown in regions or at times of year when vernalization does not occur (see below).

LONG DAYS ACCELERATE INFLORESCENCE DEVELOPMENT AFTER VERNALIZATION

Daylength can influence both the timing of inflorescence initiation and the rate of inflorescence development after vernalization. In long days vernalized plants will progress rapidly through inflorescence initiation and subsequent stages of inflorescence development until head emergence and anthesis. In short days inflorescence initiation occurs, though not as rapidly as in long days, and thereafter inflorescence development occurs slowly and there is a strong delay of head emergence and anthesis (e.g., Limin and Fowler, 2006; Sasani et al., 2009).

Although long days can accelerate inflorescence initiation in glasshouse experiments this is unlikely to occur in the field for autumn sown varieties (vernalization requiring cultivars, see comments above regarding archetypal flowering behavior), which typically undergo inflorescence initiation in short days toward the end of winter. Instead longer days are likely to coincide with the inflorescence development and stem elongation stage (Figure 1). Daylength shift experiments, where wheat plants are shifted from short to long days, or vice versa, at different time points, show that the time from inflorescence initiation to the point when the terminal spikelet develops is a critical window for the acceleration of inflorescence development by long daylengths (Miralles and Richards, 2000).

Key genes controlling the long-day flowering response are *PHOTOPERIOD1* (*PPD1*) and *FT*, the cereal *FT* equivalent (Turner et al., 2005; Shaw et al., 2012). Expression of *FT1* is induced by long days, analogous to *FT* in *Arabidopsis*, and it seems that the role for the *FT* protein as a leaf expressed mobile flowering signal is conserved in cereals (Turner et al., 2005; Tamaki et al., 2007). Long-day induction of *FT1* requires *PPD1*. *PPD1* encodes a pseudo response regulator related to components of the circadian clock and includes a CCT domain that can interact with NF-Y complexes, similar to *CO* (Turner et al., 2005; Li et al., 2011). Transcription of *PPD1* follows a distinctive diurnal profile, though current models suggest that *PPD1* does not play a role in circadian clock *per se*, and instead regulates outputs of the clock under long days to induce expression of *FT1* (Turner et al., 2005; Campoli et al., 2012).

Deletions in the promoter of the wheat *PPD1* gene are associated with elevated transcript levels of this gene at night, in short daylengths (Beales et al., 2007). These deletions are linked to elevated *FT1* expression and reduced long-day requirement

(photoperiod insensitivity), such that plants flower early irrespective of daylength (Beales et al., 2007). Mutations that alter circadian clock rhythms can also up regulate *FT1* and reduce photoperiod sensitivity, loss of *EARLY FLOWERING 3* (*ELF3*, *MAT.a8*) or *PHYTOCLOCK1/LUX1* function, for example (Faure et al., 2012; Mizuno et al., 2012; Zakhrebekova et al., 2012; Campoli et al., 2013; Gawroński et al., 2014). Genetic activation of the *FT1* gene itself reduces daylength sensitivity, triggering constitutive early flowering (Yan et al., 2006). Activation of *FT1* can also bypass the normal requirement for vernalization; a strongly active allele of this gene was first identified as *Spring Growth Habit 3*, a locus linked to reduced vernalization requirement (subsequently named *VRN3*; Takahashi and Yasuda, 1971; Yan et al., 2006). This active allele has increased *FT1* gene copy number (four copies), which might cause increased transcriptional activity (Nitcher et al., 2013). A recent study suggests that structural alterations also occur amongst the four gene copies in this allele, so the exact cause of increased *FT1* transcription remains unclear (Loscó et al., 2014).

The *FT1* protein of wheat interacts with FD-like proteins, analogous to FT and FD in *Arabidopsis* (Li and Dubcovsky, 2008). This is likely to activate expression of *AP1*-like genes at the shoot apex, including *VRN1* but also two other *AP1*-like genes (Preston and Kellogg, 2008). *FT1* also interacts with *FDL2* in leaves (Li and Dubcovsky, 2008). This potentially allows long-day induction of *VRN1* in leaves. This pathway operates in cereal varieties where activation of *FT1* triggers inflorescence initiation without prior vernalization, such as those that lack *VRN2* or that carry active *FT1* (*VRN3*) alleles (Yan et al., 2006; Hemming et al., 2008; Shimada et al., 2009). Long-day induction of *VRN1* via *FT1* is unlikely to play a major role in triggering inflorescence initiation in response to vernalization, however, since *VRN1* is expressed at high levels in leaves and at the shoot apex of vernalized plants irrespective of daylength (Sasani et al., 2009). It is possible that a FT-FD dependent pathway might activate expression *VRN1* after short-day induction, or activate expression of other *AP1*-like genes in the leaves of vernalized plants.

Gibberellins play a role in the long-day flowering response of grasses (King and Evans, 2003). A shift to long days can trigger a rapid increase of gibberellin biosynthesis in leaves and application of gibberellins can accelerate inflorescence development in short days, mimicking the effect of longer daylengths (Evans et al., 1990; King et al., 2006). Leaf produced gibberellins might act as florigens in grasses, providing a mobile florigenic signal that is transported from the leaves to shoot apex to accelerate flowering, in addition to the FT protein (King and Evans, 2003). Applying gibberellins only accelerates inflorescence development when applied to vernalized plants or to accessions that carry active alleles of *VRN1*, placing the gibberellin response downstream of vernalization (MacMillan et al., 2005; Pearce et al., 2013; Boden et al., 2014). Paclobutrazol, an inhibitor of GA biosynthesis, slows the constitutive early flowering of *ELF3* loss-of-function mutants in short days without influencing *FT1* expression (Boden et al., 2014). This shows that gibberellins likely act parallel to, or downstream of, *FT1*.

HIGH TEMPERATURES ACCELERATE INFLORESCENCE DEVELOPMENT IN LONG DAYS

Elevated growth temperatures can accelerate flowering of cereals. This occurs in long days, where elevated temperatures accelerate inflorescence development (Rawson and Richards, 1993). In short days high temperatures have the opposite effect, slowing inflorescence development (Rawson and Richards, 1993; Hemming et al., 2012). The acceleration of inflorescence development by high temperatures when days are long makes sense in a seasonal context, since this would further accelerate flowering toward the end of spring and allow grain production to occur more rapidly in warmer conditions that might accompany increased risk of heat stress and water limitation. Overall the physiology of the high-temperature flowering response in cereals is markedly different to that of *Arabidopsis*; accelerating inflorescence development post-initiation in long days, versus promoting floral initiation in short days.

Gene expression studies suggest that neither *FT1* nor other *FT*-like genes are high-temperature responsive in cereals (Hemming et al., 2012). Furthermore, activation of *FT1* by miss-expression of *PPD1* or by mutations in components of the circadian clock do not allow high-temperatures to accelerate inflorescence development in short-days (Hemming et al., 2012). This suggests that *FT1* is not the long-day activated factor that allows high-temperature acceleration of inflorescence development. Instead, it seems that an as yet unidentified long-day activated pathway allows high-temperatures to accelerate inflorescence development.

Transcriptome analyses have been used to identify high-temperature responsive developmental regulators in barley (Hemming et al., 2012). A series of genes thought to act downstream of *VRN1* in the vernalization response show altered expression at elevated temperatures, including the MADS box gene *ODDSOC2* (Hemming et al., 2012). This gene has no direct equivalent in *Arabidopsis* but might be related to the *FLC* gene family (Ruelens et al., 2013). *ODDSOC2* is down-regulated in vernalized barley plants and represses flowering when expressed constitutively, likely through down-regulation of *FLOWERING PROMOTER FACTOR1*-like genes (*FPP1*-like; Greenup et al., 2010). *ODDSOC2* is expressed at elevated levels when vernalized plants are grown at high temperatures (constant 25°C) in short-days, where inflorescence development is retarded (Hemming et al., 2012). This highlights a potentially interesting parallel with the role of *FLC*-like MADS box genes (*FLM*) in regulating temperature responses in *Arabidopsis* and other plants (Lee et al., 2013; Posé et al., 2013). *FPP1*-like genes, a potential downstream target of *ODDSOC2*, also show temperature-responsive expression in barley (Hemming et al., 2012). Further research is required to confirm a role for *ODDSOC2* and *FPP1*-like genes in regulating temperature-induced flowering responses of cereals.

MAPPING MOLECULAR NETWORKS CONTROLLING SEASONAL FLOWERING: CONSIDERATIONS FOR CEREALS VERSUS *Arabidopsis*

As outlined above, multiple pathways controlling developmental responses to seasonal cues are integrated to determine the timing of a single developmental transition in *Arabidopsis*. These

pathways can be mapped together as a single network. The situation is more complex in grasses, where seasonal cues have varying effects at different developmental stages and/or different times of year. We suggest that molecular pathways controlling seasonal developmental responses in grasses should be considered in a developmental stage and season specific manner (Figure 2). The concept of sub-dividing networks controlling developmental responses to specific phases/seasons is also important for the development of gene based models to predict flowering behavior of cereals and related grasses (Brown et al., 2013).

VARIATION IN FLOWERING BEHAVIOR ADAPTS CEREALS TO DIFFERENT GEOGRAPHICAL REGIONS

Diversity in the *VRN1* gene is a major driver of variation in vernalization requirement in temperate cereal crops. Different mutations in the promoter, insertions or deletions in the first intron, amino acid substitutions and copy number variation all occur at the *VRN1* locus (Table 1). Similar, though not identical, variation occurs in non-domesticated wheats, so variation in vernalization requirement mediated by *VRN1*

occurs independently of crop domestication (Golovnina et al., 2010).

The extent of diversity in *VRN1* suggests that variation in this gene might be useful to adapt accessions to different environments and there is strong historical evidence that this is the case. The history of the Australian wheat industry provides an excellent example. The first wheats grown in Australia were English wheats that required both vernalization and long days to flower rapidly. These wheats were ill-suited to warm Australian growing environments, taking too long to flower in the field, and suffered from end of season heat stress and water limitation, and also strong disease pressure (see Evans, 1980; Eagles et al., 2009). In the late 19th century William Farrer had the foresight to realize that selective breeding could be used to improve the adaptation of Australian wheats. He imported early flowering wheats from India, which he then crossed with European wheats to select better adapted strains. His first cultivar "Federation" was released in 1901 and proved enormously successful. Indeed, the germplasm he developed was the basis for adaptation of Australian wheat cultivars until the 1960s (see Eagles et al., 2009). Molecular characterization of the Australian wheat pedigree, using

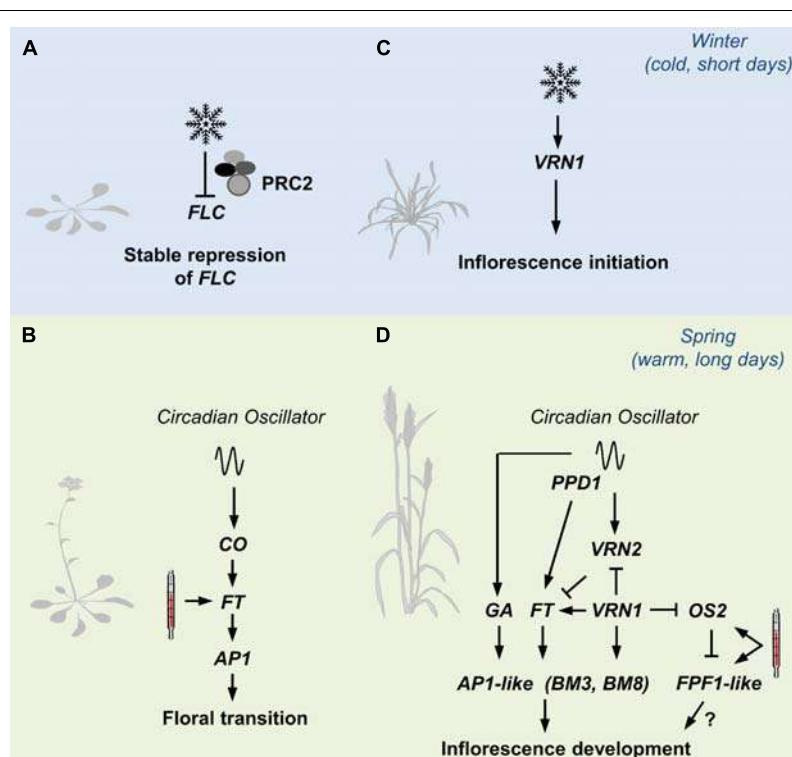


FIGURE 2 | An overview of pathways controlling seasonal flowering responses in grasses contrasted with those in *Arabidopsis*. (A) The prolonged cold of winter (snowflake) triggers lasting repression of *FLOWERING LOCUS C* (*FLC*) in *Arabidopsis*, via the Polycomb Repressor Complex 2 (PRC2). (B) The long days of spring activate expression of *FLOWERING LOCUS T*, a process mediated by the circadian oscillator via *CONSTANS* (*CO*). *FT* activates expression of genes such as a *APETALA1* that trigger floral development. High-temperatures (thermometer) also activate expression of *FT* to accelerate flowering. (C) Winter cold activates expression of *VERNALIZATION1* (*VRN1*) in cereals and related

grasses. *VRN1* promotes inflorescence initiation at the shoot. (D) *VRN1* remains active after winter and down-regulates *VRN2*, which would otherwise repress the long-day flowering response in leaves. As daylength increases after winter, expression of *FT-like 1* is activated by the circadian oscillator, via *PHOTOPERIOD1*. Long days also activate gibberellin (GA) biosynthesis. The long-day flowering response activates expression of genes at the shoot apex that promote the development of floral organs. These include other *AP1-like* genes (*BM3* and *BM8* in barley for example). High temperatures accelerate the long-day response, possibly via *FLOWERING PROMOTER1-like* genes (*FPF1*).

Table 1 | Variation in the *VRN1* gene of cultivated cereals.

| Variation | Genome | Effect | Reference |
|-----------------------------------------|----------------|--------------------------------------------------|-----------------------------------------------------------------------------|
| Promoter mutations, insertion deletions | A ^m | Elevated expression, Reduced vernalization req. | Yan et al. (2003) |
| Large deletions, first intron | A, B, D, H | Elevated expression, Reduced vernalization req. | Fu et al. (2005), Oliver et al. (2013) |
| Insertion, first intron | H | Elevated expression, Reduced vernalization req. | Cockram et al. (2007), Stockinger et al. (2007), Oliver et al. (2013) |
| Promoter SNP (and intron deletion) | D | Reduced expression, Increased vernalization req. | Zhang et al. (2012) |
| CNV (3 x), coding region SNP | A | Reduced expression, Increased vernalization req. | Chen et al. (2009), Díaz et al. (2012) |
| CNV (2 x) with promoter insertion | A | Elevated expression, Reduced vernalization req. | Yan et al. (2004b) |

^mA = *Triticum monococcum*.

H = *Hordeum vulgare*.

A, B, D = subgenomes of hexaploid bread wheat (*Triticum aestivum*).

CNV = copy number variation.

DNA extracted from seeds held in stock centers, shows that the reduced vernalization requirement of Federation was caused by an allele of *VRN1* that has a mutation in the promoter (Eagles et al., 2009).

Variation in *VRN1* can also increase the duration of vernalization required to trigger rapid flowering. A multi-copy allele of *VRN1*, with a substitution of a conserved amino acid residue in at least one copy, occurs in winter wheats grown in regions with cold winters (Chen et al., 2009; Díaz et al., 2012; Cane et al., 2013). This allele is associated with low-transcriptional activity and a slow transcriptional response to vernalization, and is linked to increased requirement for vernalization (Díaz et al., 2012). An association screen suggests that frost tolerant wheats have this allele of *VRN1* together with an expanded number of *C-REPEAT BINDING FACTOR* genes at the *FROST TOLERANCE 2* locus (Zhu et al., 2014). It seems that variation in *VRN1* allows the vegetative growth phase to be lengthened in cultivars grown in regions with extreme winters and this has been co-selected with genes that enhance frost tolerance.

There is also extensive variation in daylength sensitivity amongst cereals (Table 2). In wheat, a constitutive long-day flowering response is mediated mainly by active alleles of *PPD1* that allow crops to be grown at times of year or at latitudes where short daylengths would otherwise limit cultivation. For example, breeding programs that are located at low-latitudes and focus on rapid cycling varieties (e.g., International Maize and Wheat Improvement Centre, CIMMYT, Mexico) are likely to select cultivars with photoperiod insensitivity, which allows rapid flowering without long days. Conversely, a mutated version of the barley *PPD1* gene occurs in many European barleys (Turner et al., 2005; Jones et al., 2011). These barleys have a reduced response to long-days, which lengthens the growing season and allows more biomass to accumulate, increasing grain yield (see Cockram et al., 2007). Another example of adaptation through altered photoperiod sensitivity is the use of a loss-of-function mutation in the *ELF3* gene (MAT.a8) to breed barley cultivars that flower rapidly

irrespective of daylength, which are well suited to short summer growing seasons at high latitudes (Lundqvist, 2009; Faure et al., 2012; Zakhrebekova et al., 2012).

Variation that influences development irrespective of environmental cues, often referred to as the *EARLINESS PER SE* (EPS), has also been utilized in crop breeding. The *EPS2* gene (*HvCENTRORADIALIS*, *HvCEN*) of barley encodes a PEBP that is distantly related to the *FT* gene family (Comadran et al., 2012). An amino acid substitution in the *HvCEN* gene (Ala135 to Pro) delays flowering, and has been used to lengthen the inflorescence development phase of European spring barleys. Conversely, the wildtype allele predominates in autumn sown vernalization responsive barleys (Comadran et al., 2012). Thus, *HvCEN1* can be considered as a modifier of seasonal flowering responses.

The examples above highlight how different developmental regulators have been used to modify the crop life cycle to suit the growing environments encountered at particular location and sowing dates. These examples also show that such variation influences the duration of specific developmental phases (Figure 3). Altering the duration of different developmental phases can have profound effects on plant physiology and plant architecture, and strongly influences major components of yield such as grain number per spike and grain size (e.g., Stelmakh, 1993; Eagles et al., 2014). There is now considerable interest in understanding how different combinations of genetic variation in genes controlling flowering behavior can be used to breed cereal varieties that are suited to specific climates but that also have high yield potential (Reynolds et al., 2009).

SEASONAL FLOWERING RESPONSES OF TEMPERATE GRASSES

Temperate grasses such as poa (*Poa* sp.), ryegrass (*Lolium* sp.), fescue (*Festuca* sp.), and timothy grass (*Phleum pretense*) flower in response to vernalization and long days, though there is naturally occurring variation in vernalization and daylength requirements

Table 2 | Variation in the *PPD1* gene of cultivated cereals.

| Variation | Genome | Effect | Reference |
|--------------------------------|--------|------------------------------|-----------------------------------------------------------------|
| Promoter deletion | A, D | Reduced long day requirement | Beales et al. (2007), Wilhelm et al. (2009), Shaw et al. (2013) |
| Promoter insertion | A | Reduced long day requirement | Nishida et al. (2013) |
| CNV (2–4.5 x) | B | Reduced long day requirement | Díaz et al. (2012) |
| CCT domain mutation | H | Reduced long-day response | Turner et al. (2005) |
| Promoter deletion | A | Reduced long-day response | Shaw et al. (2013) |
| Deletion, transcribed sequence | A | Reduced long-day response | Beales et al. (2007), Shaw et al. (2013) |
| Deletion, transcribed sequence | D | Reduced long-day response | Beales et al. (2007), Shaw et al. (2013) |
| Intron insertion | D | Reduced long-day response | Beales et al. (2007), Shaw et al. (2013) |

H = *Hordeum vulgare*.

A, B, D = subgenomes of hexaploid bread wheat (*Triticum aestivum*).

CNV = copy number variation.

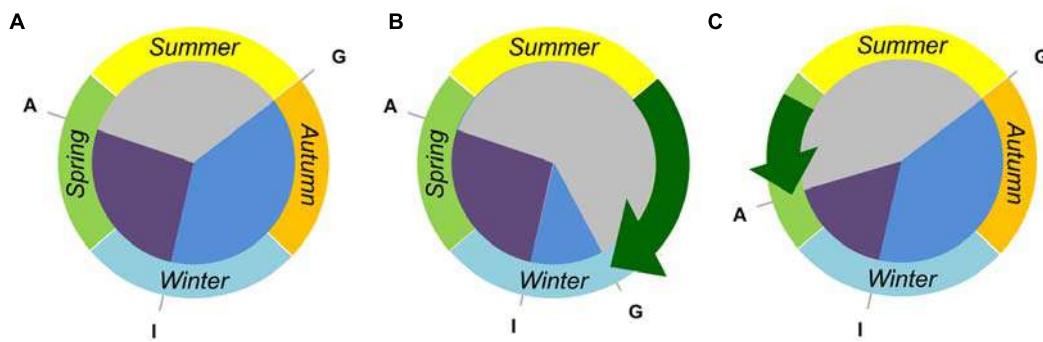


FIGURE 3 | Tailoring the crop life cycle to fit different environments by modifying the duration of discrete developmental phases. (A) A vernalization responsive and daylength sensitive cereal cultivar can be sown and germinate "G" in early autumn but will remain vegetative until late winter, when inflorescence initiation occurs "I". Anthesis "A" will occur during spring, at a date determined by the photoperiod sensitivity. **(B)** A near-isogenic line derived from the hypothetical wheat has reduced vernalization requirement. This line can be sown at a later date and will

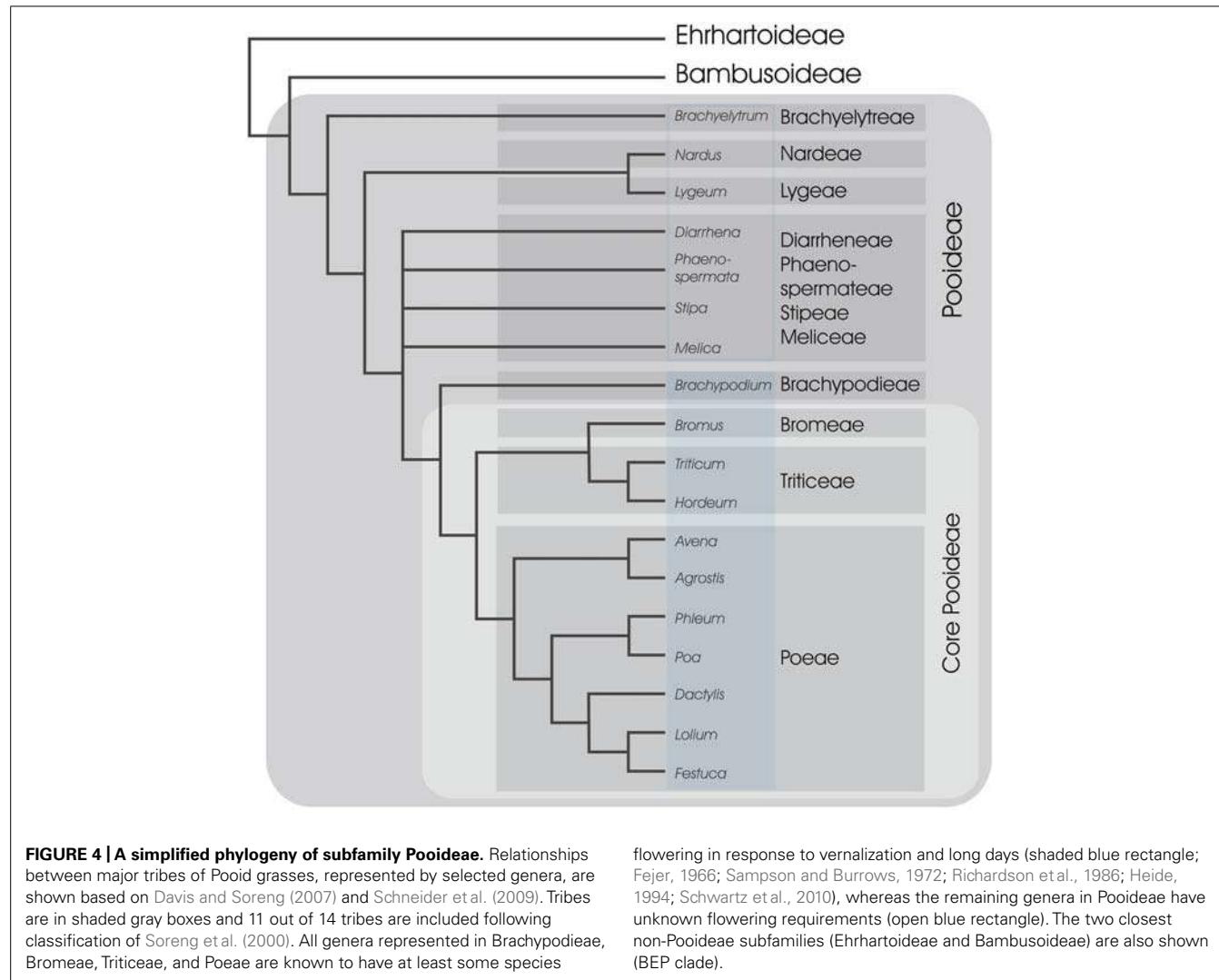
have a shortened vegetative growth phase (blue sector). Such a line might be useful in regions where there is a late onset of autumn rain, requiring a delayed sowing of crops. **(C)** A second near-isogenic line has reduced photoperiod sensitivity. This line can be sown in autumn, and still requires vernalization to flower, but will progress through inflorescence development (purple sector) more rapidly in spring. Such a line would be useful where there is a rapid onset of hot/dry conditions, allowing plants to avoid heat and drought stress during the grain filling stage.

both between species of specific genera and within populations of individual species (Evans, 1964). The molecular biology of seasonal flowering-responses has been studied in some temperate grasses. Orthologs of *VRN1* appear to function in the vernalization responses of perennial ryegrass (*Lolium perenne*), timothy grass (*Phleum pratense*), and fescue (*Festuca pratensis*; Petersen et al., 2004; Andersen et al., 2006; Seppänen et al., 2010; Ergon et al., 2013). The *VRN1* ortholog of *Brachypodium* is also likely to play a key role in maintaining a memory of winter cold (Ream et al., 2014). Similarly, *FT1*-like genes are likely to play a central role in promoting flowering in response to long days in perennial ryegrass and *Brachypodium* (King et al., 2006; Lv et al., 2014; Ream et al., 2014). Genetic variation in vernalization requirement or daylength sensitivity has also been linked to *VRN1* and *FT1*-like genes in perennial ryegrass (Jensen et al., 2005; Asp et al., 2011; Skøt et al., 2011; Shinozuka et al., 2013). Thus, knowledge of the molecular pathways controlling seasonal flowering responses in temperate cereals is relevant to Pooid grasses and can provide insights into how variation in

genes controlling vernalization and daylength requirements can adapt temperate grasses to different climates or geographical regions.

EVOLUTION OF SEASONAL FLOWERING-RESPONSES IN THE GRASSES

Most of the ~10,000 species of the grass family are members of one of two monophyletic clades (groups of species including a single common ancestor and all its descendants). The first clade includes tropical grass subfamilies and is known as the PACMAD (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, Danthonioideae), of which the Panicoideae subfamily includes crops such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), and sugar cane (*Saccharum officinarum*). The second clade includes the Pooideae subfamily, together with the subfamilies Ehrhartoideae (including rice, *Oryza sativa*) and Bambusoideae (the bamboos) and is known as the BEP clade, with Bambusoideae widely accepted as the sister group to the Pooideae (Figure 4; Grass Phylogeny Working Group [GPWG] II, 2012; Wu and Ge, 2012; Zhao et al., 2013). Of the grass subfamilies,



Pooideae is one of the most species rich and dominates the grass flora in temperate regions with as much as 90% of the grasses in northern temperate regions belonging to this subfamily (Hartley, 1973).

Current knowledge of the molecular pathways that control flowering of temperate grasses comes from a restricted selection of cereals and pasture grasses, all members of a group informally termed the “core Pooideae” (Davis and Soreng, 1993), or from *Brachypodium*, the sister group to the core Pooideae (Figure 4). The vernalization response and long-day flowering are conserved features of the core Pooideae and *Brachypodium* (Figure 4). In contrast, rice does not require vernalization and flowers in response to shorter daylengths, similar to many grasses of the Panicoideae. Thus, it has been hypothesized that the vernalization and long-day flowering responses of grasses evolved in an ancestor of the Pooideae.

THE EVOLUTION OF THE VERNALIZATION RESPONSE

As outlined above, orthologs of the cereal *VRN1* gene function in the vernalization responses of temperate grasses such as ryegrass,

fescue, and timothy grass (all core Pooids), and the *VRN1* gene of *Brachypodium* seems to have the same function. This suggests that the central role for *VRN1* in the vernalization response of temperate grasses evolved early during the radiation of the Pooideae. Vernalization-induced flowering likely co-evolved with increased freezing tolerance (see Sandve et al., 2011).

There are two regulatory features of *VRN1* that are likely to have been pivotal to the evolution of the vernalization response in temperate grasses. The first is that activity of *VRN1* is maintained at a low basal level before winter to delay inflorescence initiation. The second is low-temperature induction. Low basal activity of *VRN1* appears to be mediated by the large (~10 kb) first intron, which has expanded relative to the equivalent region in the rice ortholog of *VRN1* (*OsMADS14*, ~5 kb first intron; Fu et al., 2005; von Zitzewitz et al., 2005; Szucs et al., 2007; Hemming et al., 2009; Oliver et al., 2013). Low-temperature induction of *VRN1* seems to be controlled by the promoter, possibly by pathways that activate transcription of other cold-induced genes (Alonso-Peral et al., 2011; Oliver et al., 2013). A deeper understanding how the first intron limits *VRN1*

transcription, or of the mechanisms mediating low-temperature induction of *VRN1*, could potentially contribute to a better understanding of how the vernalization response evolved in grasses.

THE EVOLUTION OF THE LONG-DAY FLOWERING RESPONSE

One explanation for the evolution of different daylength flowering responses is that the activity of the daylength response can be inverted. For example, the short-day flowering response of rice might have arisen through inversion of activity of the long-day response pathway first identified in *Arabidopsis*. This has been suggested to occur through modification of CO activity (see Simpson, 2003). A similar inversion in the daylength response could also be suggested to account for the evolution of long-day grasses from tropical grass ancestors that flowered in short days. We suggest, however, that complete inversions of daylength responses are unlikely to occur because daylength responses influence other aspects of plant physiology aside from flowering time. For example, daylength regulates the rate of starch breakdown during the night so that starch is depleted shortly before dawn (Graf and Smith, 2012). CO strongly influences expression of genes that control diurnal starch metabolism (Ortiz-Marchena et al., 2014). Unlike flowering behavior, daylength regulation of starch metabolism is likely to be conserved between short and long day grasses, which will need similar daylength dependent regulation of starch metabolism. This potentially limits evolutionary flexibility to invert daylength-induced flowering responses by modifying CO activity.

An alternative model is that a common daylength response mechanism elicits different outputs to trigger flowering under either short or long days in different plants. According to this model a conserved daylength response mechanism operates in all plants. Then, there are both short and long day output pathways that influence flowering, but these have evolved to be stronger or weaker in different lineages (Greenup et al., 2009). For example, the temperate grasses might have evolved to flower in long days through the loss of ancestral genes that accelerated flowering under short days (Greenup et al., 2009).

There are similarities between the molecular pathways controlling daylength responses of rice and temperate cereals that suggest there has not been an inversion of the activity of pathways controlling daylength-induced flowering responses. For example, a *VRN2*-like gene, *GHD7*, is expressed in long days in rice, where it represses expression of *FT*-like genes, similar to *VRN2* in the temperate cereals (Xue et al., 2008). In rice plants that lack *GHD7* function there is expression of *FT*-like genes in long-days and this accelerates flowering (Xue et al., 2008). Similarly, the maize (*Z. mays*) ortholog of *GHD7* represses flowering in long-days and a similar function has been suggested for a Sorghum (*S. bicolor*) equivalent (Ducrocq et al., 2009; Hung et al., 2012). Thus, a role for *VRN2*-like genes in blocking flowering under long-days occurs across the grass lineage, though is not necessarily related to a vernalization requirement. A recent study of sorghum shows that CO activates expression of *FT*-like genes in long-days in lines that lack *GHD7*, consistent with the idea that CO activity has not been inverted to repress flowering in long-days in tropical grasses (Yang et al., 2014). The

weak inhibition of inflorescence initiation of temperate grasses in short-days also suggests that Pooid grasses retain ancestral pathways that allow flowering under short-days, though these pathways are weaker than the long-day response that is activated in vernalized plants (see Sections “Short Days Allow Inflorescence Initiation” and “The Prolonged Cold of Winter Promotes Inflorescence Initiation”).

Novel daylength regulators might also have evolved to suppress flowering in short-days in the temperate grasses. A novel modifier of the daylength flowering response has been identified in *Brachypodium*, where a daylength regulated microRNA (miR5200) down-regulates *FT1* and *FT2* in short days (Wu et al., 2013). This microRNA is expressed in leaves in short days, where it triggers cleavage of *FT1* and *FT2* mRNA molecules (Wu et al., 2013). Whether similar microRNA molecules are active in other temperate grasses or in cereal crops will be an interesting topic for future research.

ARE PERENNIALS THE MISSING LINK?

Perenniality occurs throughout the Pooideae (Watson and Dallwitz, 1992) and it seems that monocarpic annual grasses have evolved repeatedly from perennial ancestors. This raises the question of whether evolution of the seasonal flowering responses of the Pooideae should be considered from perennial perspective?

Molecular studies of perennial temperate grasses are less common than those focussed on annual species. The best characterized are perennial ryegrass and timothy grass, which have both been the focus of physiological and molecular studies (MacMillan et al., 2005; Seppänen et al., 2010; Jokela et al., 2013). Like other members of the core Pooideae, perennial ryegrass flowers in response to vernalization and long-days, and *VRN1* and *FT1* homologs play key roles in each of these responses, respectively (Petersen et al., 2004; King et al., 2006). The perennial growth habit arises from the retention of vegetative meristems at the base of the plant during flowering, allowing vegetative growth to continue after a period of summer dormancy. It is not clear whether these buds do not respond to vernalization or whether there is a loss of the memory of vernalization.

Timothy grass is also a member of the core Pooideae that flowers in response to vernalization then long days (Seppänen et al., 2010). Unlike annual grasses, only a percentage of tillers flower in spring, allowing vegetative growth to continue even when some tillers flower. The reason why some tillers flower while others remain vegetative is unclear, but this is an important question for understanding the perennial growth habit of this grass. Timothy grass produces a second flush of elongating tillers in summer. Unlike the tillers produced in spring, the inflorescence of these tillers does not develop fully. Expression of *VRN1* is activated by vernalization in the spring tillers but is not maintained in the summer tillers (Seppänen et al., 2010). This might be one reason that inflorescence development stalls in summer tillers. An inability to maintain *VRN1* expression might also contribute to the perennial growth habit of timothy grass.

Another interesting finding from timothy grass is that expression of a *VRN2*-like gene increases as daylength increases following vernalization (Seppänen et al., 2010). This contrasts with the expression behavior of *VRN2* in other core Pooideae, where *VRN2*

is repressed following vernalization. A similar observation has been made in *Brachypodium*, where a VRN2-like gene is activated by long days in vernalized plants (Ream et al., 2014). Perhaps VRN2-like genes have a broader role in perennial grasses to repress flowering in the long days of summer and to restore a vegetative growth habit before autumn.

FUTURE DIRECTIONS

The Pooideae are a worthwhile target for the study natural variation in seasonal flowering responses. There are large collections of different accessions from many Pooid species and this provides a rich source of genetic diversity, from wild species and domesticated crops. Rapidly developing genomic resources are allowing this diversity to be explored. Surveying diversity alone will not provide mechanistic insights into the molecular pathways controlling flowering or how variation in these pathways influences adaptation to different climates. The development and study of focussed genetic resources including mapping populations, near-isogenic lines, mutants, and transgenic plants will be a priority for future research, since these tools can resolve gene function and assess the impact of genetic variation.

Studying the molecular pathways controlling seasonal flowering responses in a broader range of Pooid species, particularly grasses outside the core Pooid group, will provide a clearer view of how these pathways evolved. Research in to basal Pooids, such as *Stipa* or *Melica* for example (Figure 4), might provide some indication of when the vernalization and long day flowering responses evolved during the radiation of the Pooideae. Additionally, a deeper understanding of the pathways controlling flowering in perennial Pooids can potentially offer further insights into the functions of key genes. Most importantly, ongoing research focussed directly on the Pooideae is essential if we are to develop a deeper understanding the reproductive biology of this important group of plants.

AUTHOR CONTRIBUTIONS

Siri Fjellheim, Scott Boden, and Ben Trevaskis wrote the manuscript.

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Recent advancements to study flowering time in almond and other *Prunus* species

Raquel Sánchez-Pérez^{1*}, Jorge Del Cueto², Federico Dicenta² and Pedro Martínez-Gómez²

¹ Plant Biochemistry Laboratory, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Copenhagen, Denmark

² Department of Plant Breeding, CEBAS-CSIC, Murcia, Spain

Edited by:

Christian Jung, Christian Albrechts University of Kiel, Germany

Reviewed by:

Adriana Garay, Universidad Nacional Autónoma de México, Mexico

Tony Millar, Australian National University, Australia

Christian Jung, Christian Albrechts University of Kiel, Germany

***Correspondence:**

Raquel Sánchez-Pérez, Plant Biochemistry Laboratory, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, Frederiksberg C, DK-1871 Copenhagen, Denmark
e-mail: rasa@plen.ku.dk

Flowering time is an important agronomic trait in almond since it is decisive to avoid the late frosts that affect production in early flowering cultivars. Evaluation of this complex trait is a long process because of the prolonged juvenile period of trees and the influence of environmental conditions affecting gene expression year by year. Consequently, flowering time has to be studied for several years to have statistical significant results. This trait is the result of the interaction between chilling and heat requirements. Flowering time is a polygenic trait with high heritability, although a major gene *Late blooming* (*Lb*) was described in "Tardy Nonpareil." Molecular studies at DNA level confirmed this polygenic nature identifying several genome regions (Quantitative Trait Loci, QTL) involved. Studies about regulation of gene expression are scarcer although several transcription factors have been described as responsible for flowering time. From the metabolomic point of view, the integrated analysis of the mechanisms of accumulation of cyanogenic glucosides and flowering regulation through transcription factors open new possibilities in the analysis of this complex trait in almond and in other *Prunus* species (apricot, cherry, peach, plum). New opportunities are arising from the integration of recent advancements including phenotypic, genetic, genomic, transcriptomic, and metabolomics studies from the beginning of dormancy until flowering.

Keywords: *Prunus dulcis*, breeding, almond, flowering time, dormancy, genome, transcription factors, molecular markers

INTRODUCTION

From a commercial point of view, flowering time is one of the most important agronomic traits in almond (*Prunus dulcis* (Miller) D. A. Webb) as it determines the vulnerability of production to late frosts, as well as the use of cultivars for cross-pollination in order to achieve successful pollination when the flowering time of two varieties must coincide (Dicenta et al., 2005).

Breeding new, late flowering almond cultivars is a very long and costly task since, due to the long juvenile period, their first flowering usually occurs in the third year after plantation in the field, or even later. In addition, the influence of climatic factors on this trait obligates the breeder to record the data for several years (Dicenta et al., 1993, 2005). In this sense, it would be very useful to have tools for early selection of the latest flowering seedlings in the nursery (after germination of seeds), which would be planted in the experimental orchards for further selection (Dicenta et al., 2005). Flowering will only happen when dormancy is broken. Endodormancy has been described as the inability of a tree to start floral or vegetative budbreak, even with moderate temperatures. Endodormancy occurs prior to ecodormancy, which happens in late winter and spring and is imposed by temperatures unfavorable to growth (Sánchez-Pérez et al., 2012).

On the other hand, almond and other *Prunus* species (apricot, cherry, peach, plum, etc.) accumulate a mono cyanogenic

glucoside (CNGLc) called prunasin in different vegetative and reproductive parts of the plant, and in the seeds a di-CNGLc called amygdalin (McCarty et al., 1952; Fehner et al., 1990; Sánchez-Pérez et al., 2008). When specific enzymes called β -glucosidases degrade CNGLcs, glucose, benzaldehyde and cyanide are released (Morant et al., 2008). Upon degradation of hydrogen cyanamide—a nitrogen-based chemical compound sprayed in the flower buds—nitroxil and cyanide are released. This brings forward the flowering time in apple, apricot, peach, persimmon, sweet cherry flower buds, etc., when compared to untreated plants (Dozier et al., 1990; George et al., 1992; Faust et al., 1997).

In this work, recent advancements to study flowering time in almond have been included at the phenotypic (observed trait), genetic (inheritance and transmission), genomic (DNA analysis), transcriptomic (gene expression analysis) and metabolomic (metabolites involved, as cyanide content) level.

PHENOTYPIC STUDIES

In general, the accuracy of phenotypic evaluation is critical for further reliable genetic and molecular studies. To diminish the significant influence of the environment, flowering time can be dissected as the sum of two traits: chilling and heat requirements. These can be determined in monitored conditions by measuring the temperatures in the field and simultaneously controlling temperatures and humidity in the growth chamber (Egea

et al., 2003; Sánchez-Pérez et al., 2012). Chill requirements have a much stronger effect on flowering time compared to heat requirements (Egea et al., 2003) with a high positive correlation between chilling requirements and flowering time (Sánchez-Pérez et al., 2012).

These results were also verified in other *Prunus* species such as apricot (*P. armeniaca* L.) (Ruiz et al., 2007) and sweet cherry (*P. avium* L.) (Alburquerque et al., 2008). However, Alonso et al. (2005) described a higher influence of heat requirements than chilling requirements on the flowering time in cold areas, using a mathematical model recording temperatures and flowering time, but not evaluating the endodormancy breaking in flower buds.

GENETIC STUDIES

Inheritance studies of the genetic control of flowering time on almond showed that flowering time is a polygenic trait (Kester et al., 1977; Dicenta et al., 1993; Sánchez-Pérez et al., 2007a). However, a major dominant gene controlling this trait was described specifically in some descendants of the almond cultivar “Tardy Nonpareil,” considered a late flowering mutant of “Nonpareil” (Kester, 1965; Socias i Company et al., 1999; Sánchez-Pérez et al., 2007a). These authors, studying some descendants of “Tardy Nonpareil,” observed a bimodal distribution for this trait, which was explained by the presence of a late blooming major gene (*late blooming*, *Lb*), quantitatively modified by minor genes.

Flowering time of almond has a high heritability (Kester et al., 1977; Dicenta et al., 1993), so crossing late-flowering parents will produce late-flowering seedlings (Dicenta et al., 2005). The best breeding strategy to obtain late-blooming almond descendants is therefore to cross parents as late-blooming as possible, and when the offspring shows a bimodal distribution, the latest-blooming genotypes should be selected, probably carrying the *Lb* allele, which could be transmitted to its descendants (Sánchez-Pérez et al., 2012).

On the other hand, studies on the genetic basis and inheritance of chilling and heat requirements to break endodormancy and ecodormancy in almond are scarcer. Sánchez-Pérez et al. (2012) described a polygenic nature of these traits in accordance with the observed flowering time of seedlings. In addition, these authors observed, in a 2-year study, that the bimodal distribution of chilling requirements in the studied progeny could be explained by the presence of the *Lb* gene hypothetically linked to these chilling requirements. In other *Prunus*, quantitative inheritance of chilling requirements for breaking endodormancy was also observed in flower buds in peach (Fan et al., 2010) and vegetative buds in apricot (Olukolu et al., 2009). In this species, a higher influence of chilling requirements on flowering time was also described, although a single recessive gene called EVERGROWING (EVG) gene (Rodríguez et al., 1994) was described as a chilling and heat requirement related gene (Bielenberg et al., 2004, 2008). Interestingly, the deletion of four out of six of the *Dormancy Associated MADS-box* (DAM1-DAM6) genes in the *evg* mutant caused the transcriptional inhibition of the other two structurally intact genes of the family (Bielenberg et al., 2008), in which no flowering occurred.

More is known about the genetic bases of flowering time in the long day plant *Arabidopsis thaliana* than *Prunus* species. More

than 60 genes were identified as regulating flowering time in *Arabidopsis* (Ehrenreich et al., 2009), and due to SNPs present in genes including *CONSTANS* (*CO*), *FLOWERING LOCUS C* (*FLC*), *VERNALIZATION INSENSITIVE 3*, *PHYTOCHROME D*, *GIBBERELLIN*, etc. or coding region deletions as in *FRIGIDA* gene, there are different phenotypes for flowering time in *Arabidopsis*. In fact, dominant alleles of *FRI* generate late flowering phenotypes while *FRI* mutants are early flowering (Johanson et al., 2000; Jung and Müller, 2009). Early flowering is also possible if *APETALA1* (*API*) is constitutively expressed (Chi et al., 2011). When *PsAPI*, *API* from cherry (*Prunus avium* L.), was over-expressed in *Arabidopsis*, it produced an early flowering phenotype, shortening the juvenile period (Wang et al., 2013). *API* belongs to the *MADS-box* gene family. *MdMADS5*, an *API*-like gene of apple (*Malus × domestica*), also caused early flowering in transgenic *Arabidopsis* (Kotoda et al., 2002), as did *MdMADS2* in transgenic tobacco (*Nicotiana tabacum*). *API* is activated by *LEAFY* (*LFY*), which is also involved in flowering time in *Arabidopsis*. Moreover, *TERMINAL FLOWER 1* (*TFL1*) and *FLOWERING LOCUS T* (*FT*) are two key regulators of flowering time. These genes belong to the *PEBP* family but they have antagonistic functions. *TFL1* interacts with b-ZIP transcription factor *FD* and represses the transcription of *FD*-dependent genes as *API* and *AGAMOUS* (*AG*), while *FT* interacting with *FD* activates *API* and *AG* (Wang and Pijut, 2013). *TFL1* homologous genes such as *PsTFL1* (*P. serotina* Ehrh., *TFL1* homolog) delay flowering when expressed in *Arabidopsis*. Homologous genes from *Arabidopsis* found in *Prunus* species are shown in Table 1.

On the other hand, the adaptation of the short day model plant rice (*Oryza sativa* L.) to different climate conditions depends on variation in flowering time, also called “heading date (hd).” A number of genes and QTLs have been identified in rice, including *Hd1/Se1*, *Ehd1*, *Hd3a*, and *RFT1* (*Rice Flowering Locus T1*) among others (Ogiso-Tanaka et al., 2013). Functional defects in *RFT1* are the main reasons for late flowering rice in an *indica* cultivar. *Hd3*, *RFT1*, and *FT* are florigen genes. *FT* protein is the long-sought florigen that activates the transcription of *FD*, forming the florigen activation complex (FAC). FAC will bind to the promoter regions of floral meristem identity genes such as e.g., *API*, so flowering will occur (Taoka et al., 2013). Therefore, flowering time is affected by florigen in *Arabidopsis* and rice.

GENOMIC STUDIES

The first genomic studies performed used RAPDs (Random Amplified Polymorphic DNA) and bulk segregant analysis in a F1 progeny from “Tardy Nonpareil,” corroborating the presence of the previously mentioned major gene *Lb* controlling late flowering time. Moreover, three RAPDs were found to be associated with *Lb* in linkage group 4 (G4) of the “Felisia” × “Bertina” (“Felisia” is a descendant from “Titan,” that is a seedling of “Tardy Nonpareil”) genetic map (Ballester et al., 2001). In addition, Silva et al. (2005) described several Quantitative Trait Loci (QTLs) linked to flowering time in an interspecific F1 almond × peach progeny using a Candidate Gene approach (CG) in G1, G2, G3, G5, G6, and G7. More recently, different works using SSR (Simple Sequence Repeat) markers in a F1 population between a seedling of “Tardy Nonpareil” (“R1000”) × “Desmayo Largueta” (RxD), also confirmed the location of *Lb* in G4 and identified other QTLs

Table 1 | Identified genes involved in the regulation of flowering time in *Prunus* species.

| Species | Gene | Gene ID | Annotation | References |
|---------------------|----------|--------------|-------------------------------------|-------------------------|
| <i>P. armeniaca</i> | PHYE | Q6SCK5 | Phytochrome E | GeneBank data |
| <i>P. armeniaca</i> | RGA | RGA | Transcription factor | Soriano et al., 2005 |
| <i>P. armeniaca</i> | SOC1 | L7Y228 | Transcription factor | Trainin et al., 2013 |
| <i>P. armeniaca</i> | TFL1 | ADL62862 | Phosphatidylethanolamine binding | Liang et al., 2010 |
| <i>P. avium</i> | APETALA1 | APETALA1 | MADS-box gene family | Wang et al., 2013 |
| <i>P. avium</i> | GA1 | GA1 | Gibberellin biosynthesis | Blake et al., 2000 |
| <i>P. avium</i> | TFL1 | AB636121.1 | Phosphatidylethanolamine binding | Mimida et al., 2012 |
| <i>P. domestica</i> | FT1 | FT1 | Transcription coactivator | Tränkner et al., 2010 |
| <i>P. dulcis</i> | GA20 | BU574794 | Gibberellin 20-oxidase | Silva et al., 2005 |
| <i>P. dulcis</i> | LFY | PrdLFY | AFL2 | Silva et al., 2005 |
| <i>P. dulcis</i> | MADS1 | PrdMADS1 | MdMADS10 | Silva et al., 2005 |
| <i>P. dulcis</i> | PHYA | Q94EK7 | Phytochrome A | GeneBank data |
| <i>P. dulcis</i> | TFL | BU574411 | Flowering locus T-like protein | Silva et al., 2005 |
| <i>P. mume</i> | AGL 24 | AB437345.1 | dormancy-associated MADS-box | Yamane et al., 2008 |
| <i>P. mume</i> | FT | AM943979 | Flowering locus T protein | GeneBank data |
| <i>P. serotina</i> | AGAMOUS | EU938540 | Formation of stamens and carpels | Liu et al., 2010 |
| <i>P. persica</i> | AGL 2 | BU048398 | MdMADS 8 | Silva et al., 2005 |
| <i>P. persica</i> | AP1 | BU039475 | MdMADS 2 | Silva et al., 2005 |
| <i>P. persica</i> | AP2 | BU046298 | AHAP2 | Silva et al., 2005 |
| <i>P. persica</i> | ATM YB33 | XM_007218900 | myb domain protein 33 | Zhu et al., 2012 |
| <i>P. persica</i> | CDF1 | XM_007215192 | cycling DOF factor 2 | GeneBank data |
| <i>P. persica</i> | CO | BU042239 | Constans like protein | Silva et al., 2005 |
| <i>P. persica</i> | DAM 5 | AB932551 | MADS-box 5 | Yamane et al., 2011 |
| <i>P. persica</i> | DAM 6 | DQ863252 | MADS-box 6 | Bielenberg et al., 2008 |
| <i>P. persica</i> | FAR 1 | BU047045 | Far-red-impaired responsive protein | Silva et al., 2005 |
| <i>P. persica</i> | FPF 1 | XM_007225892 | Flowering promoting factor | Romeu et al., 2014 |
| <i>P. persica</i> | FRL 1 | DY640223.1 | FRI-related gene | Silva et al., 2005 |
| <i>P. persica</i> | FT | G3GAW0 | Flowering locus T protein | GeneBank data |
| <i>P. persica</i> | FT | BU044758 | Flowering locus T protein | Silva et al., 2005 |
| <i>P. persica</i> | LEAFY | EF175869 | Activator of AP1 | An et al., 2012 |
| <i>P. persica</i> | PHYA | Q945F7 | Phytochrome A | GeneBank data |
| <i>P. persica</i> | PHYB | Q945T4 | Phytochrome B | GeneBank data |
| <i>P. persica</i> | TFL1 | ADL62867 | Phosphatidylethanolamine binding | Liang et al., 2010 |

to flowering time in G1, G6, and G7 (Sánchez-Pérez et al., 2007b; Martínez-Gómez et al., 2012) (**Figure 1**). In the first study (2007), carried out in this RxD population, the SSR UDP-96003 was located very close to the *Lb* gene in G4 of the map. When QTL analysis was performed, this major QTL (*Lb*) in G4 was able to explain between 56.5 and 86.3% of the variance in “R1000,” which is supposed to carry the *Lb* gene, and 54.5–67.7% of the variance in the mapped RxD population.

In other *Prunus* species, QTLs associated with *flowering time* were also described in peach, apricot and cherry, confirming the polygenic nature. In peach, Fan et al. (2010) described different QTLs linked to flowering time in G1, G2, G4, G6, G7, and G8. Campoy et al. (2011) and Salazar et al. (2013) described a QTL linked to flowering time in G1, G5, and G7 in apricot. Finally, Wang et al. (2000); Dirlewanger et al. (2012), and Castede et al. (2014) also identified a QTL linked to flowering time in G1, G2, and G4 of sour and sweet cherry.

Silva et al. (2005) and Sánchez-Pérez et al. (2012) described several QTLs linked to *chilling* (G1, G3, G4, G5, G6, and G7) and *heat* (G2 and G7) requirements in almond (**Figure 1**), confirming again its polygenic nature. Fan et al. (2010) also described several

QTLs linked to chilling and heat requirements in G1, G2, G4, G6, G7, and G8 in peach. QTLs linked to chilling requirements of vegetative buds were described in apricot by Olukolu et al. (2009) in G1, G2, G3, G5, and G8. In sweet cherry (*P. avium* L.), Dirlewanger et al. (2012) and Castede et al. (2014) also found one QTL in G4 in sweet cherry.

Moreover, functionally characterized homologs from *Arabidopsis* were used as a CG approach with 10 genes from almond or peach (*PrdLFY*, *PrdMADS1*, *PrpAP1*, *PrpFT*, *PrpAGL2*, *PrpFAR1*, *PrdTFL*, *PrdGA20*, *PrpAP2*, and *PrpCO*) (Silva et al., 2005). These 10 genes were mapped in the *Prunus* reference map (Texas x Earlygold) and can be found in G1, G2, G3 G5, G6, and G7 (**Figure 1**, **Table 1**). However, none of them co-localized with *Lb* gene in G4. One reason for this is that there are more than 60 genes involved in flowering time in *Arabidopsis*, so other CGs should be analyzed. The other reason could be that the flowering time trait is due to different mechanisms in perennial plants than in annual plants.

The other recent CG analysis study was performed in an area of 3.7 Mbp in G4 (around the *Lb* gene) and showed 429 genes in the peach Lovell genome (Castede et al., 2014). Based on

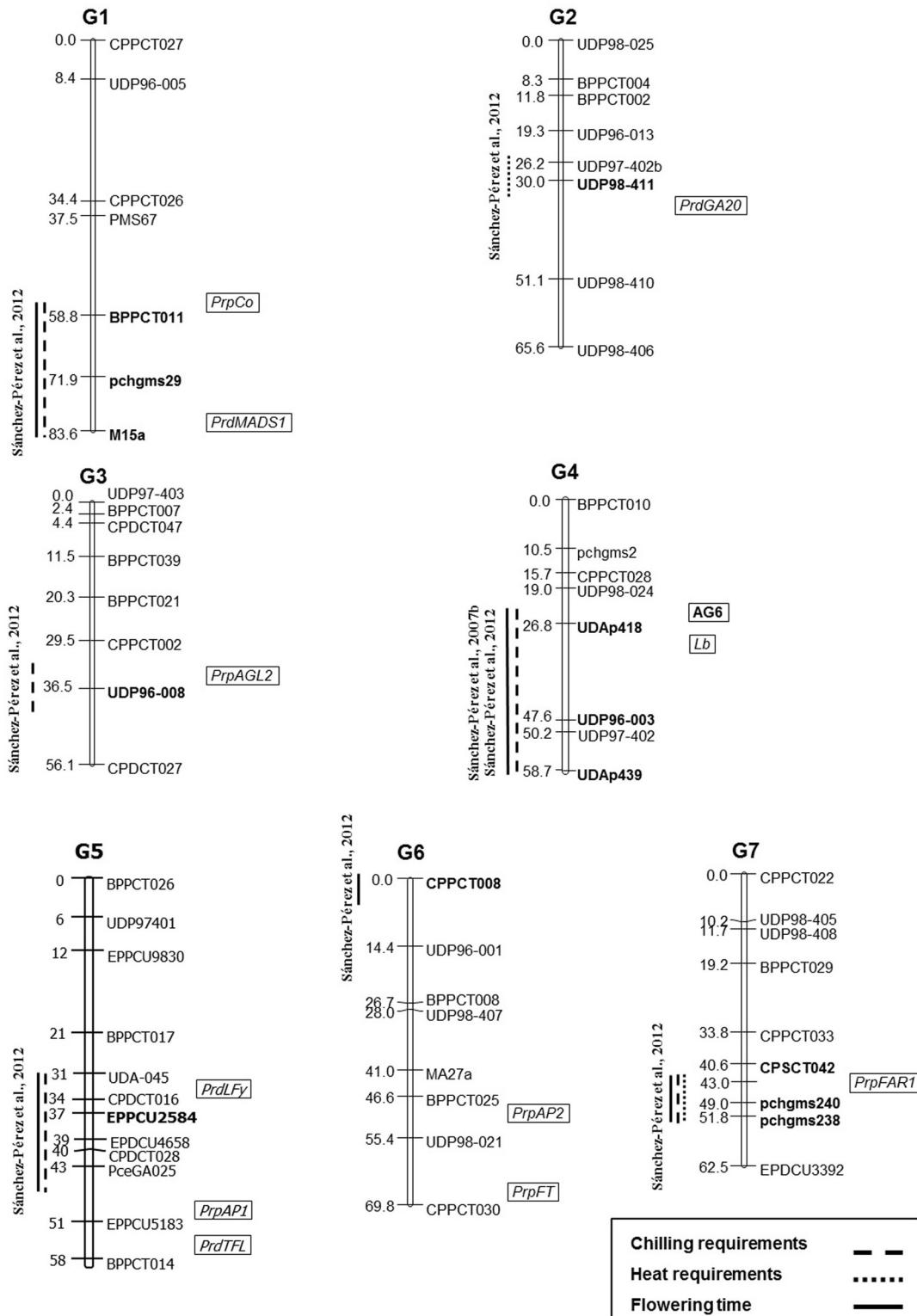


FIGURE 1 | Location of QTLs linked to flowering time and chilling and heat requirements in the almond map from the population R1000 × Desmayo Langueta performed by Sánchez-Pérez et al. (2007b, 2012). The closest SSR marker linked to the QTL is marked with bold lettering on this map. The approximately location of the RAPD marker (AG6) (Ballester et al.,

2001) and candidate genes (*in italics*) (Silva et al., 2005) linked to flowering time in other almond populations, are indicated inside the boxes. The integration of this information from different genetic maps has been performed using the centimorgan (cM) distances indicated by the different authors in each linkage group and each map.

predicted function of proteins, they selected nine CGs. One of these, ppa002685m, is an *embryonic flower 2* gene involved in the vernalization response and a negative regulator of the flower development through histone methylation. It would be interesting to see the analysis of this gene in varieties with different flowering times in almond.

Recently, Zhebentyayeva et al. (2014) developed a comprehensive program to identify genetic pathways and potential epigenetic mechanisms involved in control of chilling requirement and flowering time in peach. These authors described the *TFL1*, which regulates the vegetative to reproductive transition, and the *PcG* (*polycomb group*) genes, which are involved in the epigenetic regulation of flowering in *Arabidopsis*. It is worth noting that these authors failed to identify a direct *FLC* gene ortholog and its regulator *FRI*, suggesting that control of flowering time in *Prunus* species has a complex genetic architecture.

The recent release of the complete peach genome sequence (Verde et al., 2013) together with four almond genome sequences (Koepke et al., 2013) and the sweet cherry genome publicly available since 2013 (Carrasco et al., 2013), offer new possibilities for integrating genetic and genomic approaches to find new CGs for flowering time in perennial plants (Martínez-Gómez et al., 2012).

TRANSCRIPTOMIC STUDIES

Almond transcriptomic studies have not been performed to date. The only transcriptomic study performed in other *Prunus* species has been using flower buds of Japanese apricot (*Prunus mume* Sieb. et Zucc.) at different dormant stages (Habu et al., 2012). In this species, varying flowering time is caused by irregular bud endodormancy release (Zhuang et al., 2013). The transcriptome analysis of flower buds showed 25 endodormant-specific up-regulated unigenes. *DAM6* was one of them although, in most of the unigenes, no hit was found. As we have previously mentioned, many of the *MADS* family genes, such as *DAM6*, are involved in different steps of flower development including flowering time (Riechmann and Meyerowitz, 1997). At this moment, more than 50 *MADS-Type* Transcription Factors (TFs) have been identified in the peach genome, so further studies should be done to identify more gene products involved in flowering.

In *Arabidopsis*, flowering time is dependent on intricate genetic networks to perceive and integrate both endogenous and environmental signals (Khan et al., 2014). In the aging pathway, it has been found that the role of five microRNAs (miRNAs) families called *miR156*, *miR172*, *miR159/319*, *miR390*, and *miR399* is important in flowering time (Spanudakis and Jackson, 2014). Recently, miRNAs differently expressed in chilled peach vegetative buds have been identified co-localizing with known QTLs for chilling requirement and flowering time traits (Barakat et al., 2012; Ríos et al., 2014). A cascade of miRNAs such as *miR156*, *miR172* and their respective targets *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*, and *AP2* like genes are involved in modulating flowering induction in *Arabidopsis* through *FT* and other flowering related genes (Khan et al., 2014; Spanudakis and Jackson, 2014). Transcriptomic studies in poplar and leafy spurge have shown a differential expression of *SPL* genes and *miR172* during dormancy induction, suggesting that this miRNA pathway may also play a regulating role in dormancy processes that affect flowering time (Ríos et al., 2014).

Application of new high-throughput RNA sequencing (RNA-seq) technologies (Flintoft, 2008; Martínez-Gómez et al., 2011) could greatly clarify the TFs involved in the regulation of flowering time, allowing the determination of transcripts from a particular region of the genome.

METABOLOMIC STUDIES

The common by-product upon degradation of hydrogen cyanamide and cyanogenic glucosides is cyanide, which is not only involved in bringing forward flowering time but also in breaking seed dormancy by inducing formation of Reactive Oxygen Species (ROS). ROS activates a cascade in which *ETHYLENE RESPONSE FACTOR 1* is implicated, producing germination-associated proteins (Oracz et al., 2007). CBF proteins belong to the *CBF/DRE* binding sub-family of the *APETALA2-ETHYLENE* responsive factor (Nakano et al., 2006). The action mechanism of nitrogen-based chemical treatments could involve the regulation of the effect of these TFs.

Further, in stone-fruit species (e.g., *Prunus* species), the presence of common regulatory mechanisms between the chilling requirements for seed and bud dormancy release have been suggested (Leida et al., 2012). Moreover, secondary metabolites as cyanogens were suggested to be involved in the germination of cocklebur seeds (*Xanthium pensylvanicum* Wallr.) in response to various nitrogenous compounds (Esashi et al., 1996). Other cyanogens such as the CNGlc prunasin have been described in flower parts in eucalyptus (*Eucalyptus cladocalyx* F. Muell.), seeing that young flower buds were the most cyanogenic, when reproductive organs were analyzed at various stages of development (Gleadow and Woodrow, 2000).

The integrated analysis of these well-known mechanisms reveals accumulation of cyanogenic glucosides and regulation of flowering time through TFs, which open new possibilities in the analysis of this complex trait in almond and the rest of *Prunus*.

NEW PERSPECTIVES

Almond is not only the earliest fruit tree to break dormancy but also shows the widest range of flowering time among all fruit and nut species (Socias i Company and Felipe, 1992), making it a suitable candidate for studying this important trait within perennial plants. There are many genes that are conserved during the evolution of flowering plants. However, there are other mechanisms i.e., miRNAs regulation or metabolite signaling, which could be also included in new studies to deepen the analysis of gene regulation of the flowering time in almond. The final objective continues to be the development of efficient molecular markers for selection in breeding programs. This would enable breeders to select the late flowering individuals in the nursery which would allow them to avoid yield losses due to frosts, which currently occurs in early flowering genotypes.

AUTHOR CONTRIBUTIONS

Raquel Sánchez-Pérez and Pedro Martínez-Gómez participated in the coordination of the study. Federico Dicenta and Jorge Del Cueto collected and revised the genetic information. Pedro Martínez-Gómez and Raquel Sánchez-Pérez collected and revised genomic and transcriptomic information. Raquel Sánchez-Pérez and Jorge Del Cueto collected metabolomics information.

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Genetic architecture of main effect QTL for heading date in European winter wheat

Christine Zanke¹, Jie Ling¹, Jörg Plieske², Sonja Kollers³, Erhard Ebmeyer³, Viktor Korzun³, Odile Argillier⁴, Gunther Stiewe⁵, Maike Hinze⁵, Sebastian Beier¹, Martin W. Ganal² and Marion S. Röder^{1*}

¹ Department of Cytogenetics and Genome Analyses, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany

² TraitGenetics GmbH, Gatersleben, Germany

³ KWS LOCHOW GmbH, Bergen, Germany

⁴ Syngenta Seeds S.A.S., Orgerus, France

⁵ Syngenta Seeds GmbH, Bad Salzuflen, Germany

Edited by:

Klaus Pillen, Martin-Luther-University Halle-Wittenberg, Germany

Reviewed by:

Dongying Gao, University of Georgia, USA

Tobias Würschum, State Plant Breeding Institute, Germany

*Correspondence:

Marion S. Röder, Department of Cytogenetics and Genome Analyses, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, Gatersleben 06466, Germany
e-mail: roder@ipk-gatersleben.de

A genome-wide association study (GWAS) for heading date (HD) was performed with a panel of 358 European winter wheat (*Triticum aestivum* L.) varieties and 14 spring wheat varieties through the phenotypic evaluation of HD in field tests in eight environments. Genotyping data consisted of 770 mapped microsatellite loci and 7934 mapped SNP markers derived from the 90K iSelect wheat chip. Best linear unbiased estimations (BLUEs) were calculated across all trials and ranged from 142.5 to 159.6 days after the 1st of January with an average value of 151.4 days. Considering only associations with a $-\log_{10} (P\text{-value}) \geq 3.0$, a total of 340 SSR and 2983 SNP marker-trait associations (MTAs) were detected. After Bonferroni correction for multiple testing, a total of 72 SSR and 438 SNP marker-trait associations remained significant. Highly significant MTAs were detected for the photoperiodism gene *Ppd-D1*, which was genotyped in all varieties. Consistent associations were found on all chromosomes with the highest number of MTAs on chromosome 5B. Linear regression showed a clear dependence of the HD score BLUEs on the number of favorable alleles (decreasing HD) and unfavorable alleles (increasing HD) per variety meaning that genotypes with a higher number of favorable or a low number of unfavorable alleles showed lower HD and therefore flowered earlier. For the vernalization gene *Vrn-A2* co-locating MTAs on chromosome 5A, as well as for the photoperiodism genes *Ppd-A1* and *Ppd-B1* on chromosomes 2A and 2B were detected. After the construction of an integrated map of the SSR and SNP markers and by exploiting the synteny to sequenced species, such as rice and *Brachypodium distachyon*, we were able to demonstrate that a marker locus on wheat chromosome 5BL with homology to the rice photoperiodism gene *Hd6* played a significant role in the determination of the heading date in wheat.

Keywords: genome wide associations, *Triticum aestivum* L., photoperiodism, environmental adaptation, flowering time

INTRODUCTION

Heading date (HD) is one of the critical traits for the adaptation of bread wheat (*Triticum aestivum* L.) to diverse climatic environments and the cultivation in various regions and cropping seasons. The adaptability of wheat to a wide range of environments has been favored by allelic diversity in genes regulating growth habit and photoperiod response. Differences in the vernalization genes (*Vrn*) determine spring and winter wheat habits. The photoperiod genes (*Ppd*) play a major role in determining the flowering time and the sensitivity to photoperiodism. Earliness *per se* (*Eps*) genes influence flowering time independently from photoperiodism.

On a molecular level, regulation networks for heading and flowering are conserved between model species, such as *Arabidopsis* (Andrés and Coupland, 2012), as well as in dicotyledonous and monocotyledonous crop plants (Jung and Müller,

2009) including the temperate cereals (Cockram et al., 2007; Trevaskis et al., 2007; Distelfeld et al., 2009).

Positional cloning identified *Ppd-H1*, the major determinant of barley photoperiod response, as a pseudo-response regulator, which is an ortholog of the *Arabidopsis* photoperiod pathway gene *CONSTANS* (Turner et al., 2005). In wheat, an orthologous gene was identified as the *Ppd-D1* gene on chromosome 2D (Beales et al., 2007). A semi-dominant mutation, *Ppd-D1a* widely used in the “green revolution,” converts wheat from a long day (LD) to a photoperiod insensitive (day neutral) plant, providing adaptation to a broad range of environments. Recently it was shown that the bolting locus B of sugar beet, distinguishing annuals from biennials, is also a pseudo-response regulator gene named *BOLTING TIME CONTROL 1* (*BvBTC1*) with similarity to the *CONSTANS* gene of *Arabidopsis* and *Ppd-H1* in barley (Pin et al., 2012). Another photoperiodism gene, *Ppd-B2*, which was

detected when plants were exposed to a long photoperiod, was mapped on chromosome 7BS in wheat (Khlestkina et al., 2009).

Similarly, the molecular mechanisms for the requirement of vernalization have been identified (Trevaskis et al., 2007; Distelfeld et al., 2009) in wheat. Natural variation in vernalization requirement in the temperate cereals is mainly associated with allelic differences in the *VRN1*, *VRN2*, and *VRN3* vernalization genes. *VRN1* encodes a MADS-box transcription factor with high similarity to *Arabidopsis* meristem identity genes *APETALA1*, *CAULIFLOWER* and *FRUITFUL* (Yan et al., 2003; Distelfeld et al., 2009). *VRN2*, a dominant repressor of flowering, is down-regulated by vernalization. The *VRN2* region includes two similar ZCCT genes encoding proteins with a putative zinc finger and a CCT domain that have no clear homologs in *Arabidopsis* (Yan et al., 2004; Distelfeld et al., 2009). The vernalization gene *VRN3* encodes a RAF kinase inhibitor like protein with high homology to *Arabidopsis* protein *FLOWERING LOCUS T (FT)* (Yan et al., 2006; Distelfeld et al., 2009).

The presence of earliness *per se* genes (*Eps*) has been demonstrated by QTL-mapping studies in barley and wheat since a long time (Laurie et al., 1995; Worland, 1996). Only recently the molecular identification of two *EARLY MATURITY* genes, *eam8* and *eam10*, has been reported in barley (Faure et al., 2012; Zakhrabekova et al., 2012; Campoli et al., 2013). Earliness *per se* genes have been fine-mapped in diploid or hexaploid wheat on chromosomes 1A and 3A (Faricelli et al., 2010; Gawronski and Schnurbusch, 2012).

Several QTL and meta-QTL mapping studies showed that in wheat, besides the known major loci, a wealth of additional chromosomal regions affect the flowering time (Sourdille et al., 2000; Hanocq et al., 2004, 2007; Griffiths et al., 2009; Rousset et al., 2011). Co-location of QTLs for agronomic traits, such as post-anthesis leaf senescence, grain yield or grain protein concentration with QTL for flowering time indicated pleiotropic effects of anthesis date (Bogard et al., 2011). Also in barley a number of flowering time QTL were associated with agronomic traits (Wang et al., 2010).

While with bi-parental mapping studies only a limited number of parental lines can be investigated, genome wide association studies (GWAS) are suitable for the monitoring of a larger germplasm panel (Zhu et al., 2008). The method is based on the meiotic events which occurred during the entire development of the lines and which results in an increased genetic resolution determined by the extent of linkage disequilibrium (LD) of the species under investigation (Hamblin et al., 2011). Whole-genome association mapping was applied in wheat for ear emergence (Le Gouis et al., 2012), as well as for yield and agronomic traits (Neumann et al., 2011; Reif et al., 2011; Wang et al., 2012) and resistance to pathogens (Crossa et al., 2007; Maccaferri et al., 2010; Miedaner et al., 2011; Yu et al., 2011, 2013; Letta et al., 2013; Kollers et al., 2013a,b).

The goal of our study was to map marker-trait associations (MTAs) for HD in a panel of European winter wheat varieties and to identify markers suitable for marker assisted selection. We were interested to compare the MTAs detected with genome wide SSR (simple sequence repeat) markers to the pattern of MTAs detected by a SNP (single nucleotide polymorphism) array.

Finally, we exploited the synteny of the SNP marker sequences to other grass species with complete genome sequence, such as rice and *Brachypodium distachyon*, in order to detect relationships to already described genes connected to the regulation of photoperiodism and flowering time.

MATERIALS AND METHODS

PLANT MATERIAL AND PHENOTYPING

The plant material, consisting of 358 European winter wheat varieties plus 14 spring wheat varieties as an outgroup, is described in more detail in Kollers et al. (2013a). Field trials were conducted in the season 2008/2009 in Andelu/France (09.AND), Seligenstadt/Germany (09.SEL) and Wohlde/Germany (09.WOH) and in the season 2009/2010 in Andelu/France (10.AND), Janville/France (10.JAN), Saultain/France (10.SAU), Seligenstadt/Germany (10. SEL) and Wohlde/Germany (10.WOH) by applying an alpha design with two replications per site. Both winter and spring varieties were sown in autumn and HD was recorded as the developmental stage at that time, by counting days from the 1st of January, when ears of approximately half of the genotypes were fully visible (Supplemental Table 1).

MOLECULAR DATA ANALYSIS, GENETIC MAPPING AND ANALYSIS OF SYNTENY

For marker-trait analysis a set of 732 microsatellite markers, resulting in 770 different loci spread across all chromosomes of wheat was used. Of these 770 loci, 635 loci were mapped and 135 loci were unmapped. Since the microsatellites are multi-allelic, they amounted to 3176 alleles. More details about this data set and the description of LD and population structure are provided in Kollers et al. (2013a). For SNP-analysis a novel 90k Infinium chip (90k iSELECT) was genotyped on all 372 varieties (Cavanagh et al., 2013; Wang et al., 2014). This resulted in a total of 21742 scorable and polymorphic markers on our association panel by considering all polymorphic markers with a minor allele frequency (MAF) >0.03. Of these markers, only the 7934 mapped markers were included in the association analysis, while the unmapped markers were not used for association analysis. The SSR-markers were mapped on the ITMI-population (International Triticeae Initiative) based on recombinant inbred lines between the parents W7984 (synthetic wheat) × Opata M85 (Röder et al., 1998; Ganal and Röder, 2007), while the SNP markers were mapped on 138 lines of a newly created doubled-haploid population of the same parents (Sorrells et al., 2011; Poland et al., 2012). Map construction was performed using the software package Joinmap 4.1. Both maps have different recombination values, and currently only few common markers are available, which makes comparisons difficult. For display a reduced version of the SNP-map was used containing all relevant markers with MTAs for HD.

In order to establish the synteny of interesting MTA loci to rice, a BLAST X (cutoff: *e*-value of 10E-2) was conducted against the data base of MSU Rice Genome Annotation Project Release 7.0 (<http://rice.plantbiology.msu.edu/>) for all SNP markers with significant ($-\log_{10} (P\text{-value}) \geq 3.0$) MTAs for HD. For the blast search the flanking sequences of the SNP markers (101–201 bp in length) according to Wang et al. (2014) were used. The resulting

3877 syntenic relationships were filtered for chromosomal synteny as described by Salse et al. (2009) resulting in 956 syntenic relationships. For comparison to literature data in some cases the ID converter (<http://rapdb.dna.affrc.go.jp/tools/converter/run?type=rap-msu;id=Os11g0157100>) was used in order to compare to locus designations of the RAP-DB rice annotation project database (<http://rapdb.dna.affrc.go.jp/>).

For detecting the synteny to *Brachypodium distachyon* a BLAST X (cutoff: *e*-value of 10E-2) was conducted against version 1.2 of the MIPS annotation (<http://mips.helmholtz-muenchen.de/plant/brachypodium/download/index.jsp>) resulting in 3404 syntenic relationships. Those were filtered according to the expected chromosomal synteny (The International Brachypodium Initiative, 2010) resulting in 1575 syntenic relationships.

As candidate genes the photoperiodism gene *Ppd-D1* (Beales et al., 2007) and the vernalization genes *Vrn-B1* and *Vrn-D1* (Zhang et al., 2008) were genotyped on all varieties.

STATISTICAL ANALYSIS AND ASSOCIATION MAPPING

Each year-location combination was considered as an environment in our study. For each environment and genotype the adjusted mean of two replications was calculated as the phenotypic data using GenStat 13th edition as

$$y = \mu + \text{replication} + \text{genotype} + \text{block} + e$$

with replication and genotype as fixed factors and block as random factor and block nested within replication; μ represents an overall mean and e is a residual term.

In addition, best linear unbiased estimations (BLUEs) across all eight environments were calculated using the software package GenStat 14th edition (VSN International, Hemel Hempstead, Hertfordshire, UK) as described in Kollers et al. (2013a) with

$$y = \mu + \text{genotype} + \text{environment} + e$$

with genotype and environment as fixed effects; μ represents an overall mean and e is a residual term. Since the datasets for all environments were complete and balanced, the BLUEs, in fact, equaled the arithmetic means across environments.

For calculating genotype-phenotype associations a minor allele frequency (MAF) threshold of 3% (equaling 11 varieties) was applied to all markers. A mixed model for association mapping was calculated using the software package GenStat 14th edition as described in Kollers et al. (2013a) by applying a kinship matrix as relationship model.

$$P_i = \mu + x_i\alpha + G_i + e$$

$$\text{with } G_i \sim N(0, 2K\sigma_g^2), \text{ error } \sim N(0, \sigma^2)$$

x_i is the marker score for cultivar i , α is the marker fixed effect, μ represents an overall mean, e is a residual term and G_i represents the score of genotype corrected by kinship matrix (K) to structure random genotypic effects.

The Loiselle kinship matrix was calculated for 155 SSR markers, equally distributed on the genome, by using the software package SPAGeDi (Hardy and Vekemans, 2002). This kinship

matrix was applied to correct for false positives for calculating MTAs with SSR as well as with SNP markers as described by Matthies et al. (2012). The threshold of Bonferroni correction for multiple testing was calculated by dividing $P < 0.01$ with the number of SSR or SNP markers used for the analysis.

Additive effects and marker effects (r^2) were estimated using the software package TASSEL 3.0.

Spearman rank order correlations and ANOVA using the adjusted means of the eight environments were calculated with the software package SigmaPlot 11.0. The heritability was calculated from the variance components according to the formula: $H^2 = \text{Var}(\text{genotype}) / (\text{Var}(\text{genotype}) + \text{Var}(\text{error}) / \text{no. of locations})$ with variance components calculated with the software package SPSS v. 19. This software was also used to conduct a trait *Post-hoc* test according to Tukey B.

RESULTS

DESCRIPTION OF PHENOTYPIC DATA

The phenotypic data for 358 European winter wheat varieties plus 14 spring wheat varieties were based on field evaluations in eight environments. The resulting best linear unbiased estimations for heading time across all environments ranged from 142.5 to 159.6 days after 1st of January with an average of 151.4 days (Supplemental file 1). All 14 spring varieties, which had been sown at the same time as the winter varieties were found in the early segment of HD (Figure 1A). Also all 53 varieties carrying the photoperiodism insensitive mutant of gene *Ppd-D1* on chromosome 2DS (Beales et al., 2007) were in the first half of the phenotypic distribution, with the exception of winter wheat variety “Paledor,” which was found in the second half of the phenotypic distribution (Figure 1B). The Spearman Rank Order correlation coefficients of the HD scores among the environments and with the BLUEs ranged from 0.843 to 0.973 ($P < 0.001$), indicating

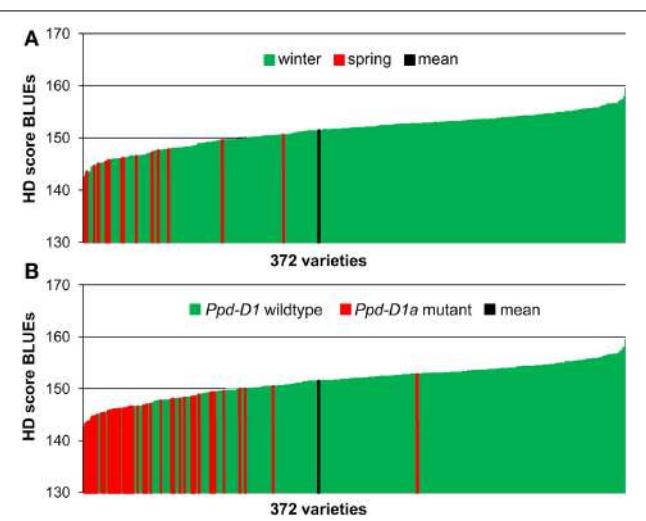


FIGURE 1 | Phenotypic distribution of HD score BLUEs in 372 wheat varieties.

The BLUEs of the HD score were calculated across eight environments. A low score indicates earlier heading date. HD score BLUEs were arranged according to the growth habit (A) or to the distribution of the *Ppd-D1* wildtype or *Ppd-D1a* mutant gene (B).

a high reproducibility of the ranking of varieties grown in different locations (Supplemental file 2). The analysis of variance (ANOVA) was significant for genotype as well as environment (Supplemental file 3). A Tukey *B*-test detected six different class means for the environments ranging from 144.5 to 161.5 days, which is also reflected in a broad sense heritability of $H^2 = 0.609$ (Supplemental file 4).

DETECTION OF MARKER-TRAIT ASSOCIATIONS (MTAs)

MTAs were calculated separately for each environment plus the resulting BLUEs by employing a mixed linear model with a kinship matrix. Two sets of genotypic data were used: First, a set of 732 microsatellite markers (SSRs) resulting in 770 loci spread across the 21 chromosomes of wheat, and secondly, a set of 7934 SNP markers coming from the 90K wheat iSELECT array. While the SNP markers represent a bi-allelic marker system, the microsatellites provide multiple alleles per locus resulting in a total number of 3176 alleles. The microsatellite data were described in former whole genome association studies (Kollers et al., 2013a,b) and provide good genomic coverage of all chromosomes. The SNP data were mapped to all chromosomes, but due to the lack of polymorphism, the chromosomes of the D-genome were less covered than those of the genomes A and B (Supplemental file 5).

A total of 340 SSR and 2983 SNP MTAs reached a standard threshold of $\log_{10}(P\text{-value}) \geq 3.0$ (corresponding to a $P\text{-value} < 0.001$). These included 42 BLUEs for the SSRs and 326 BLUEs for the SNPs. After applying a Bonferroni correction for multiple testing (with $\alpha = 0.01$), a $-\log_{10}(P\text{-value}) \geq 4.82$ for SSR and a $-\log_{10}(P\text{-value}) \geq 5.89$ for SNP were considered as significant. After this correction, a total of 72 SSR and 438 SNP MTAs remained significant (Table 1, Figure 2, Supplemental files 5–7), which included 10 BLUEs for the SSRs and 51 BLUEs for the SNPs. A total of 79 different marker loci were involved in MTAs detection for the SSR markers and 758 marker loci for SNP markers corresponding to a $-\log_{10}(P\text{-value}) \geq 3.0$ (Supplemental file

5). Since many marker loci co-segregated or were closely linked in the genetic map, marker loci with distances ≤ 1.0 cM were combined. When considering only the BLUEs with $\log_{10}(P\text{-value}) \geq 3.0$, the number of combined marker loci was 30 for the SSRs and 92 for the SNPs (Supplemental file 5).

Many marker loci were significant for several environments (Figure 3, Supplemental file 8) with up to nine significant MTAs per marker locus (eight environments plus BLUEs). The number of significant MTAs varied considerably among the various chromosomes. For both marker types the highest number of significant MTAs was detected on chromosome 5B before Bonferroni correction, while after Bonferroni correction most SSR loci were significant on chromosome 6D (Supplemental file 5).

The comparison of the SSR map with the SNP map is still difficult, because the two maps were constructed on different mapping populations and contain only few common markers. Based on the mapping positions, some MTAs can be matched between SSR and SNP markers. An example is the MTA with SSR marker GWM1130 at the distal end of chromosome 1BS and the SNP marker Kukri_c38553_173. Overall, many additional marker loci were significant for the SNPs as compared to the SSR markers.

Highly significant MTAs were detected for the photoperiod sensitivity gene *Ppd-D1* on chromosome arm 2DS. In all environments and the BLUEs the photoperiod insensitive mutant allele *Ppd-D1a* led to a decreased HD score, which means earlier heading (Figure 4, Supplemental file 9). The mutant allele of *Ppd-D1a* was detected in a total of 53 varieties including five spring varieties (Supplemental file 1). Additionally, the candidate gene markers for *Vrn-B1* and *Vrn-D1* were genotyped. While *Vrn-D1* was monomorphic for all winter varieties and a second allele detected in only two spring varieties (Supplemental file 1), *Vrn-B1* had a dominant allele for three spring varieties, but also three winter varieties (Buteo, Discus and Lona). Since both markers were below the threshold of minor allele frequency, they were not included in the regular analysis for MTAs. When they were tested for associations without setting a MAF, no significant association results were detected for *Vrn-B1* and significant MTAs in two environments were detected for *Vrn-D1* (Supplemental file 10).

Table 1 | Number of MTAs per environment for the SSR marker and the SNPs on the 90K iSelect chip.

| Environments | SSR | | 90K iSelect | |
|---------------------|---------------------------------------|----------------------------------------|---------------------------------------|----------------------------------------|
| | $-\log_{10}(P\text{-value}) \geq 3.0$ | $-\log_{10}(P\text{-value}) \geq 4.82$ | $-\log_{10}(P\text{-value}) \geq 3.0$ | $-\log_{10}(P\text{-value}) \geq 5.89$ |
| Andelu (2009) | 45 | 9 | 325 | 60 |
| Seligenstadt (2009) | 29 | 5 | 254 | 43 |
| Wohlde (2009) | 30 | 9 | 337 | 39 |
| Andelu (2010) | 34 | 4 | 253 | 27 |
| Janvielle (2010) | 23 | 3 | 232 | 33 |
| Saultain (2010) | 44 | 8 | 290 | 47 |
| Seligenstadt (2010) | 52 | 13 | 583 | 94 |
| Wohlde (2010) | 41 | 11 | 383 | 44 |
| BLUEs | 42 | 10 | 326 | 51 |
| Sum | 340 | 72 | 2983 | 438 |

ADDITIONAL EFFECTS FOR FAVORABLE AND UNFAVORABLE ALLELES

In the following section, marker alleles with a negative additive effect leading to earlier heading will be referred to as “favorable alleles” and vice versa marker alleles leading to later heading as “unfavorable alleles.” We are aware, that earlier heading is not favorable in all circumstances; the designation is mainly meant to facilitate the following description of the allele effects.

Considering the SSR markers, the varieties contained between zero to 25 favorable alleles and between six to 28 unfavorable alleles (Figure 5). A significant Spearman Rank Order correlation of $R = -0.697$ ($P = 0.00000020$) existed between the HD BLUEs score and number of favorable alleles; for the HD BLUEs score and the number of unfavorable alleles the Spearman Rank correlation coefficient was $R = 0.642$ ($P = 0.00000020$). Linear regression showed a dependence of the HD BLUEs score from the number of favorable alleles with $R^2 = 0.577$ and $Y = 155.0 - 0.4X$; for the unfavorable alleles $R^2 = 0.503$ and $Y = 141.6 + 0.5X$ was observed (Figure 6). This means that varieties with a

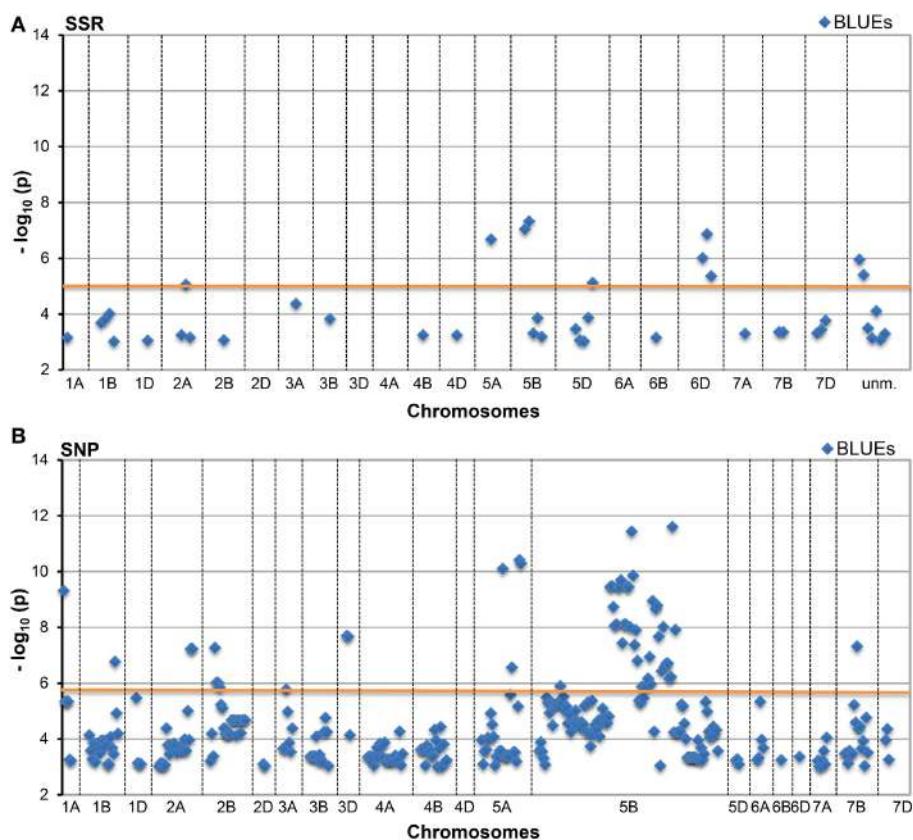


FIGURE 2 | Manhattan Plots of (A) SSR and (B) SNP marker alleles associated with HD BLUEs. This plot presents significant alleles associations at threshold $-\log_{10}$ (P -value) ≥ 3.0 for BLUEs sorted according

to their chromosomal location. The red line indicates the threshold $-\log_{10}$ (P -value) ≥ 4.82 (SSR) and ≥ 5.89 (SNP), respectively, for Bonferroni correction.

higher number of favorable alleles and a lower number of unfavorable alleles have an earlier heading time. The regression of favorable minus unfavorable alleles against the HD BLUEs score was $Y = 148.5 - 0.3X$ with $R^2 = 0.603$ (Supplemental file 11).

We calculated the same regressions by taking the best or worst 20, 10 or 5 marker alleles into account (Figure 6, Table 2). These included the SSR markers with significant associations with a $-\log_{10}(P\text{-value}) \geq 3.0$ and the candidate gene *Ppd-D1*. The selection was based on the mean additive effect as described in Supplemental file 5. Even with only five marker alleles, significant regressions with $R^2 = 0.372$ for the favorable alleles and $R^2 = 0.326$ for the unfavorable alleles were observed. Therefore the chosen marker alleles are good candidates for adapting the HD in breeding programs by marker assisted selection.

EXPLOITATION OF SYNTENY TO RICE AND BRACHYPODIUM DISTACHYON

After conducting a BlastX to the rice genomic sequence and filtering for the synteny relationships between wheat and rice described by Salse et al. (2009) a total of 956 synteny relationships between significant wheat SNP markers and the rice genome were established (Supplemental file 12). For *Brachypodium distachyon*, a total of 1575 synteny relationships to the wheat markers were found after filtering for synteny according to the described

chromosomal relationships (The International Brachypodium Initiative, 2010) (Supplemental file 13).

In the publication of Higgins et al. (2010) all known genes related to flowering time pathways were blasted to *Brachypodium* and rice. A comparison of our list of syntenic rice loci (Supplemental file 12) to their detected homologs gave two direct hits for the wheat marker Kukri_c10016_369 to two rice loci at LOC_Os03g10940.1 and LOC_Os03g55490.1. Both genes are coding for expressed putative protein casein kinase II subunit alpha-2, which both have homology to the rice gene *Hd6* located at LOC_Os03g55389 (Takahashi et al., 2001). Also for *Brachypodium* homologs four direct hits with the same wheat marker Kukri_c10016_369 were found (Bradi1g07750.1, Bradi1g07810.1, Bradi1g59010.1, Bradi1g70690.1), with all four genes belonging to the *Hd6* gene family. *Hd6* was cloned as a rice quantitative trait locus involved in photoperiod sensitivity and is thought to be involved in the plant phototransduction pathway. Wheat marker Kukri_c10016_369 was highly significant, even after Bonferroni correction, in all eight environments plus BLUEs and is part of a cluster of significant markers on chromosome 5B. In an analysis of LD it was shown, that LD existed between Kukri_c10016_369 and the highly significant SSR markers WMC160 and BARC232, especially the alleles discovering MTAs, WMC160_137 bp, WMC160_190 bp,

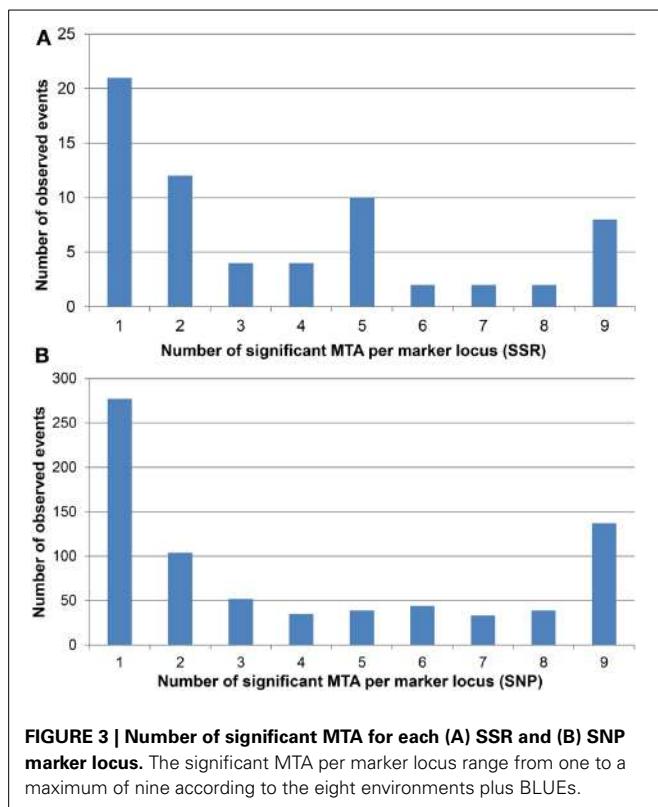


FIGURE 3 | Number of significant MTA for each (A) SSR and (B) SNP marker locus. The significant MTA per marker locus range from one to a maximum of nine according to the eight environments plus BLUEs.

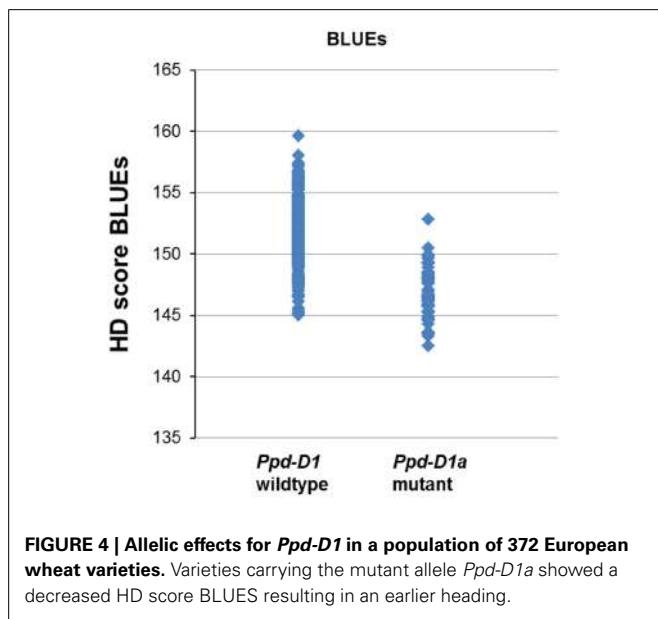


FIGURE 4 | Allelic effects for Ppd-D1 in a population of 372 European wheat varieties. Varieties carrying the mutant allele *Ppd-D1a* showed a decreased HD score BLUES resulting in an earlier heading.

BARC232_197 bp, and BARC232_232 bp, while no LD existed with *Vrn-B1* (Supplemental file 14). Therefore the MTAs discovered by those SSR alleles are not based on LD to *Vrn-B1* but most likely on LD to an *Hd6* related gene in wheat. It can be concluded, that the gene from which Kukri_c10016_369 was derived, is an *Hd6*-related gene in wheat, which itself has a significant impact on HD or is in LD with another gene affecting HD.

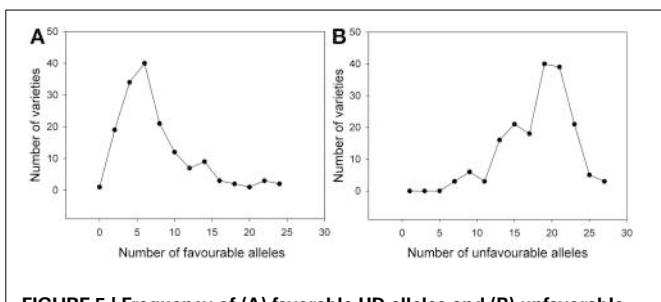


FIGURE 5 | Frequency of (A) favorable HD alleles and (B) unfavorable HD alleles from SSR markers in individual varieties. Most of the varieties carried between zero to ten favorable alleles decreasing the heading date and between ten to 25 unfavorable alleles increasing the heading date.

DISCUSSION

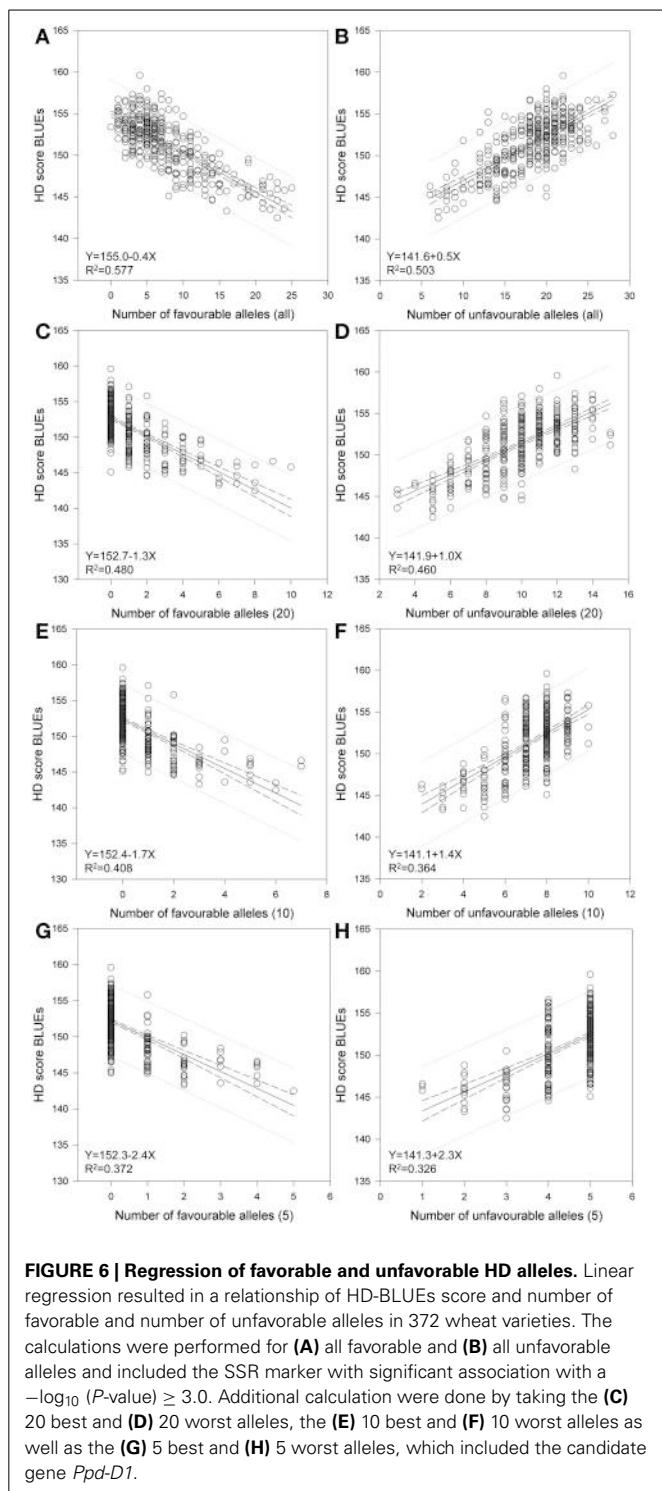
COMPARISON OF MTAs DISCOVERED WITH SSR AND SNP MARKERS

The chosen approach led to the discovery of a number of highly significant MTAs for HD in European winter wheat. In comparison to other traits, which were analyzed in the same set of varieties and molecular markers, the number of significant MTAs for HD was lower and less loci were involved. For resistance to *Fusarium* head blight a total of 794 significant MTAs [$-\log_{10}(P\text{-value}) \geq 3.0$], which included 323 SSR alleles, were detected in four environments (Kollers et al., 2013a), while for resistance to *Septoria tritici* blotch 115 MTAs were significant [$-\log_{10}(P\text{-value}) \geq 3.0$] involving 68 microsatellite loci in two environments (Kollers et al., 2013b). For HD, 340 MTAs detected by 79 SSR loci were significant [$-\log_{10}(P\text{-value}) \geq 3.0$] in eight environments (Table 1). In a previous genome-wide association study involving a 227-wheat core collection and 760 molecular markers, consisting of mainly DArT markers, 62 markers individually associated to earliness components corresponding to 33 chromosomal regions, were identified (Le Gouis et al., 2012). This number corresponds well to the 30 loci identified in our study by SSRs, when considering the BLUEs only and when adjacent markers were combined to unified loci (Supplemental files 5, 6B, 7B). A meta-QTL analysis of the genetic control of ear emergence in elite European winter wheat germplasm discovered 19 meta-QTL regions (Griffiths et al., 2009).

Many marker loci were detected in two or more environments (Figure 3). This observation indicates the impact of major genes in shaping the genetically determined pattern of HD in winter wheat. It is also an indicator of a high reproducibility of the ranking of varieties considering the phenotypic data, which was confirmed by the high correlations observed between the environments (Supplemental file 2), though the environments covered a range of geographical latitudes (48.2 to 54.4°N; Supplemental file 1) and various micro-climates in France and Germany.

Overall, the used number of SNP markers was higher with 7934 SNP markers compared to 770 SSR loci with a total of 3176 SSR alleles. After Bonferroni correction, 90 SSR markers remained significant as compared to 438 for the SNPs. These included 10 BLUEs for SSR and 51 BLUEs for SNPs (Supplemental files 5, 6B, 7B).

Though the overall number of SNP markers was higher than the SSR markers, there was less coverage for specific



chromosomes like 4D and 6D, and many co-segregating loci resulted in a reduced number of haplotypes. Like the SSRs, the SNP markers often detected significant MTAs for HD in various environments with 137 SNP markers detecting all eight environments plus BLUEs (Figure 3B). Often SNPs, which co-segregated in the genetic map, were all involved in MTA detection, resulting in clusters of significant markers (Supplemental file 8).

The prerequisite for a detailed comparison of the significant SSR and SNP loci is a highly integrated map for both marker systems, which currently is not available yet. By comparing the chromosomal locations of the SNP and SSR maps (Supplemental file 8), it becomes obvious that several novel chromosomal locations were detected by the SNPs compared to the SSRs. Examples are a cluster of significant SNP markers at the distal end of chromosome 1AL (RAC875_c21411_162, wsnp_BE444305A_Td_2_1, wsnp_RFL_Contig3542_3718200, RAC875_c12348_720) and a cluster of highly significant markers on the distal end of chromosome 3DS (Excalibur_c19658_127, Kukri_c24488_431, Kukri_rep_c94244_223).

CANDIDATE GENES FOR MTAs WITH SSR

The presence of detailed mapping information of the SSR markers in various maps (Somers et al., 2004; Ganal and Röder, 2007; <http://wheat.pw.usda.gov/GG2/index.shtml>) allowed the comparison of our association results to the mapping positions of known candidate genes. MTAs most likely corresponding to the series of photoperiodism genes *Ppd* on the short arms of the homeologous group 2 chromosomes were detected for chromosome 2A (markers WMC177 and WMC522) and chromosome 2B (marker GWM4167). *Ppd-B1* was previously mapped in the interval of GWM257 and GWM148 (Mohler et al., 2004), which includes marker GWM4167 in our map. The marker for candidate gene *Ppd-D1* was the most significant marker based on the observed additive effects, however no significant SSR markers in the expected region on chromosome 2DS in the vicinity of marker GWM261 (Pestsova and Röder, 2002) were observed. One possible reason may be the existence of a 21 centiMorgan gap in the genomic region between WMC112 and BARC168. If *Ppd-D1* is located in this gap, the extent of LD may not reach the flanking markers. An LD plot showed no LD with $r^2 > 0.1$ between the alleles of markers GWM261, WMC112 or BARC168 and the *Ppd-D1* candidate gene (Supplemental file 15). The agronomic effects described for *Ppd-D1* depended very much on the trial sites. In the UK, the 2D chromosome carrying *Ppd-D1* reduced yield about 5–10%, while in Yugoslavia the same genotypes increased yield about 30% (Worland et al., 1998). The advantages of earlier heading of *Ppd-D1* insensitive varieties in Southern European countries were attributed to an escape of heat and drought during summer. The genotyping of the candidate marker for *Ppd-D1* indeed showed that the insensitive mutant allele is mainly present in varieties originating from South France (Supplemental file 1). *Ppd-B1* (old nomenclature *Ppd2*) was described as a weaker gene for photoperiod insensitivity than *Ppd-D1* with a strong influence of the environmental conditions on the agronomic effects (Worland et al., 1998). For central European varieties, where the effects of *Ppd-D1* are too strong, *Ppd-B1* may provide a moderate gene for the adaptation to hot and dry summers. An epistatic interaction between *Ppd-B1* and *Ppd-D1* was described in a doubled haploid mapping population (Hanocq et al., 2004). We found in our list of the markers with the strongest additive effects besides the *Ppd-D1* candidate gene also GWM4167 associated with *Ppd-B1* and WMC522 associated with *Ppd-A1* (Table 2), emphasizing the presence and importance of these genes in the Central European varieties.

Table 2 | List of the best favorable and worst unfavorable alleles.

| Marker alleles | Chromosome (linked genes) | Position | Alleles belong to the | | | | | |
|-----------------|------------------------------------|----------|-----------------------|---------|--------|----------|----------|---------|
| | | | 20 best | 10 best | 5 best | 20 worst | 10 worst | 5 worst |
| GWM1130_109bp* | 1B | 0 | x | | | | | |
| GWM1130_115bp* | 1B | 0 | | | | x | x | x |
| BARC0240_231bp | 1B | 36.1 | | | | x | | |
| GWM3166_153bp | 1B | 175.7 | x | | | | | |
| WMC0732c_295bp | 1D | 132.4 | x | | | | | |
| WMC0522_200bp | 2A (<i>Ppd-A1</i>) | 88.3 | x | x | | | | |
| GWM4167_217bp | 2B (<i>Ppd-B1</i>) | 40 | | | | x | | |
| BARC0160_111bp | 2B | 80.4 | | | | x | x | |
| CFD0056c_250bp | 2D | 20.2 | | | | x | | |
| GWM0988_180bp | 2D | 84.5 | | | | x | | |
| CFD0168_256bp | 2D | 160.3 | | | | x | x | x |
| Ppd_insensitive | 2D | unm. | x | x | x | | | |
| Ppd_sensitive | 2D | unm. | | | | x | x | x |
| WMC0264_141bp* | 3A ¹ | 131.3 | x | x | | | | |
| WMC0264_148bp* | 3A ¹ | 131.3 | | | | x | | |
| WMC0808_147bp | 3B ¹ | 67.5 | x | | | | | |
| GWM0160a_181bp | 4A | 186.4 | x | | | | | |
| GWM4636_233bp | 4B ¹ | 59.4 | x | | | | | |
| WMC0285_293bp | 4D | 0 | x | x | | | | |
| GWM0291_176bp | 5A (<i>Vrn-A2</i>) | 231 | x | x | x | | | |
| WMC0160b_137bp | 5B ¹ (Hd6-related gene) | 158.4 | | | | x | x | |
| WMC0783_179bp | 5B | 219.8 | | | | x | | |
| WMC0215_208bp | 5D | 200.5 | x | | | | | |
| GDM0063_147bp | 5D | 265.4 | | | | x | | |
| WMC0161b_184bp | 5D | 301.3 | | | | x | | |
| GWM4047_194bp | 6B | 0 | | | | x | x | |
| GWM0825b_122bp | 6B | 34.3 | | | | x | x | |
| GWM1391_158bp* | 6D | 0 | | | | x | x | x |
| GWM1391_160bp* | 6D | 0 | x | x | x | | | |
| CFD0019c_313bp | 6D | 130.9 | x | x | x | | | |
| BARC0204b_500bp | 6D | 194 | x | | | | | |
| GWM0983b_130bp* | 7B | 54 | | | | x | x | x |
| GWM0983b_133bp* | 7B | 54 | x | x | | | | |
| BARC0182_118bp | 7B | 176 | | | | x | x | |
| GWM0428_145bp | 7D | 228.2 | | | | x | | |
| WMC0014_267bp | 7D | 274.2 | | | | x | | |
| BARC0261_170bp | Unmapped | - | x | x | x | | | |
| CFA2263_123bp | Unmapped | - | x | | | | | |
| WMC0327_209bp | Unmapped | - | x | | | | | |
| WMC0349_118 | Unmapped | - | x | x | | | | |

*Markers with positive and negative additive effects, ¹coincides with meta-QTL described by Griffiths et al. (2009).

A series of vernalization genes determining the growth habit of wheat, has been described and functionally characterized (Trevaskis et al., 2007; Distelfeld et al., 2009). These include the series of VRN-1 genes on homeologous chromosomes 5A, 5B, and 5D (Yan et al., 2003), the *Vrn-A2* gene on the distal end of chromosome 5AL (Yan et al., 2004), the *Vrn-B3* gene on chromosome arm 7BS (Yan et al., 2006) and the *Vrn-D4* gene in the centromeric region of chromosome 5D (Yoshida et al., 2010). In

winter wheat usually all four genes *Vrn-A1*, *Vrn-B1*, *Vrn-D1*, and *Vrn-B3* are present in recessive state, while the presence of one or more dominant alleles was only detected in spring wheat varieties (Zhang et al., 2008). This assumption did not verify for *Vrn-B1* in our set of varieties, which had a dominant allele for three spring varieties, but also three winter varieties (Buteo, Discus, and Lona). No significant associations were found for this rare *Vrn-B1* allele, which indicated that the highly significant association

of SSR markers WMC160 and BARC232 on chromosome 5BL was not caused by LD to *Vrn-B1*, but probably by the presence of another gene. Also on the respective chromosomal locations for *Vrn-A1* on chromosome 5A and *Vrn-B3* on chromosome 7BS no significant SSR markers were detected. The highly significant MTAs detected by marker GWM291 on the distal end of chromosome 5A in all environments and BLUES coincided with the location of *Vrn-A2*. *Vrn-A2* has been described as floral repressor that delays flowering until plants are vernalized. Loss of function of *Vrn-A2* results in spring types (Yan et al., 2004; Trevaskis et al., 2007). Allele GWM291_176 bp was among the five best markers based on the additive effects (Table 2). The vernalization gene *Vrn-B3* is linked completely to a gene similar to Arabidopsis *FLOWERING LOCUS T* (*FT*). Transcript levels of the barley and wheat orthologs, designated as *HvFt* and *TaFT*, respectively, are significantly higher in plants for the dominant *Vrn3* alleles (early flowering) than in plants homozygous for the recessive *vrn3* alleles (late flowering) (Yan et al., 2006). It was shown that nucleotide polymorphisms on A and D copies of the wheat *FT* gene were associated with variations for HD in a collection of 239 diverse lines (Bonnin et al., 2008). Gene copy *TaFT-7D* was mapped in the region of marker GWM44 in the central region of chromosome 7D (Bonnin et al., 2008). We detected three significant markers (GWM4335, GWM3062, BARC126) located distal to GWM44, which may or may not be in LD with *TaFT-7D*.

Several of our MTAs coincided with published meta-QTL regions for HD (Hanocq et al., 2007; Griffiths et al., 2009). Besides the already described genomic regions on homeologous groups 2 and 5, the marker WMC264 on chromosome 3A detecting multiple MTAs coincided with a meta-QTL described by Griffiths et al. (2009). Two alleles of WMC264 with opposing effects are included in our table of best and worst alleles (Table 2). On chromosome 3B, QTL for HD were described for the genomic region proximal to GWM493 (Pánková et al., 2008; Griffiths et al., 2009), which may coincide with the MTAs detected by WMC808 in our study. The studies of Griffiths et al. (2009) as well as Hanocq et al. (2007) describe QTLs linked to GWM251 on chromosome 4B. Marker GWM4636, which detected multiple MTAs in our study, is the neighboring marker in our map. In the Charger × Badger population a QTL was described in the interval GWM408 to BARC140 on chromosome 5BL. This interval includes WMC160 and BARC232 which detected both highly significant MTAs in multiple environments in our study. We assume that this MTA is independent of *Vrn-B1*, since the candidate markers for *Vrn-B1* were not significant. A second QTL was described by Griffiths et al. on chromosome 5B located in the interval GWM540 to GWM544. This interval includes WMC376 in our map, which detected multiple MTAs. Markers for both QTL regions on chromosome 5B (WMC160 and WMC783) are included in our selected list of markers (Table 2). Marker WMC14 on chromosome 7DL detected both QTL in the studies of Griffiths et al. (2009) and Hanocq et al. (2007) as well as MTAs in our study. A QTL extending distal to GWM44 on chromosome 7DS in the Savannah × Rialto population (Griffiths et al., 2009) coincided with the MTAs detected by markers GWM4335, GWM3062, and BARC126 in our study. On chromosome 1BL, the QTL in the interval WMC44 to BARC80 detected in the Avalon × Cadenza

population (Griffiths et al., 2009) coincided with MTAs detected by markers GWM3166 and GWM1364 in our study. The QTL detected in the region of GWM18 on chromosome 1BS (Griffiths et al., 2009) covered BARC240 showing a MTA in our study, however the highly significant GWM1130 further distal seems not to be included in the described meta-QTL region. In the association study of Le Gouis et al. (2012) marker GWM 642 detected an association for HD in non-vernalized plants. This marker is in close vicinity to WMC732 detecting multiple MTAs in our study. The detailed comparison to the other associations described by Le Gouis et al. (2012) is difficult due to the lack of common markers.

ASSOCIATIONS DETECTED WITH SNPs AND EXPLOITATION OF SYNTENY

The SNP markers on the array are mostly new and therefore no literature data on MTAs involving these markers are available. While the SSR markers are mainly based on genomic sequences, the SNPs were mostly derived from genes and can therefore be used to establish the synteny to rice and other grasses, where full genome sequences are available (International Rice Genome Sequencing Project, 2005; The International Brachypodium Initiative, 2010).

Our results indicated, that a wheat gene on chromosome 5B, which is related to the *Hd6* gene family of rice, has a major impact on heading time in wheat. Several earliness *per se* QTL on chromosome 5B were described in the Cutler × Barrie spring wheat population (Kamran et al., 2013). The earliness *per se* QTL *QFlt.dms-5B.1* inducing earlier flowering could help to elongate the grain filling duration for higher grain yield (Kamran et al., 2013). The SSR marker GWM371 linked to *QFlt.dms-5B.1* is located in some distance from the location of WMC160 and BARC232 according to Ganal and Röder (2007), indicating that *QFlt.dms-5B.1* is different from the *Hd6* related SNP marker association of marker Kukri_c10016_369.

The synteny to rice can also be used to indirectly compare the mapping of our significant wheat markers to published literature data. An example is a cluster of three highly significant wheat SNP markers on chromosome 1AL which was not discovered by SSR markers (Supplemental file 8). On chromosome 1AL the fine mapping of the earliness *per se* gene *Eps-A^{m1}* was reported (Valárik et al., 2006; Lewis et al., 2008). After establishing the synteny of rice of our significant loci (LOC_Os05g45930 for wsnp_BE444305A_Td_2_1 and for wsnp_RFL_Contig3542_3718200; LOC_Os05g45900 for RAC875_c21411_162) it was possible to compare to the location of *Eps-A^{m1}* established by Valárik et al. (2006) between markers *Adk1* (LOC_Os05g51560) and *Pp2c* (LOC_Os05g51510). Based on the rice syntenic loci our locus appears to be different from gene *Eps-A^{m1}*. A similar example exists for chromosome 3A for which the presence of earliness *per se* locus *Eps-3A^m* was reported (Gawronski and Schnurbusch, 2012). The syntenic locus of the significant wheat marker wsnp_ex_c8884_14841846 (LOC_Os01g64490) in our map did not match the location of the markers PAV_295_296 (LOC_Os01g740300), CAPS_zt4_zt5 (LOC_Os01g741100) and CAPS_281_282 (LOC_Os01g741400) reported to be linked to *Eps-3A^m* (Gawronski and Schnurbusch, 2012).

In barley, the circadian clock gene *early maturity 8* (*eam8*) was identified as an ortholog of the *Arabidopsis thaliana* circadian clock regulator *early flowering* (*elf3*) (Faure et al., 2012; Zakhrebekova et al., 2012). The reported syntenic region in rice, ranging from LOC_Os5g51560 to LOC_Os05g51650 did not include any significant markers in our list, for which synteny to rice could be established. For the barley *early maturity 10* (*eam10*) gene the *Hvlux1* gene, an ortholog to the *Arabidopsis* circadian gene *LUX ARRHYTHMO*, was proposed as a candidate (Campoli et al., 2013) with orthologs in rice (LOC_Os01g74020) and *Brachypodium* (Bradi2g62070). For none of these orthologous sites candidates were found in our wheat association panel.

CONCLUSIONS

Genome wide associations for HD in European winter wheat were established for SSR as well as SNP markers. It could be shown that a number of known regulatory photoperiodism genes, such as *Ppd-A1*, *Ppd-B1*, *Ppd-D1* and the vernalization gene *Vrn-A2* have a major impact in shaping the genetic architecture of HD. The distribution of MTAs in multiple environments led however to the conclusion, that many more major genetic loci are involved. We were able to demonstrate the significance of an *Hd6* related gene marker on chromosome 5BL, which indicated the importance of the *Hd6* related gene for HD in wheat.

The dependence of the number of favorable alleles of SSR markers in a variety in relation to the HD-BLUEs indicated the strong genetic component in HD. By considering only five markers, it was possible to obtain a regression with $R^2 = 0.372$. Therefore, the described list of markers (Table 2) could be used for the stacking of alleles by marker assisted breeding and for the development of well adapted varieties for specific environments and geographical locations.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00217/abstract>

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Inflorescence development in tomato: gene functions within a zigzag model

Claire Périlleux^{*†}, Guillaume Lobet[†] and Pierre Tocquin

Laboratory of Plant Physiology, PhytoSYSTEMS, Department of Life Sciences, University of Liège, Liège, Belgium

Edited by:

George Coupland, Max Planck Society, Germany

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Mariana Benítez, Universidad Nacinal Autónoma de México, Mexico

***Correspondence:**

Claire Périlleux, Laboratory of Plant Physiology, PhytoSYSTEMS, Department of Life Sciences, University of Liège, Boulevard du Rectorat 27, 4000 Liège, Belgium
e-mail: cperilleux@ulg.ac.be

[†]These authors have contributed equally to this work.

Tomato is a major crop plant and several mutants have been selected for breeding but also for isolating important genes that regulate flowering and sympodial growth. Besides, current research in developmental biology aims at revealing mechanisms that account for diversity in inflorescence architectures. We therefore found timely to review the current knowledge of the genetic control of flowering in tomato and to integrate the emerging network into modeling attempts. We developed a kinetic model of the tomato inflorescence development where each meristem was represented by its "vegetativeness" (V), reflecting its maturation state toward flower initiation. The model followed simple rules: maturation proceeded continuously at the same rate in every meristem (dV); floral transition and floral commitment occurred at threshold levels of V ; lateral meristems were initiated with a gain of V (ΔV) relative to the V level of the meristem from which they derived. This last rule created a link between successive meristems and gave to the model its zigzag shape. We next exploited the model to explore the diversity of morphotypes that could be generated by varying dV and ΔV and matched them with existing mutant phenotypes. This approach, focused on the development of the primary inflorescence, allowed us to elaborate on the genetic regulation of the kinetic model of inflorescence development. We propose that the lateral inflorescence meristem fate in tomato is more similar to an immature flower meristem than to the inflorescence meristem of *Arabidopsis*. In the last part of our paper, we extend our thought to spatial regulators that should be integrated in a next step for unraveling the relationships between the different meristems that participate to sympodial growth.

Keywords: *Solanum lycopersicum*, flowering, morphogenesis, sympodial growth, biological model, AGL24

INTRODUCTION

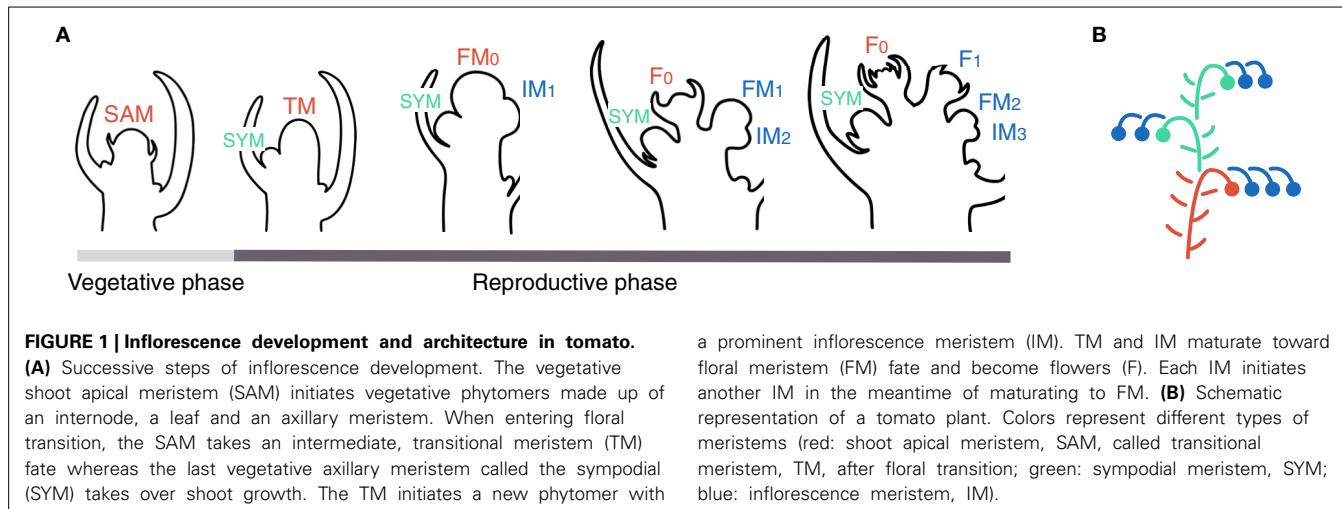
Essentially all cultivated forms of the tomato belong to the species *Solanum lycopersicum*. A large variability exists between cultivars in the form of the plant and leaves, in the number of flowers, or in the shape and color of the fruits. The number and size of the inflorescences are key traits determining potential productivity of the plant and hence understanding the mechanisms that regulate inflorescence architecture is critical.

Historically, inflorescence development in tomato has been studied by a classical forward genetic approach focused on a limited number of mutants, some of which having been found accidentally in the field and selected for traits that increased yield or facilitated fruit harvest (Emmanuel and Levy, 2002). The different genetic backgrounds in which the mutations had appeared as single alleles and the high plasticity of the flowering process in tomato have impeded research. More recently, tools have been developed for large scale studies in reference genotypes, including generation of mutant populations (Minoia et al., 2010), phenotyping platforms (Ecarnot et al., 2013; Polder et al., 2013) and genome sequencing (Tomato Genome Consortium, 2012), and these progress will undoubtedly accelerate functional genomic research. However, carrying out comparative analyses of inflorescence development among different species, either on a gene-by-gene basis or in a modeling attempt, can still be highly

constructive today. At the genetic level, the current knowledge obtained in tomato contains sufficient functional and epistasis information that allow to draw a regulatory network of flowering, inspired by what is known in *Arabidopsis*. The first aim of this paper is to give an overview of the knowledge on the subject, based on literature survey. Besides, conceptual frameworks have been recently explored to understand the diversity of inflorescence structures in nature and identify the underlying rules. The second objective of our paper is to exploit these concepts toward modeling the tomato inflorescence, and to test how the model can produce the known mutant phenotypes. This approach allowed us to reciprocally assess the significance of the model and of the genetic network behind.

GENETIC CONTROL OF INFLORESCENCE DEVELOPMENT

Floral transition of the shoot apical meristem (SAM) is a switch from the production of vegetative phytomers to the initiation of reproductive phytomers. Each vegetative phytomer is made up of an internode, a leaf and an axillary meristem. After the initiation of 6–12 vegetative phytomers forming the initial segment of the plant, the SAM of tomato enters floral transition (Figure 1A) (Sawhney and Greyson, 1972). The last vegetative phytomer is called the sympodial (SYM) because it takes pole position and continues shoot growth after transformation of



the SAM into the first inflorescence (Figure 1B). The transitional SAM (transitional meristem, TM), while maturing toward a flower meristem (FM) fate, initiates a new phytomer where, in contrast to vegetative phytomers, the meristematic zone (called inflorescence meristem, IM) is much prominent whereas the subtending leaflike phyllome—or bract—is completely repressed. The IM will reproduce the TM programme, maturing toward the FM fate and initiating a second IM in the meantime. This reiterative process allows endless formation of flowers, providing that maturation and initiation of successive meristems keep in pace.

FLORAL TRANSITION AND FLOWER MERISTEM (FM) FATE

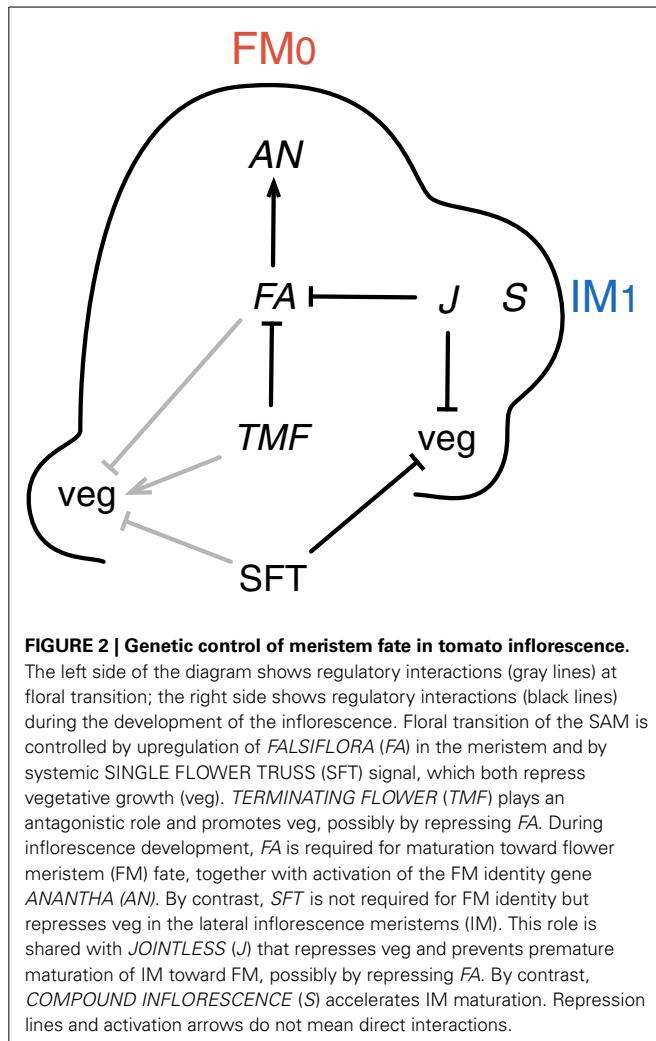
There are several excellent reviews on the genetic control of flowering in tomato (Quinet and Kinet, 2007; Samach and Lotan, 2007; Lozano et al., 2009); our focus here will be on functional data updated from the recently published literature and on epistasis studies from which genetic interactions can be inferred. It is worth mentioning first that there is no clear distinction in the literature between regulation of “flowering time” and “inflorescence development” in tomato because mutants that are late- or early-flowering according to the number of vegetative phytomers formed in their initial segment also show abnormalities in their first inflorescence. This is a first indication that the termination of the initial segment by floral transition of the SAM and termination of the lateral branches initiated in the inflorescence obey to the same rules. It must keep in mind, however, that the mutants investigated so far were isolated from modern cultivars, and are the result of strong selection leading to the acquisition of rapid growth cycle and alleviation of environmental requirements for floral transition (Kinet and Peet, 1997) and hence more variability in flowering time might be found in the future by larger exploration of diversity.

Interestingly, late-flowering mutants of tomato show an increased propensity to return to vegetative functioning in the inflorescence indicating that common mechanisms are involved in repressing vegetative growth in the SAM and in the lateral meristems initiated afterwards in the inflorescence. This is the case of the *falsiflora* (*fa*) and *single flower truss* (*sft*) mutants that produce more vegetative phytomers before floral transition

of the SAM, and where leaf production resumes in the inflorescence (Allen and Sussex, 1996; Molinero-Rosales et al., 1999, 2004). On the opposite, overexpression of *FA* or *SFT* accelerates flowering of the initial segment, which produces 3–5 leaves only, and can transform its multi-flowered inflorescence into a single flower (Lifschitz et al., 2006; MacAlister et al., 2012). It can be concluded therefore that *FA* and *SFT* are potent promoters of floral transition in tomato (Figure 2). The late-flowering phenotypes of *fa* and *sft* mutants are additive (Molinero-Rosales et al., 2004; Thouet et al., 2012), indicating that the genes act in parallel pathways.

Only recently, an early flowering mutant of tomato was studied in detail: *terminating flower* (*tmf*) shows the same reduction in vegetative phytomer number in the initial segment and solitary flower phenotypes than plants overexpressing *FA* or *SFT* (MacAlister et al., 2012). Interestingly, *fa* but not *sft* mutation is epistatic to *tmf*, indicating that *TMF* acts upstream of *FA* but independently of *SFT* (Figure 2). Consistently, *FA* is prematurely activated in the *tmf* mutant, although the TM still expresses molecular markers of vegetative meristem fate (MacAlister et al., 2012). The function of *TMF* would thus be to maintain a vegetative SAM.

The promotive role of *FA* and *SFT* genes on floral transition is fully consistent with the fact that they are the tomato orthologs of the *Arabidopsis* *LEAFY* (*LFY*) and *FLOWERING LOCUS T* (*FT*) genes, respectively (Molinero-Rosales et al., 1999; Lifschitz et al., 2006). *FA*, like *LFY* in *Arabidopsis*, is expressed in the leaf primordia before floral transition, and its expression increases in the meristem at TM stage (Molinero-Rosales et al., 1999; Park et al., 2012; Thouet et al., 2012). *SFT*, like *FT* in *Arabidopsis*, is expressed in the leaves and encodes a systemic florigenic signal (Lifschitz and Eshed, 2006; Lifschitz et al., 2006). The *SFT* signal is graft transmissible and induces early flowering in tomato and other day-neutral or photoperiodic species (Lifschitz et al., 2006). Floral transition of the SAM thus appears to be regulated in tomato, as in *Arabidopsis* (Blazquez et al., 1997; Corbesier et al., 2007) by two limiting factors at least: the expression level of *FA* in the SAM and the dosage of systemic *SFT* (Figure 2). Flowering time, as measured by the number of leaves in the initial segment, is



rather stable under various environmental conditions, the major effect being due to the amount of light (Kinet and Peet, 1997) and hence endogenous clues should be responsible for upregulation of *FA* and *SFT*. An age-dependent increase in expression of *FA* in the SAM was reported (Park et al., 2012) as well as a higher activity of *SFT* in expanded mature leaves than in younger leaves (Shalit et al., 2009). By contrast, *TMF* is expressed predominantly at the periphery of vegetative meristems, extending into initiating vasculature, and decreases slightly at the TM stage (MacAlister et al., 2012). How *TMF*, which encodes a member of the ALOG (*Arabidopsis LIGHT-SENSITIVE HYPOCOTYL 1*, *Oryza G1*) family of proteins, might regulate *FA* in the SAM is not known.

FA shares with *LFY* the key function of being a FM identity gene. Indeed, the inflorescences of the *fa* mutant are very leafy and made of a combination of vegetative axes with elongated internodes and clumps of indeterminate meristems that are blocked in their development (Allen and Sussex, 1996; Molinero-Rosales et al., 1999). This phenotype is even stronger than that of *lfy* in *Arabidopsis* which shows replacement of flowers by leafy branches but, unlike *fa*, may eventually produce some abnormal flowers (Schultz and Haughn, 1993). A second FM identity gene

identified in tomato is *ANANTHA* (*AN*) whose mutation leads to the formation of cauliflower-like masses of meristems where leaves, although still present, are highly suppressed (Allen and Sussex, 1996). The *AN* gene encodes an F-box protein orthologous to *UNUSUAL FORMATION OF ORGANS* (*UFO*) which, in *Arabidopsis*, acts as a cofactor of *LFY* for upregulation of homeotic genes in petal and stamen whorls of the flower (Lee et al., 1997). Thus *AN* and *FA* form a conserved floral specification complex that hallmarks FM fate (Figure 2) (Moyroud et al., 2010). The *fa* mutation is completely epistatic to *an* (Allen and Sussex, 1996) and the expression of *AN* is undetectable in *fa* mutants, indicating that *FA* functions upstream of *AN* (Lippman et al., 2008). Consistently, the expression of *FA* after floral transition of the SAM is higher in maturing FM than in IMs (Thouet et al., 2012) while activation of *AN* occurs later in the FM (Lippman et al., 2008).

INFLORESCENCE MERISTEM (IM) FATE

Contrary to *FA* and *AN*, the loss of *SFT* function does not prevent formation of flowers but hampers continuation of their initiation in the inflorescence: in the *sft* mutant, the reappearance of vegetative axes follows the formation of one or a few normal flowers (Molinero-Rosales et al., 2004; Quinet et al., 2006b), indicating that the *SFT* gene is not required for floral identity but for the maintenance of the floral switch. Consistently, overexpression of *SFT* in different non-allelic flowering mutants caused early termination of the primary segment after 3–4 leaves but did not rescue morphogenetic defects (Shalit et al., 2009). On an *sft* receptor, 35S:*SFT* donor complemented the inflorescence phenotype as long as the graft was maintained, indicating that permanent emission of *SFT* signal is required for proper formation of the inflorescence (Lifschitz et al., 2006).

Mutation of the *JOINTLESS* (*J*) gene, like loss of *SFT* function, allows the resumption of vegetative growth in the inflorescence after a few flowers are formed (Szymkowiak and Irish, 1999; Mao et al., 2000; Quinet et al., 2006b), indicating that both genes are required to confer IM identity on meristems that arise after floral transition of the SAM (Figure 2). This is supported by the fact that a very robust one-flower phenotype is obtained by the combination of *sft* with *j* mutation (Thouet et al., 2012). The *J* gene encodes a MADS-box protein of the SHORT VEGETATIVE PHASE (SVP)/AGAMOUS LIKE 24 (AGL24) clade (Mao et al., 2000). *In situ* hybridization and transcriptomic analyses showed that *J* is expressed in the SAM at floral transition (Park et al., 2012) and is later more active in the IMs than in FM (Thouet et al., 2012). Because MADS-box proteins act in complexes, it was previously hypothesized that *J* interacts with a MADS-box protein induced by systemic *SFT* protein, and that this complex represses vegetative growth in the newly initiated meristems of the inflorescence (Thouet et al., 2012). Thus, identification of the MADS-box partners of *J* is important for further functional analyses. Leseberg et al. (2008) found interaction in yeast between *J* and several other MADS-box proteins of the same sub-families as SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1), APETALA1/FRUITFULL (AP1/FUL), and SEPALLATAS (SEPs) in *Arabidopsis*. Functional evidence was obtained for the interaction of *J* with the MACROCALYX (MC) protein of the

AP1/FUL clade, since the expression of an antisense *MC* gene phenocopies the *j* mutation, including the leafy inflorescence phenotype (Vrebalov et al., 2002; Nakano et al., 2012). Interestingly, repression of *MC* also causes conversion of sepals to leaf-like structures, a morphological trait that is also observed in one-flowered *sft* and *j sft* mutants where one of the leafy-sepals is much larger than the others (Molinero-Rosales et al., 2004; Quinet et al., 2006b; Thouet et al., 2012).

FROM IM TO FM

Gene clusters that are dynamically expressed during meristem maturation have been identified and define a “maturation clock” that can be used to capture the relative maturation state of the meristems in the inflorescence and evaluate their “maturation rate” (Park et al., 2012). This tool offered an explanation to the very early-flowering and single-flower phenotype of the *tmf* mutant, due to premature activation of the FM molecular network, including *FA* and *AN* in the SAM (MacAlister et al., 2012).

In contrast to *tmf*, the *compound inflorescence (s)* mutant forms highly branched inflorescences containing tens or hundreds of flowers (Quinet et al., 2006b). At seemingly identical stages, the TM and IM are delayed in maturation in the *s* mutant as compared with WT inflorescences and consequently the time window during which they can initiate a higher order branch is extended (Lippman et al., 2008; Park et al., 2012). This finding indicates that, in WT inflorescence, the *S* gene promotes TM and IM maturation toward the FM fate. The expression pattern of *S* is consistent with this hypothesis, *S* being transiently expressed in the TM and the IM and followed by activation of the FM identity gene *AN* (Figure 2) (Lippman et al., 2008; Park et al., 2012), but the mechanism is not understood. The *S* gene encodes a homeobox domain protein of the WUSCHEL (WUS) family, *WOX9*, involved in *Arabidopsis* in stem cell maintenance (Wu et al., 2005; Lippman et al., 2008) and hence one possible scenario is that *S* modulates the rate of maturation via the regulation of meristem size. *FA* expression shows little change in expression in the *s* mutant whereas activation of *AN* is much delayed, suggesting that *S* acts downstream of *FA* and upstream of *AN* (Park et al., 2012).

Unexpectedly, the *j* mutation was found to completely override the highly branched phenotype of *s*, indicating that *J* acts antagonistically to *S* and represses early maturation of IM (Thouet et al., 2012). This hypothesis is supported by the fact that in *Arabidopsis*, a MADS-box protein complex that includes the homologs of *J*, *AGL24* and *SVP*, represses premature activation of FM identity genes (Liu et al., 2009). In this way, *J* would be essential in the IM to prevent both return to leaf production and premature differentiation (Figure 2). Such a role is consistent with the expression pattern of *J* which is more highly expressed in the IM than in the FM, complementarily to the pattern of the FM identity gene *FA* (Thouet et al., 2012).

VEGETATIVENESS GAIN AND LOSS GENERATE A ZIGZAG MODEL

MODEL DESCRIPTION

Modeling helps to reveal rules underlying repetitive processes such as the construction of the plant body. Two recently proposed

models help to comprehend the development of the tomato inflorescence, based on the fact that the arrangement of flowers reflects the spatiotemporal balance between maintenance of meristem indeterminacy and acquisition of floral meristem identity. In their model, Prusinkiewicz et al. (2007) postulate that an inflorescence is built from different meristems that lose their initial “vegetativeness” to become flowers at different times and rates. In the Solanaceae model proposed by Lippman et al. (2008), the branching of the inflorescence depends on the maturation rate of the IM toward FM fate. Both models thus describe meristem development as a continuum—seen alternatively as vegetativeness loss or maturity gain—from initiation to floral commitment.

Our aim here was to construct a simple kinetic model of inflorescence development in tomato. Therefore, we used the term “vegetativeness” after Prusinkiewicz et al. (2007) since it seemed appropriate to describe the frequent resumption of leaf production in the inflorescence, as observed in the *fa*, *sft*, and *j* mutants (see above). In our model, vegetativeness is a complex variable representing the meristem state, with high levels of vegetativeness corresponding to shoot meristem identity and low levels to flower meristem identity. Transcriptomic analyses of individual meristems in tomato allowed to capture gene regulatory networks of different maturation stages and showed that a “molecular clock” drives meristem maturation as a continuous process (Park et al., 2012). We then assumed that vegetativeness could be represented as a continuous function.

In Figure 3, the ontogeny of the tomato inflorescence (see Figure 1) is schematized with each line showing the vegetativeness decline of meristems initiated sequentially at one-plastochron intervals. Flowering of cultivated tomato occurs autonomously and hence we assumed that the vegetativeness of the SAM decreases continuously during the vegetative phase of the plant until it passes—at the TM stage—below a permissive threshold for flowering. The last leaf bears an axillary meristem, which is the SYM. Maturation of the TM toward FM fate defines a second phase of vegetativeness decrease during which it initiates a lateral IM that will go through the same program: mature toward FM fate and initiate a lateral meristem. This means that maturation of TM and of successive IMs to FM is slow enough to permit initiation of one IM before they lose indeterminacy. Since IMs are produced iteratively, we postulated that each lateral meristem is initiated at a lower maturity level (i.e., has a higher vegetativeness) than the one from which it was produced. This was expressed by adding ΔV to the vegetativeness level of the previous-order meristem at each new meristem initiation event, creating a link between successive meristems. This gave a “zigzag” shape to the meristem dynamics building-up the inflorescence and allowed to simulate the inflorescence of WT plants.

Based on the zigzag dynamics shown in Figure 3, we constructed a mathematical model (Figure S1) in which every meristem was subjected to the same rules:

- Meristem vegetativeness decreases with time following the equation:

$$V_i = V_{i-1} - \frac{dV}{V_{i-1}},$$

where V_i is the current vegetativeness level of the meristem at plastochrone i , V_{i-1} is its vegetativeness one plastochrone before (or at initiation (V_0) see Figure S1) and dV is the rate of vegetativeness decrease. We found that a non-linear decrease of the vegetativeness (simply obtained with dV/V_{i-1}) was necessary to stop the production of flowers in WT inflorescences and to account for the vegetative reversions observed in some mutant inflorescences (see below). dV can take different values before and after the floral transition. Changes before the transition affects flowering time while changes after the transition have an effect on the architecture of the inflorescence.

- Leaf production (vegetative functioning) is repressed below the floral transition threshold.
- At fixed time points (plastochrons), meristems are allowed to produce a new phytomer, which includes an axillary meristem, unless their vegetativeness is below the floral commitment threshold.
- At initiation, a lateral meristem has a higher vegetativeness level (V'_0), than the meristem that produced the phytomer:

$$V'_0 = V_p + \Delta V,$$

where V_p is the vegetativeness (V_i) of the previous-order meristem and ΔV is the gain of vegetativeness at lateral meristem initiation.

Thus, this simple model is based on two vegetativeness threshold values (floral transition and floral commitment) and two variables: the rate of maturation or vegetativeness decrease

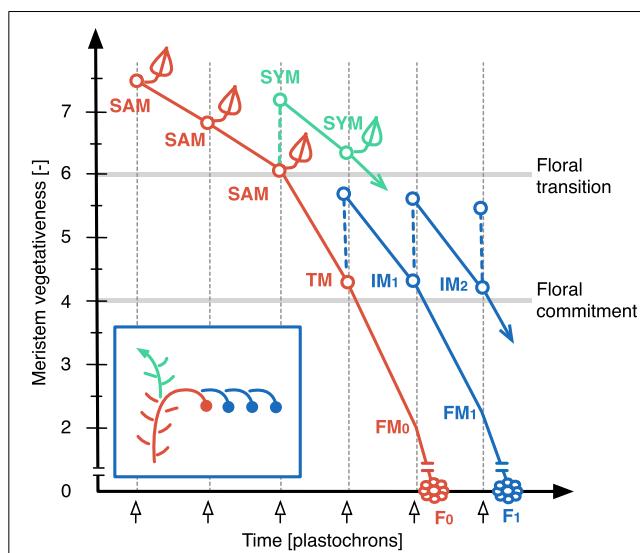


FIGURE 3 | The zigzag model. Plots show vegetativeness decline of successive meristems initiated at one-plastochron intervals. Colors represent different types of meristems (red: shoot apical meristem, SAM, called transitional meristem, TM, after floral transition; green: sympodial meristem, SYM; blue: inflorescence meristem, IM). TM and IM mature toward floral meristem (FM) fate and become flowers (F). See Figure 1 for spatial visualization. Note that the vegetativeness level of the SYM should be higher than shown, since it usually produces more than one leaf.

(dV) and the vegetativeness gain of newly initiated meristems (ΔV). In the framework of this study, dV was changed after floral transition only, in order to focus on inflorescence development.

Each output of the model, for any given dV and ΔV value, is an inflorescence, that was characterized by three metrics describing its topology: the number of flowers before the first occurrence of vegetative reversion if any (Figure 4A), the branching level, i.e., the number of phytomers initiated by the TM before being committed to make a flower (Figure 4B) and the number of vegetative axes (Figure 4C). These metrics were arbitrarily discretized, i.e., threshold values were fixed (Table 1) in order to divide the range of morphological variation created by the model (the “morphospace”) in a reduced number of inflorescence types (“morphotypes”) (Figures 4D–G). In order to test whether the model was able to generate known mutant phenotypes, it was run for a range of ΔV (from 0 to 3) and dV (from 0 to 20) values for a total of 1200 simulations. These ranges were chosen to capture the largest variation of simulation outputs (Figure S2).

THE ZIGZAG MODEL GENERATES A MORPHOSPACE WHERE KNOWN MUTANTS FIND THEIR PLACE

Single mutant morphotypes

In order to test the plausibility of our model, morphotypes were assigned to known mutant phenotypes (Figure S3). Highly branched inflorescences, such as *s*, *fa* and *an* are found on the left side of the morphospace: they are generated by the model when the vegetativeness of the meristems forming the inflorescence decreases slowly (Figure 5). The slow maturation rate of the IMs allows them to initiate other IMs before being committed to make a flower (Lippman et al., 2008). This hold true for the initial TM as well, so that branched inflorescences always show a proximal fork.

In contrast to *s* mutant, *an* and *fa* never form flowers (Allen and Sussex, 1996; Molinero-Rosales et al., 1999; Quinet et al., 2006b). In our model, the absence of flowers and high branching of the *an* and *fa* inflorescences are explained by an almost null maturation rate of IMs (dV value close to zero; Figure 5), reflecting the fact that the meristems never acquire the FM identity. In addition, the return to leaf production in the inflorescence of *fa*

Table 1 | Combination of metrics describing the first inflorescence topology and used to distinguish eight different morphotypes generated by the model.

| Morphotype | Number of flowers | Branching level | Number of vegetative axes |
|------------|-------------------|-----------------|---------------------------|
| 1 | ≥ 2 | 2–5 | =0 |
| 2 | =0 | ≥ 6 | =0 |
| 3 | =0 | ≥ 6 | ≥ 1 |
| 4 | ≥ 2 | 2–5 | ≥ 1 |
| 5 | =1 | =1 | ≥ 1 |
| 6 | ≥ 2 | =1 | ≥ 1 |
| 7 | =1 | =0 | =0 |
| 8 | ≥ 2 | =1 | =0 |

Morphotypes are illustrated in Figure 4.

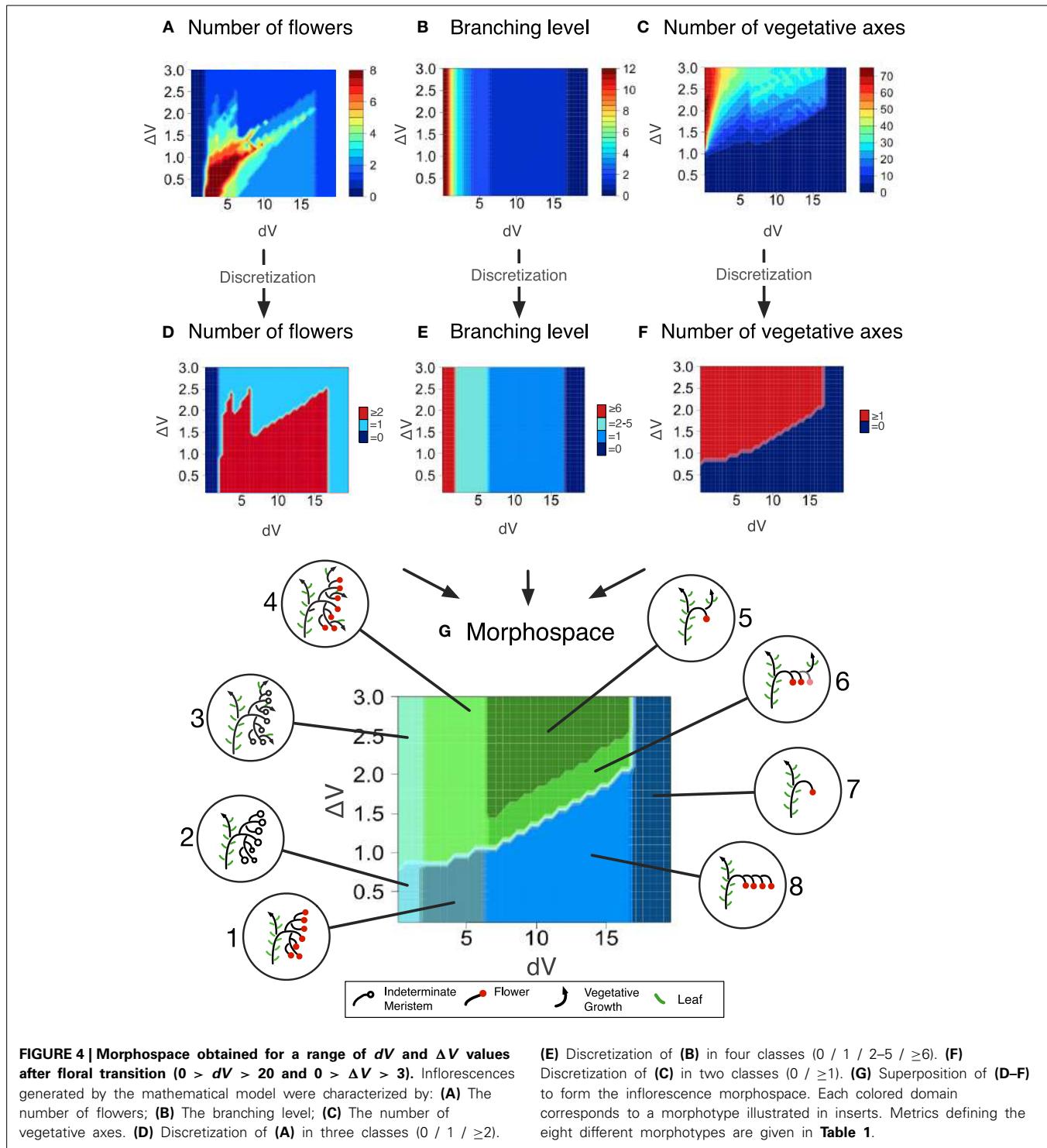


FIGURE 4 | Morphospace obtained for a range of dV and ΔV values after floral transition ($0 > dV > 20$ and $0 > \Delta V > 3$). Inflorescences generated by the mathematical model were characterized by: (A) The number of flowers; (B) The branching level; (C) The number of vegetative axes. (D) Discretization of (A) in three classes (0 / 1 / ≥ 2). (E) Discretization of (B) in four classes (0 / 1 / 2–5 / ≥ 6). (F) Discretization of (C) in two classes (0 / ≥ 1). (G) Superposition of (D–F) to form the inflorescence morphospace. Each colored domain corresponds to a morphotype illustrated in inserts. Metrics defining the eight different morphotypes are given in Table 1.

mutant would result from an increase in ΔV which ultimately causes the vegetativeness level of newly formed lateral meristems to exceed the threshold value for vegetative vs. reproductive programs (floral transition threshold, Figure 3).

On the opposite side of the morphospace created by our model stand mutants with reduced branching and flower numbers, the more extreme one being *tmf* (Figure 5). In our model,

its single flower phenotype is obtained by an acceleration of TM maturation (dV values $>$ WT), possibly combined with a decrease in ΔV . While this increase in dV in *tmf* reflects the precocious floral commitment of this mutant, an additional ΔV contribution is supported by the vegetative reversions and higher branching observed in *TMF* overexpressors (MacAlister et al., 2012). It is important to emphasize that this interpretation of *tmf*

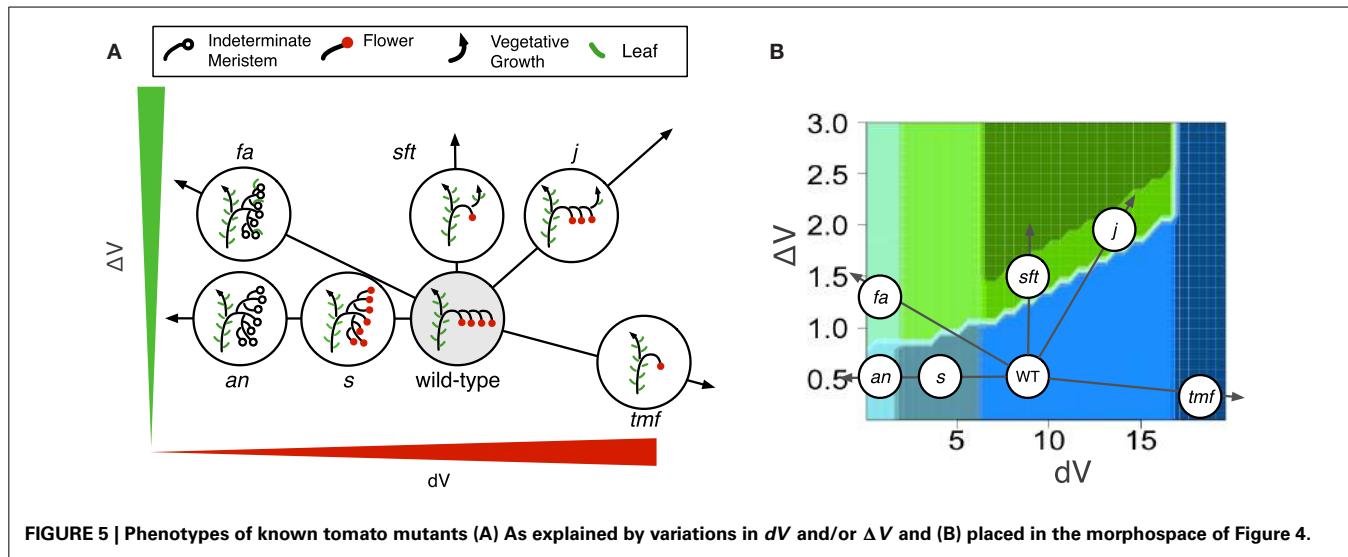


FIGURE 5 | Phenotypes of known tomato mutants (A) As explained by variations in dV and/or ΔV and (B) placed in the morphospace of Figure 4.

is valid for the SAM of the initial segment only since inflorescences formed later are more or less normal (MacAlister et al., 2012).

Other single-flower mutants of tomato are late flowering and their single-flower phenotype is due to a return to vegetative functioning in the inflorescence, as known for the *sft* mutant (Molinero-Rosales et al., 2004). In our model, the *sft* morphotype is generated by an increase in ΔV , which results in the initiation of one or a few flowers before the vegetativeness level of the newly initiated lateral meristem exceeds the threshold value for vegetative functioning. The same phenotype is observed in the inflorescence of the *j* mutant (Szymkowiak and Irish, 1999) but, as explained earlier and unlike *sft*, *j* mutation was shown to accelerate IM maturation (Thouet et al., 2012). That is consistent with our model showing that the morphotype corresponding to *sft* and *j* can be generated by an increase in ΔV combined or not with an increase in dV .

Additivity of dV and ΔV contributions in double mutants

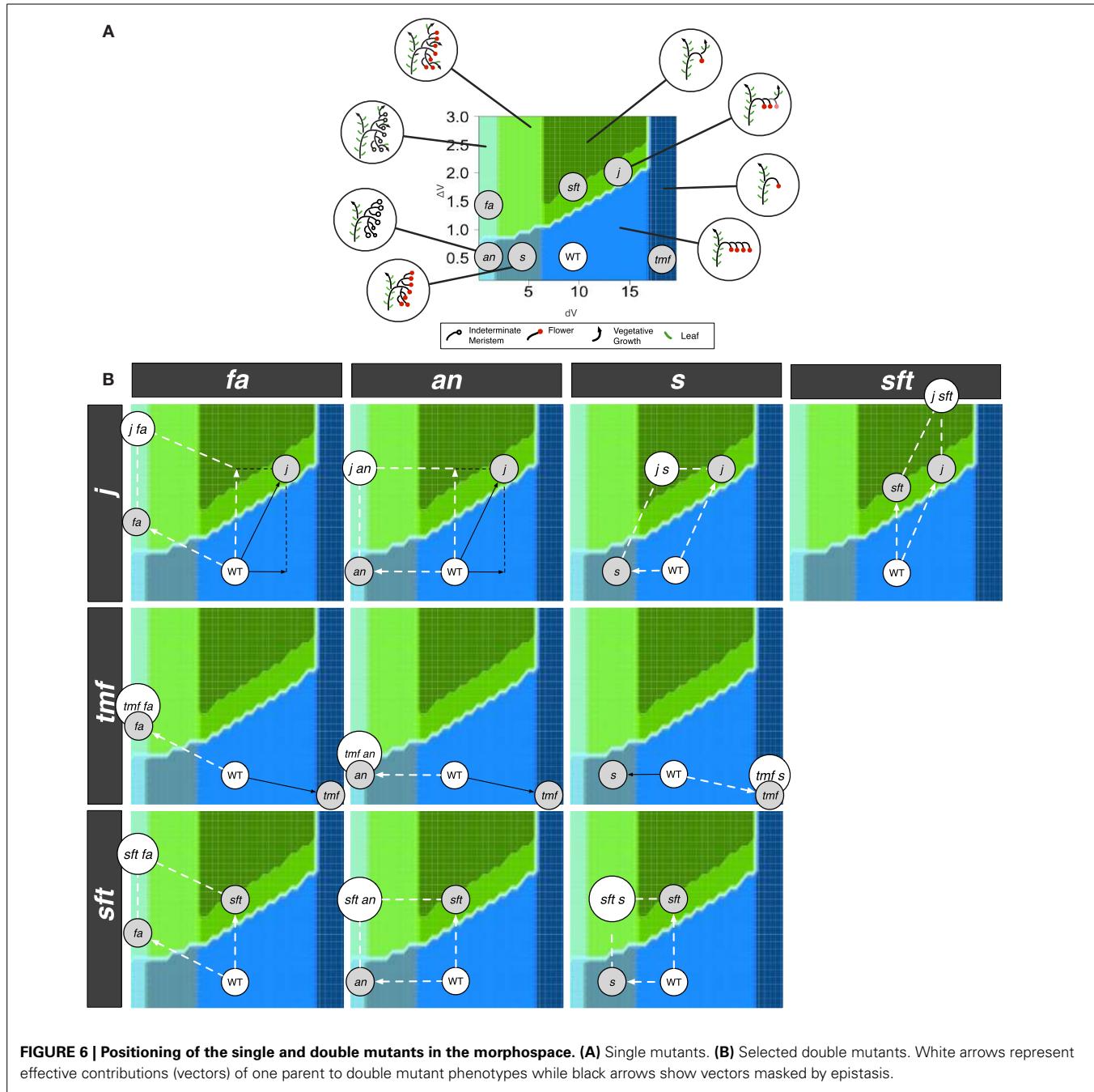
Double mutant analyses provide additional data to test the consistency of the model and to examine the relative contributions of the two variables dV and ΔV to deviation from WT inflorescence. For each single mutant, these deviations, or translations, were materialized in the morphospace by vectors (Figure 6A). We therefore evaluated whether double mutant phenotypes could be explained by summing dV and/or ΔV variations attributed to the single mutations, i.e., if their position in the morphospace (as deduced from their phenotype, Figure S4) could be predicted by the vector resulting from the addition of single mutant vectors. Such an analysis clearly depends on the position attributed to each single mutant in the area of its own morphotype but reciprocally, the phenotype of the double mutants actually provides experimental data to refine the mapping of their parents. Further testing could be performed by combining allelic series, which are not available yet.

We found that, in most cases, our assumption was correct (Figure 6B) since many double mutants fell in the morphotype

area pointed by the resultant vector. In these cases, we qualified the interaction between the two genes as “additive.” The most representative example is given by the *s sft* double mutant, combining the high branching of the *s* parent (lower dV) and the propensity to return to leaf production of *sft* (higher ΔV). This cross produces a morphotype with numerous flowers and vegetative axes that is not found in single mutants (Lippman et al., 2008; Thouet et al., 2012), but was correctly predicted by our model. Additivity was also reported between the flowerless *an* mutant (null dV) and *sft*, generating branched flowerless inflorescences with vegetative axes (Lippman et al., 2008) like the *an j* double mutant (Szymkowiak and Irish, 2006). This phenotype belongs to the same morphotype as the *fa* single mutant, supporting that our interpretation was correct. We indeed assumed that the leafy phenotype of *fa* inflorescences was due to the fact that IM are initiated at a higher vegetativeness level, which was translated in our model by a higher ΔV . The *fa an* double mutant has the *fa* phenotype, suggesting that *FA* acts upstream of *AN* (Allen and Sussex, 1996), *fa* imposing its ΔV contribution over *an* (not illustrated).

Unexpectedly, we also found that the *j s* double mutant phenotype matched the morphotype pointed by the vector resulting of the addition of the two single mutant contributions. Indeed, on the basis of phenotypic analyses, *j* mutation was described as epistatic to the *s* mutation since the inflorescences of the double mutant are indistinguishable from that of the *j* single mutant (Thouet et al., 2012). However, our model shows that this epistatic phenotype could be alternatively explained if we consider that the high dV of *j* is able to counterbalance the low dV of *s*.

Mutations that alter ΔV were also found to be additive, suggesting that “vegetativeness” is a quantitative feature. On the right side of the morphospace, two mutations stimulate a return to vegetative functioning in the inflorescence: *sft* and *j*. Their effect is additive since the double *j sft* mutant returns to vegetative functioning after initiation of a single flower while the single mutants may produce more flowers (Thouet et al., 2012). Moreover, both *sft* and *j* stimulate the development of leaves in *fa*



and *an* inflorescences (Szymkowiak and Irish, 2006; Thouet et al., 2012). In *fa* background, the increased ΔV due to *sft* or *j* adds on the increment due to *fa* mutation and hence less branches are formed in the double mutants before the vegetativeness of the new meristems exceed the threshold for leaf production (Thouet et al., 2012).

Masking effect of extreme dV values

By contrast, mutations that changed dV to extreme values, i.e., laying at the extreme left and right sides of the morphospace, did not show additive interactions with dV contributions of other

mutants: minimum (*fa*, *an*) and maximum (*tmf*) dV values were not counterbalanced by intermediate dV values of other mutants. This masking effect, hereafter qualified as epistatic with respect to the phenotypic traits due to dV variations (branching and flower number), can be deduced by the non-coincidence in the morphospace between the double mutant phenotype and the resultant vector obtained from the single mutants.

Epistasis was observed for *fa*, in *fa s* (Thouet et al., 2012), *fa j* (Thouet et al., 2012), and *fa tmf* (MacAlister et al., 2012) double mutants showing highly branched, flowerless inflorescences like *fa*. The *an* mutation was also reported to be epistatic to *s*

(Lippman et al., 2008), to *j* (Szymkowiak and Irish, 2006) and to *tmf* (MacAlister et al., 2012) for the same traits. Epistasis of *an* and *fa* finds its biological significance in the fact that *FA* and *AN* genes are indispensable FM identity genes and hence mutations completely block maturation to FM fate.

On the right side of the morphospace, the high *dV* *tmf* mutant is epistatic to *s* with respect to first inflorescence architecture (MacAlister et al., 2012). Epistasis of *tmf* over *s* mutation is due to the early activation of *FA* and *AN* (MacAlister et al., 2012), forcing maturation and preventing any branching of the inflorescence. The double *j tmf* mutant has not been described so far but can be predicted to have *tmf* phenotype as well.

INTEGRATING GENES WITHIN THE ZIGZAG MODEL

In *Arabidopsis*, modeling of inflorescence architecture focused on two genes considered as master regulators: *LFY*, which reduces vegetativeness in meristems and *TERMINAL FLOWER 1* (*TFL1*) which increases vegetativeness (Prusinkiewicz et al., 2007). The “transient model” proposed by Prusinkiewicz et al. postulated that lateral meristems are initiated at a transient state of vegetativeness and thereafter become a flower or revert to produce a branch. It yielded different types of inflorescence according to the relative time length different meristems take to achieve flowering and it was therefore used to address the adaptive and evolutionary value of inflorescence architectures. This model was deeply discussed because alternative rules could yield the same observed types of inflorescences and because the reduction of a complex developmental process to a pair of antagonistic genes seemed oversimplified (Alvarez-Buylla et al., 2007; Winther, 2012). However, although the transient model excluded some special cases of inflorescence architecture (Prenner et al., 2009), its unifying goal has been largely acknowledged (e.g., Castel et al., 2010). More recently, another modeling approach was used considering groups of genes or “hubs” that contribute to the function of key regulators of floral transition in *Arabidopsis* (Jaeger et al., 2013). This approach allowed to generate the racemose inflorescence, providing that the *TFL1* hub is upregulated in proportion of the floral inductive signal FT. Feedback loops then establish a stable state with *TFL1* repressing flowering and maintaining indeterminacy at the center of the SAM (called IM) whereas *LFY* is expressed and flowers are initiated on the flanks of the meristem.

Our tomato model uses the same terminology of vegetativeness as Prusinkiewicz et al. (2007) and describes meristem maturation as a continuous decrease of vegetativeness (*dV*) but the overall dynamics are different. While Prusinkiewicz et al. introduced a transient state in lateral meristem fate, a key feature of our model is that the maturation state of a lateral meristem depends on the meristem from which it derives. This link is expressed by the variable ΔV and might be established in the meristems by the diffusive properties of some regulators, as postulated by other authors (Alvarez-Buylla et al., 2007).

At the genetic level, our model does not incorporate *TFL1* because, as observed in other species forming cymose inflorescences, the tomato homolog of *TFL1* is not expressed in the SAM at floral transition or during inflorescence development (Thouet et al., 2008; see below). However, the mapping of known inflorescence mutants into the morphospace created by the model

(Figures 5, 6) allowed us to infer the contribution of the corresponding genes in the regulation of *dV* and/or ΔV , summarized in Table 2. The emerging view is undoubtedly simplified since the activity of each gene is likely to reflect system-level changes in planta but it incorporates, without a priori assumption, all genes affecting inflorescence architecture that have been characterized so far in tomato.

TMF is the only gene in Table 2 that increases vegetativeness and this was shown by MacAlister et al. (2012) to occur by repression of a subset of genes regulating floral commitment, including *FA*. The *TMF* gene could thus play in tomato the role of *TFL1* repressing *LFY* in *Arabidopsis* but this role would be limited to the SAM (MacAlister et al., 2012). Floral transition is marked by the upregulation of *FA* in meristem, but unlike *LFY* in *Arabidopsis*, the activation of *FA* is not limited to subdomains (Thouet et al., 2012) and terminates vegetative growth. Thus, repression of *FA* by *TMF* is temporal and not spatial, and the role of *TMF* is to maintain the vegetative fate and not “just” indeterminacy, unlike *TFL1* in *Arabidopsis*.

The ontogeny of the inflorescence in tomato proceeds by iterative initiation of new lateral meristems and the vegetativeness in these meristems is lowered by *FA*, *J*, and *SFT* (Table 2): if any of these genes is not functional, the tomato inflorescence contains leaves (Molinero-Rosales et al., 1999, 2004; Szymkowiak and Irish, 2006). Their lowered vegetativeness justifies that lateral meristems in the inflorescence are called IM, since they are intermediate between the two categories of meristems: the vegetative meristem which primarily produces leaves and stems and the FM which produces only floral organs (Prenner et al., 2009). Importantly, IM fate determines in tomato the time window during which meristems have the ability to branch and hence there is a close relationship between the duration of the IM fate and the number of branches in the inflorescence (Lippman et al., 2008) as shown by the large impact of varying *dV* on the morphology. We discussed above the limitation of our model for expressing epistasis relationships (extreme *dV* values) and hence we will point here the functions of two genes: *S*, which accelerates the transition from IM to FM fate and *J*, which has the opposite effect (Table 2). The function of genes such as *J* is critical to built

Table 2 | Contribution of genes to regulation of the two variables used for modeling the tomato inflorescence, as inferred from the position of the loss-of-function mutants in the morphospace.

| Gene | Vegetativeness gain of newly initiated meristems (ΔV) | Rate of vegetativeness decrease (<i>dV</i>) |
|------------|-----------------------------------------------------------------|-----------------------------------------------|
| <i>AN</i> | | + |
| <i>FA</i> | - | + |
| <i>S</i> | | + |
| <i>SFT</i> | - | |
| <i>J</i> | - | - |
| <i>TMF</i> | + | - |

(-) means that the gene activity has a negative impact on the parameter; (+) means that the gene activity has a positive impact on the parameter; lack of sign means that no correlation was found.

multiflowered inflorescences since premature achievement of FM fate would lead to termination of the inflorescence. This function of *J* in the IM was suggested to proceed through negative feedback from *J* to *FA* (Thouet et al., 2012) and must be transient as maturation proceeds toward FM fate. Thus a *J/FA* balance might have a pivotal role in the regulation of inflorescence development in tomato. How the flowering signal *SFT* regulates this balancing remains to be clarified but the facts that it promotes floral transition independently of *FA* (Molinero-Rosales et al., 2004) and interacts with *J* which refrains flower development (Thouet et al., 2012) establish clear parallels with the mechanism of interlocking loops disclosed by Jaeger et al. (2013).

Interestingly, the homologs of *J* in *Arabidopsis*, the MADS box genes *AGL24* and *SVP*, are involved in repressing differentiation at the early stages of FM formation and are therefore, together with FM identity genes, parts of regulatory loops timing meristem maturation (Liu et al., 2009; Wagner, 2009). It is worth emphasizing the importance of the “rate of maturation” (our *dV* variable) in this step. We then propose that the IM in tomato behaves as an immature FM in *Arabidopsis* whereas the IM in *Arabidopsis* (regulated by *TFL1*) is more similar to a vegetative meristem in tomato. This hypothesis could be tested by searching for conserved genes, interactions and dynamics within the gene regulatory networks of these meristems; we believe that this approach could provide novel insights into the understanding of inflorescence architectures.

PERSPECTIVES FROM A SIDE VIEW

Our reasoning has so far been focused on the temporal regulation of meristem fate by a developmental programme, but spatial regulation is intricately linked to the timing. At floral transition indeed, three meristems of a different fate are adjacent to each other: the vegetative SYM, the TM and the first IM (**Figure 1A**).

Importantly, the SYM does not enter floral transition at the same time as the SAM but will first initiate 3 vegetative phytomers before forming an inflorescence itself; the growth of the plant will then be continued by a second order sympodial segment and so on, indefinitely. This regular iteration of 3-leaf sympodial segments is regulated by the *SELF PRUNING* gene, the closest homolog of *TERMINAL FLOWER 1* in *Arabidopsis* (Pnueli et al., 1998), which is expressed in the SYM and other vegetative axillary meristems but not in the SAM at floral transition (Thouet et al., 2008). Consistently, *sp* mutation does not affect floral transition of the initial segment and does not have any impact on inflorescence architecture (Pnueli et al., 1998). In *sp* mutant, termination of successive sympodial segments occurs with less leaves and ends with two consecutive inflorescences, leading to a determinate growth. This trait facilitates mechanical harvesting of the fruits and hence the *sp* mutation was introduced for breeding “determinate” cultivars used in tomato industry. This phenotype however depends on *SFT* dosage: in *sft/+* heterozygote background, early termination of sympodial units due to *sp* mutation is overcome (Jiang et al., 2013) and in *sft/sft* homozygote background, sympodial growth is suppressed as in *sft* single mutant (Molinero-Rosales et al., 2004). The effect of *sft/+* heterozygosity in *sp* cultivars leads to a dramatic increase in inflorescence number per plant and thereby to yield (Krieger et al., 2010). By

contrast to the fact that the SYM is more sensitive than the SAM to *SP* inactivation, the opposite is observed in the differential response to *SFT*: flowering of plants overexpressing *SFT* is indeed much accelerated in the initial segment but the sympodial segments still initiate 2 or 3 leaves (Lifschitz et al., 2006; Shalit et al., 2009). It was therefore concluded that the *SP/SFT* balance regulates shoot architecture and sympodial development in tomato (Shalit et al., 2009). It is interesting to note that the *tmf* mutation, like *35S:SFT*, affects only the initial segment of the plant (MacAlister et al., 2012) suggesting that *TMF* is also checked by *SP* in the sympodial segments.

Although WT inflorescence architecture is not affected by *sp* mutation, overexpression of *SP* results in the replacement of flowers by leaves (Pnueli et al., 1998), indicating that ectopic expression of *SP* in the inflorescence promotes vegetative functioning. Consistently, vegetative meristems that arise in mutant inflorescences returning to leaf initiation after formation of normal flowers share regulatory features with the SYM as shown for *j* (Szymkowiak and Irish, 2006) and express *SP* as shown for *sft* (Thouet et al., 2008). Consequently, these vegetative axes can usurp the pole position to the canonical SYM forming a “pseudo-shoot” that continues the initial segment. This occurs when the inflorescence forms a single flower before the vegetative axis is initiated, as observed in strong *sft* mutant (Molinero-Rosales et al., 2004), otherwise the SYM remains dominant. Variability in the number of flowers in the inflorescence of *sft* may be due to allele strength, but also to the influence of the environment since the one-flower phenotype is more frequent in winter than in summer (Quinet et al., 2006b; Park et al., 2012). Interestingly, the environmental conditions that reveal the plasticity of the *sft* phenotype, light quantity and quality, are also those that are known to influence the correlative influence and dominance relationships between lateral meristems.

Another mutant where pseudo-shoots originating from the inflorescence were described is *uniflora* (*uf*) (Lifschitz et al., 2006). In this late flowering mutant, however, no lateral meristem is formed after conversion of the SAM into a flower (Dielen et al., 1998) and hence the origin of the meristem that continues the primary shoot is not clear. The incapacity of *uf* mutant to initiate lateral meristems in the inflorescence explains that the solitary flower phenotype is epistatic to mutations that affect IM fate, such as *s* and *j* (Quinet et al., 2006a). However, *uf* also shows a strong light-dose dependent flowering: the mutant is much delayed when the light integral is low (Dielen et al., 2004). Interestingly, when *uf* plants are transferred from favorable to unfavorable conditions, the number of leaves below the first flower does not show a continuous but a step increase, as if a sympodial segment was recruited in the main axis. Consistent with this hypothesis, *sp* mutation partially compensates late-flowering of *uf* (Quinet et al., 2006a). We therefore hypothesize that *uf* mutation causes a general defect in lateral meristem initiation and/or development rather than affecting flowering *per se*. Other mutants indeed illustrate the basic link between plant branching and inflorescence development in tomato. For example the *blind* (*bl*) mutant fails to initiate axillary meristems, including the SYM, so that sympodial growth is completely suppressed, and shows dramatic reduction in inflorescence branching (Schmitz

et al., 2002). Most interestingly, the *bl* inflorescence consists of one to a few flowers that tend to be fused and fasciated suggesting incomplete separation of meristems. The *Bl* gene encodes a Myb transcription factor and is expressed in prospective and actual boundaries separating lateral meristems from the SAM (Schmitz et al., 2002; Busch et al., 2011). This pattern emphasizes the importance of proper separation of adjacent meristems for specification of different fates: in *bl* mutant, the FM fate obviously “invades” the lateral IM so that siamese flowers are formed.

At later stages, a separation remains between flowers and the rest of the inflorescence in tomato: the abscission zone. The jointless pedicel character gave its name to the *j* mutants but this was considered as a side effect of the mutation since expression of *J* was not detected in the flower pedicel (Szymkowiak and Irish, 2006). Only recently were contradictory patterns published, showing expression of *J* in the pedicel primordium at early stages of flower development (Liu et al., 2014). Amazingly, transcriptomic analyses showed that branching genes such as *Bl*, boundary genes such as *Goblet* (*Gob*) that is homologous to the *Arabidopsis CUP SHAPED COTYLEDON* genes (Berger et al., 2009) and meristematic genes of the *WUS* family contribute to the development of the abscission region, which supports the idea that the cells of the abscission zone are arrested meristematic cells (Nakano et al., 2013). Interestingly, members of this genetic network are also involved in regulating compound leaf development (Blein et al., 2008; Busch et al., 2011). These findings provide novel insight into the pleiotropic effects of flowering genes on abscission zone development and leaf morphology (Shalit et al., 2009) and suggest that in tomato, partition of adjacent meristems during inflorescence formation, disjunction of flowers at a later stage of development and leaflet formation are cell separation processes sharing common regulatory pathways.

CONCLUSION

We presented here a first attempt to link tomato flowering genes into a coherent network. Such network was supported by a mathematical model that was able to generate the phenotypes of a large range of single and double inflorescence mutants. The model is based on the maturation kinetics of the successive meristems that elaborate the inflorescence and create a zigzag dynamics. Spatially, the formation of the inflorescence requires territorialization of adjacent meristematic domains to allow separation of meristem identities. This seems to involve in tomato conserved mechanisms regulating cell separation processes such as axillary meristem initiation, abscission zone development and leaflet formation. A challenging question for the future will be to integrate the spatial dynamics into the temporal models of inflorescence development, and to identify the signaling molecules that orchestrate the morphogenetic plan.

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SUPPLEMENTARY MATERIAL

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Attainment of reproductive competence, phase transition, and quantification of juvenility in mutant genetic screens

Ianis G. Matsoukas^{1,2*}

¹ Engineering, Sports and Sciences Academic Group, The University of Bolton, Bolton, UK

² Institute for Renewable Energy and Environmental Technologies, The University of Bolton, Bolton, UK

*Correspondence: i.matsoukas@bolton.ac.uk

Edited by:

Christian Jung, Christian Albrechts University of Kiel, Germany

Reviewed by:

Matthias Fladung, Johann Heinrich von Thuenen Institute (vTl), Germany

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INTRODUCTION TO JUVENILITY

Plant development between seedling emergence and flowering is characterized by a series of successive qualitative phases: (1) a post embryonic photoperiod-insensitive phase, during which plants are insensitive to photoperiod; (2) a photoperiod-sensitive inductive phase, in which plants require a number of short day (SD) or long day (LD) inductive cycles, depending on their age for rapid flowering, and (3) a photoperiod-insensitive post-inductive phase, in which plant development is no longer influenced by photoperiod (Figure 1; Matsoukas et al., 2013).

The early phase of development during which the plants cannot be induced to flower and are effectively insensitive to environmental influences of photoperiod and/or vernalization has been called the juvenile phase (Thomas and Vince-Prue, 1997). This period differs from plant to plant from a period of a few days, for small herbaceous annual plant species, through to periods that may last longer than 20 years, as is evident for many tree species. From a physio-ecological perspective, by having a juvenile phase, plant species avoid the low seed yields that would occur if they were to flower precociously while still small and with limited photosynthetic capacity (Thomas and Vince-Prue, 1997).

THE IMPORTANCE OF THE JUVENILE PHASE STUDIES

Studies on the juvenile-to-adult phase transition have significant scientific and

economic implications. Juvenility has long attracted interest as an aspect of the fundamental topic of aging and also has practical implications, especially in the growth and development of those species in which it is striking and prolonged (Matsoukas et al., 2012). From a commercial perspective, understanding the factors that affect the timing and duration of the juvenile phase length is critical for scheduling in commercial horticulture and arable crops. In addition, the long juvenile phase length of some species is one of several features limiting efficient breeding programs. For example, the efficiency of trait selection and genetic improvement in breeding programs is inversely related to the period of the breeding cycle. Thus, the exploitation of genotypes with short juvenile phase is very important. On the other hand, in many countries fast-growing tree species are being increasingly used for pulp and bioenergy production. In such cases, it may be equally important to explore molecular methods to prevent flowering and prolong juvenility (Matsoukas et al., 2012). Therefore, improving our knowledge of the ways, by which abiotic conditions and genetic factors influence juvenility and floral induction could help with crop scheduling, decrease time to flowering, and reduce waste with resulting benefits for the environment through lower inputs and energy required per unit of marketable product (Matsoukas et al., 2012, 2013).

MOLECULAR GENETICS OF THE JUVENILE-TO-ADULT PHASE TRANSITION

Molecular genetic analyses have provided insights into mechanisms that regulate the juvenile-to-adult and vegetative-to-reproductive phase transitions in several plant model systems (reviewed in Jansson and Douglas, 2007; Albani and Coupland, 2010; Huijser and Schmid, 2011; Andres and Coupland, 2012; Bolouri Moghaddam and Den Ende, 2013). MicroRNA156 (miR156), an ambient temperature-responsive miR (Lee et al., 2010) and strong floral inhibitor, is one of the central regulators of the juvenile-to-adult and vegetative-to-reproductive phase transitions in several species (Wu and Poethig, 2006; Chuck et al., 2007, 2011; Wang et al., 2011). Functional analysis of the *hasty1* (*hst1*) mutant of *Arabidopsis thaliana* revealed the function of the contrasting transcriptional pattern of the phloem-transmitted miR156 (Lee et al., 2010) and miR172 (Lauter et al., 2005; Martin et al., 2009; Varkonyi-Gasic et al., 2010) in regulation of phase transitions (Wu and Poethig, 2006; Chuck et al., 2007; Jung et al., 2007; Mathieu et al., 2009). It has been shown that the juvenile-to-adult phase transition is accompanied by a decrease in miR156/miR157 abundance and a concomitant increase in abundance of miR172, as well as the SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) transcription factors (TFs; Shikata et al., 2009; Wang et al., 2009; Jung et al., 2011; Shikata et al., 2012). Expression of

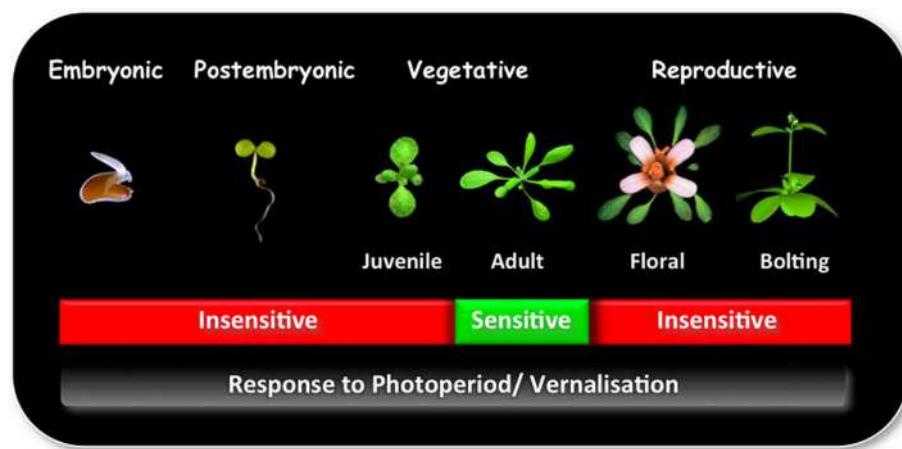


FIGURE 1 | The ability to sense and respond to photoperiod and/or vernalization varies in different phases of development. Plants undergo a series of qualitative transitions during their life-cycle in response to environmental and endogenous factors. One of the most distinguishable is the transition from a vegetative to reproductive phase of development, also known as the transition to flowering. This stage is preceded by the

juvenile-to-adult phase transition within the vegetative phase. During the juvenile phase plants are incompetent to initiate reproductive development and are effectively insensitive to photoperiod and/or vernalization. With the change to adult phase, plants attain competence to respond to floral inducers, which is required for the transition to the reproductive phase. Photoperiod is perceived in the leaf, whereas vernalization at the shoot apical meristem.

miR172 in leaves activates *FLOWERING LOCUS T* (*FT*; Aukerman and Sakai, 2003; Jung et al., 2007), the final output of the photoperiodic pathway (Corbesier et al., 2007), through repression of AP2-like transcripts *SCHLAFMÜTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*) and *TARGET OF EAT 1–3* (*TOE1–3*; Jung et al., 2007; Mathieu et al., 2009), whereas the increase in *SPLs* at the shoot apical meristem (SAM), leads to the transcription of floral meristem identity (FMI) genes (Wang et al., 2009; Yamaguchi et al., 2009). The FMI genes trigger the expression of floral organ identity genes (Causier et al., 2010), which function in a combinatorial fashion to specify the distinct floral organ identities.

The juvenile-to-adult phase transition is genetically regulated, although, as with most genetic traits, there are interactions with abiotic factors (Telfer and Poethig, 1998; Mohamed et al., 2010; Bergonzi et al., 2013). *Arabidopsis* genotypes impaired in sugar signaling, starch anabolism and catabolism, and floral repressor mutants show altered juvenile phase lengths compared to their respective wild types (Matsoukas et al., 2013). In addition, examination of diurnal metabolic changes in starch deficient and starch excess mutants indicates that their altered

juvenile phase length may be due to lack of starch turnover, which influences carbohydrate availability (Matsoukas et al., 2013). Interestingly, miR156a and miR156c, the major sources of miR156 in *Arabidopsis*, are significantly down regulated by sugars (Yang et al., 2013; Yu et al., 2013). Furthermore, it has been shown that trehalose-6-phosphate (Tre6P) acts as a local signal that links sugar availability to the juvenile-to-adult and vegetative-to-reproductive phase transitions (Wahl et al., 2013). *Arabidopsis* plants impaired in Tre6P signaling pathway are late flowering. This late flowering phenotype was found to be due to reduced expression levels of *FT*, the elevated levels of miR156, and reduced levels of at least three miR156-regulated transcripts, *SPL3*, *SPL4*, and *SPL5* (Wahl et al., 2013).

BIOCHEMICAL INFLUENCE

A number of biochemical changes have been proposed to mark the juvenile-to-adult phase transition in different plant species. For example, differences in peroxidase and esterase isozymes (Brand and Lineberger, 1992) and in protein phosphorylation (Huang et al., 1992). Furthermore, while various hormones have been shown to affect the juvenile-to-adult phase transition, their responses sometimes differ. The hormones auxin (De

Zeeuw and Leopold, 1955), abscisic acid (Rogler and Hackett, 1975), cytokinin (Mullins et al., 1979) and ethylene (Beyer and Morgan, 1971) have been demonstrated to be involved in the juvenile-to-adult phase transition. In addition, gibberellic acid (GA) has promotional and repressive effects depending on plant species (Wilson et al., 1992; Chien and Sussex, 1996; Telfer et al., 1997; Telfer and Poethig, 1998). In *Arabidopsis*, GA mutations that affect GA biosynthesis (*ga1-3*, *ga4-1*, and *ga5-1*) and GA sensitivity (*spindly4*) lengthen and shorten the vegetative phase transition, respectively (Telfer et al., 1997). However, it is unclear whether alterations in various hormone levels directly control the juvenile-to-adult phase transition. The action of hormones could be indirect, for instance, by controlling partitioning or mobilization of photosynthates, and/or interacting with other hormones (Domagalska et al., 2010) and sugar signals (Zhou et al., 1998; Moore et al., 2003).

MORPHOLOGICAL, HISTOLOGICAL, AND PHYSIOLOGICAL MARKERS

In some species, the juvenile-to-adult phase transition has also been associated with several morphological, histological, and physiological traits. For instance, leaves may change in shape, size,

phyllotaxy, and thickness. Other features associated with the developmental stage may relate to pigmentation, rooting ability, growth habit, orientation of vascular bundles, cold and disease resistance, and the physiological status of the plant (Poethig, 1990, 2003; Brunner and Nilsson, 2004; Itoh et al., 2005). However, these features are not totally reliable, since they are usually affected by different factors such as water availability, temperature, photoperiod, light quality and irradiance. In addition, the morphological, histological, and physiological changes are often less distinct in herbaceous than in woody species, and in many cases no clear association exists (Jones, 1999; Brunner and Nilsson, 2004).

In *Arabidopsis* the appearance of trichomes marks the juvenile-to-adult phase transition (Telfer et al., 1997). Leaves of plants in their juvenile phase of growth produce trichomes only on the adaxial surface, whereas leaves of plants in their adult phase produce trichomes on both the adaxial and abaxial surfaces. However, mutations in *Arabidopsis* affecting trichome development can alter trichome distribution in ways that are not phase specific (Telfer et al., 1997). This complicates the use of trichome distribution as a phase marker in mutant genetic screens.

The term “vegetative phase transition” is currently being used to characterize both heteroblasty and attainment of reproductive competence, since the two developmental events occur during the vegetative growth that precedes the transition to the reproductive phase (Poethig, 1990, 2009). However, by assessing morphological characteristics, several plant species undergo the vegetative-to-reproductive phase transition while still displaying juvenile traits, and others in which floral induction does not occur, even if adult traits are appeared and the plants are treated with photo- and/or thermo-inductive conditions (Brunner and Nilsson, 2004; Poethig, 2010). This could indicate that estimation of the length of the juvenile phase based on morphological traits does not necessarily provide a reliable indication of when juvenility ends. Therefore, the use of the terms “juvenile vegetative phase” and “adult vegetative phase” in defining both the heteroblastic transition as well as the

state of floral competence may lead to perplexity.

COMPETENCE TO RESPOND TO FLORAL INDUCTIVE SIGNALS: A RELIABLE DETERMINANT THAT CAN BE USED TO QUANTIFY THE LENGTH OF THE JUVENILE PHASE

The juvenile-to-adult phase transition is affected by several abiotic conditions (Matsoukas et al., 2013) and so chronological time (or the number of dormancy cycles) does not necessarily provide a reliable indication of when juvenility ends. Floral competence is the most robust determinant that can be used to distinguish between the juvenile and adult vegetative phases of plant development. However, non-flowering plants are not necessarily in their juvenile phase of development; they might be floral competent but have not been exposed to photo- and/or thermo-inductive conditions for flowering.

A simple method of quantifying the length of the juvenile phase accurately and reproducibly is to conduct reciprocal transfer experiments (Mozley and Thomas, 1995; Matsoukas et al., 2013). This approach involves transferring plants at regular intervals between conditions that are inductive and non-inductive for flowering, for example between LDs and SDs (or between different levels of temperature; response to vernalization), and assess leaf number and flowering time responses (Adams et al., 2001, 2003). This approach enables the analysis of reciprocal transfer experiments data in terms of the following parameters: (1) the photoperiod-insensitive juvenile phase; (2) the photoperiod-sensitive floral inductive phases in both SDs and LDs; and (3) the photoperiod-insensitive post inductive phase (Adams et al., 2003). Plants transferred from non- or less inductive conditions to inductive conditions before the end of juvenility will exhibit similar flowering times (and for terminal flowering plant species, have the same leaf numbers), as those grown constantly in the inductive conditions (Adams et al., 2003). On the other hand, floral induction will be delayed in plants that remain under non-inductive conditions after juvenility has ended. Experimental data sets obtained by the reciprocal transfer approach can

be analyzed by fitting models such as those described by Adams et al. (2001, 2003). The reciprocal transfer approach has the advantage that it can be used on small seedlings, where grafting techniques are impractical, and in species where genetic analyses are not possible as little is known about the genetic regulation of the juvenile-to-adult phase transition.

CONCLUDING REMARKS

The juvenile-to-adult and vegetative-to-reproductive phase transitions regulated by multiple pathways, which show different responses to external and internal stimuli. Much of the evidence for the various factors involved in the juvenile-to-adult phase transition can be subject to multiple interpretations. However, it can be proposed that the prolonged juvenile-to-adult and vegetative-to-reproductive phase transitions might be due to a plethora of antiflorigenic signals, which affect the transcription levels of *FT* and *SPLs*. Therefore, juvenility can be defined as the period during which the abundance of antiflorigenic signals such as miR156/miR157 is sufficiently high to suppress the expression of *FT* and *SPL* genes.

Determination of the length of the juvenile phase is a complex issue. The estimation of juvenility based on morphological, physiological, histological and biochemical markers does not necessarily provide a reliable indication of when juvenility ends. The exploitation of a single and simple experimental system to obtain accurate and reproducible estimates regarding the length of juvenility in different plant species is of crucial importance. Reproductive competence is a robust determinant that can be used to distinguish between plants that are juvenile or adult. This can be determined by conducting reciprocal transfer experiments. The simplicity of this approach enables its application in diverse plant species with comparative ease, including on young seedlings, and in genotypes where the practice of grafting is unfeasible.

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plantDARIO: web based quantitative and qualitative analysis of small RNA-seq data in plants

Deblina Patra^{1,2}, Mario Fasold^{2,3}, David Langenberger^{2,3}, Gerhard Steger⁴, Ivo Grosse^{1,5} and Peter F. Stadler^{2,5,6,7,8,9,10*}

¹ Institut für Informatik, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), Germany

² Bioinformatics Group, Department of Computer Science, Interdisciplinary Center for Bioinformatics, University Leipzig, Leipzig, Germany

³ ecSeq Bioinformatics, Leipzig, Germany

⁴ Institut für Physikalische Biologie, Heinrich-Heine-Universität, Düsseldorf, Germany

⁵ German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Leipzig, Germany

⁶ Max Planck Institute for Mathematics in the Sciences, Leipzig, Germany

⁷ Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany

⁸ Department of Theoretical Chemistry of the University of Vienna, Vienna, Austria

⁹ Center for RNA in Technology and Health, University of Copenhagen, Frederiksberg, Denmark

¹⁰ Santa Fe Institute, Santa Fe, USA

Edited by:

Klaus Pillen,
Martin-Luther-University
Halle-Wittenberg, Germany

Reviewed by:

Asa Ben-Hur, Colorado State
University, USA
Matthew R. Willmann, University of
Pennsylvania, USA

***Correspondence:**

Peter F. Stadler, Bioinformatics
Group, Department of Computer
Science, University Leipzig,
Härtelstrasse 16-18,
D-04107 Leipzig, Germany
e-mail: studla@bioinf.uni-leipzig.de

High-throughput sequencing techniques have made it possible to assay an organism's entire repertoire of small non-coding RNAs (ncRNAs) in an efficient and cost-effective manner. The moderate size of small RNA-seq datasets makes it feasible to provide free web services to the research community that provide many basic features of a small RNA-seq analysis, including quality control, read normalization, ncRNA quantification, and the prediction of putative novel ncRNAs. DARIO is one such system that so far has been focussed on animals. Here we introduce an extension of this system to plant short non-coding RNAs (sncRNAs). It includes major modifications to cope with plant-specific sncRNA processing. The current version of plantDARIO covers analyses of mapping files, small RNA-seq quality control, expression analyses of annotated sncRNAs, including the prediction of novel miRNAs and snoRNAs from unknown expressed loci and expression analyses of user-defined loci. At present *Arabidopsis thaliana*, *Beta vulgaris*, and *Solanum lycopersicum* are covered. The web tool links to a plant specific visualization browser to display the read distribution of the analyzed sample. The easy-to-use platform of plantDARIO quantifies RNA expression of annotated sncRNAs from different sncRNA databases together with new sncRNAs, annotated by our group. The plantDARIO website can be accessed at <http://plantedario.bioinf.uni-leipzig.de/>.

Keywords: non-coding RNA, microRNA, snoRNA, tRNA, high-throughput sequencing, expression analysis, ncRNAome

1. INTRODUCTION

Plant sncRNAs from seedlings and the inflorescences have been shown to have a broad range of biological functions in the model plant *Arabidopsis thaliana* (Lu et al., 2005). The universe of plant sncRNAs is much more complex and diverse than its counterpart in animals. Longer, approximately or perfectly double-stranded RNA (dsRNA) precursors are cut by Dicer-like (DCL) proteins into small RNA duplexes (Axtell, 2013). The precursors of siRNAs consist of dsRNA molecules (see Bologna and Voinnet, 2014 for a recent review) rather than more or less heavily structured single-stranded RNAs that serve as the precursors of microRNAs (Liu et al., 2014). The small RNA duplexes can be loaded onto different classes of Argonaute (AGO) proteins present in complexes of different functions that mediate the interaction of the incorporated small RNAs with their targets. For e.g., AGO1 acts mainly in microRNA (miRNA) pathways for post-transcriptional gene silencing (PTGS) (Wang et al., 2011a). In case of miRNA duplexes, while the guide strands are incorporated into AGO1 of the

RNA-induced silencing complex (RISC), the passenger strands called miRNA star (miRNA*) are mostly degraded (Wang et al., 2011b). Small RNAs loaded onto other Argonaute-containing complexes have different functions, e.g., heterochromatin maintenance.

In animals, detailed analyses of small RNA-seq samples, which were primarily produced with the aim of measuring miRNA expression (Hafner et al., 2008; Creighton et al., 2009), revealed that small, roughly microRNA-sized products, are derived from virtually all of the housekeeping ncRNAs including tRNAs (Lee et al., 2009; Sobala and Hutvagner, 2011), snoRNAs (Ender et al., 2008; Falaleeva and Stamm, 2013), and snRNAs (Langenberger et al., 2010; Li et al., 2012b), as well as from many previously undescribed genomic loci including promoters and transcriptional termini of most protein-coding genes (Kapranov et al., 2007). In plants, even more extensive groups of sncRNAs have been described, comprising in addition a variety of distinct types of small interfering RNAs (siRNAs) such as trans-acting siRNAs

(ta-siRNAs), natural antisense siRNAs (nat-siRNAs), and double-strand break interacting RNAs (diRNAs) (Mallory and Vaucheret, 2006; Ramachandran and Chen, 2008; Wei et al., 2012; Yoshikawa, 2013). Heterochromatic (hc-)siRNAs are the most abundant class of small RNAs in many plants. The transcripts yielding hc-siRNAs are transcribed by the plant-specific RNA polymerase IV and enter the RNA-directed DNA methylation (RdDM) pathway, comprising first the synthesis of dsRNA by RDR2 and subsequent cleavage by DCL3. The resulting 24 nt long hc-siRNAs are then bound to AGO4 (Matzke and Mosher, 2014). In contrast to miRNAs whose genomic loci are conserved between species, hc-siRNAs genomic loci are not, because they overlap with transposable elements (TEs), which are known to rapidly change their position and copy number in the genomes during plant evolution (Axtell, 2013).

The advent of protocols for preparing small RNA libraries and subsequently sequencing these using Next-Generation Sequencing (NGS) leads to a deluge of small RNA-seq datasets. For the analysis of these RNA-seq data, a large array of computational tools has been developed and published. Most tools focus on the prediction and quantification of sncRNA genes, like ShortStack (Allen et al., 2013), mirDeep (Friedländer et al., 2008), miRanalyzer (Hackenberg et al., 2009), CPSS (Zhang et al., 2012), miRNAkey (Ronen et al., 2010), and omiRas (Müller et al., 2013). Tools such as PsRobot (Wu et al., 2012) combine plant small RNA annotation and target analysis, while psRNATarget (Dai and Zhao, 2011) and SoMART (Li et al., 2012a) are mostly concerned with target prediction. miRanalyzer and omiRas are the only web tools that allow the upload of raw small RNA-seq data in fastq format, while for CPSS and PsRobot the data needs to be formatted to fasta format manually. The other sncRNA prediction tools need to be downloaded, installed and run locally, requiring more than basic computer skills. A drawback of all these tools are the integrated adapter clipping and read mapping steps. Although convenient, this can be problematic since different library preparations and sequencing runs result in sequencing data that should be handled independently. Given the differences in the performance of read mappers, in particular regarding sequences mapping multiple times and the handling of mismatches arising from polymorphisms (Zorc et al., 2012) or editing (Alon et al., 2012), it is desirable, to empower the researcher to use the tools of his/her choice. Furthermore, the sheer size of the raw sequencing data (several gigabyte) compared to their mapping coordinates (some megabyte) and abundances suggests the conclusion, that for a web-tool mapping coordinates are the upload format of choice.

In 2011, DARIO a web server for the analysis of small RNA-seq data in animals was introduced (Fasold et al., 2011). It was designed to perform quality control of input samples, expression analyses of annotated and user-defined sncRNAs, as well as a prediction of new non-coding RNAs. It provides exploratory analyses for mapped, but unannotated reads. Here we present a modified version of this versatile web service specifically tailored to plants. The differences between animal and plant sncRNAs (Bologna et al., 2013) resulted in several modifications in the workflow. Plant pre-miRNAs are much more heterogeneous than

their animal counterparts and have a different distribution of genomics contexts in which they reside (Axtell, 2004; Carthew and Sontheimer, 2009; Kim et al., 2009). Hence they are more difficult to annotate (Coruh et al., 2014). In contrast to most animals, plant genomes (with the exception of *Arabidopsis thaliana*) are poorly annotated for ncRNAs and thus a careful and manual annotation of their sncRNAs was essential. A classification of different sncRNAs solely based in their read patterns, as it has been used in DARIO (Fasold et al., 2011), was not possible in plants. Hence, plantDARIO uses third-party tools that also consider sequence and structure information for their predictions. Furthermore, due to a lack of one genome browser covering all plants, it was necessary to adapt and utilize different ones, allowing the researcher to take a look on the read distribution of the known and newly predicted sncRNAs.

2. MATERIALS AND METHODS

The current version of plantDARIO handles data for *A. thaliana* (TAIR9 and TAIR10)¹, *B. vulgaris* (RefBeet-1.1)² (Dohm et al., 2014), and *S. lycopersicum* (SL2.40)³ (Tomato Genome Consortium, 2012), and we plan to extend the service to include most of the available plant genomes.

2.1. WORKFLOW

The user input to the plantDARIO web service is a list of sequencing read positions mapped to one of the supported reference genomes. Data originating from any sequencing platform and mapped with the user's read alignment tool of choice can be used. However, only data originating from experiments prepared with the small RNA-seq protocol and thus predominantly covering read lengths of about 21–26 nt can be analyzed. Mapped reads can be uploaded in either BAM or bed format. We provide the PERL script map2bed.pl for converting mapped reads to bed format and for merging reads to tags, unique reads. These are represented as coordinate pairs rather than sequences for upload. This reduces the volume of data to be transferred over the internet to a manageable amount: 1 GB of SAM formatted mapper output is converted to about 15 MB of compressed bed file that can be uploaded to plantDARIO. User-defined annotations can easily be added to the annotation information stored in plantDARIO's internal database by uploading a list of loci, again in bed format.

Figure 1 summarizes plantDARIO's workflow, which is similar to that of its animal cousin (Fasold et al., 2011). The usage of plantDARIO is deliberately very similar to its animal cousin and detailed on the separate help page <http://plantdario.bioinf.uni-leipzig.de/help.py>. Instead of featuring a big extensive pipeline in the workflow, we have collated several analytical works as one step in the workflow. The first component of the pipeline performs a global statistical analysis of the input and provides the aggregate data for several quality control tools. The second component is concerned with the quantitative expression

¹<ftp://ftp.arabidopsis.org>

²<http://bvseq.molgen.mpg.de>

³http://solgenomics.net/organism/Solanum_lycopersicum/genome

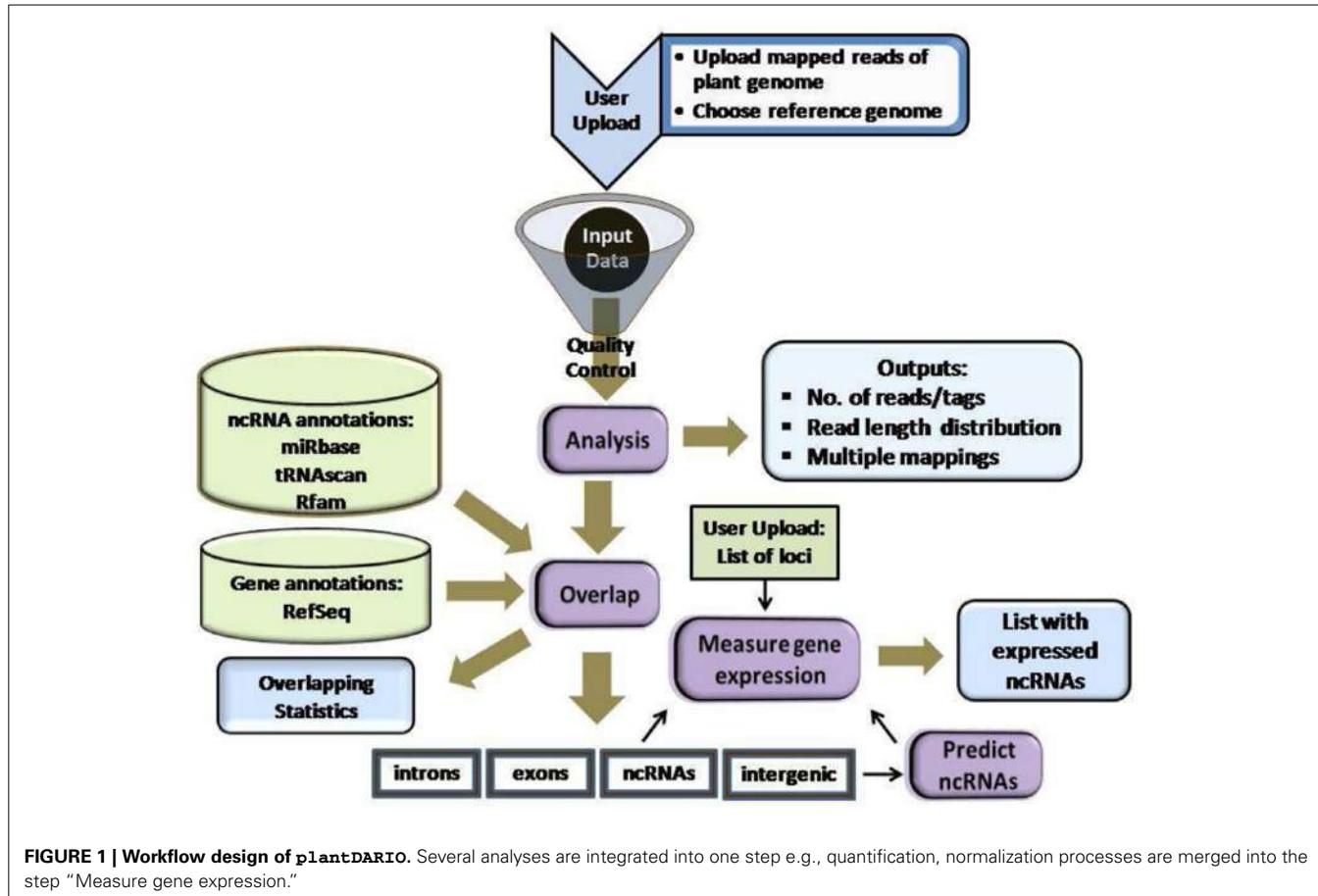


FIGURE 1 | Workflow design of plantDARIO. Several analyses are integrated into one step e.g., quantification, normalization processes are merged into the step “Measure gene expression.”

analysis of known and user-defined loci. The third component supports the discovery of novel miRNAs, snoRNAs, and tRNA-like loci. Output is displayed as HTML web pages and provided as machine-readable text files for download. A single job typically takes between 1 and 2 h.

2.2. QUALITY CONTROL

A wide variety of errors and biases have been described in high-throughput sequencing data, which may originate from sample handling, library preparation, or the sequencing itself. It is thus necessary to assess the quality and integrity of the experimental data before they are analyzed for biological content (Dohm et al., 2008; Linsen et al., 2009; Hansen et al., 2010). Important measures include the number of mappable reads and the number of tags (distinct read sequences), the distribution of read length, and the sequence composition of mapped reads.

A set of plots provides a convenient overview of the dataset (**Figure 2**). plantDARIO also computes a summary of the distribution of reads among annotation items such as introns and exons and the major classes of annotated non-coding RNAs such as miRNA, snRNA, rRNA, tRNA, ta-siRNA, and snoRNAs.

2.3. RNA QUANTIFICATION

Mapping loci are overlapped with annotated ncRNAs. To this end, plantDARIO includes an internal database of ncRNAs comprising miRNAs from miRBase (Kozomara and Griffiths-Jones,

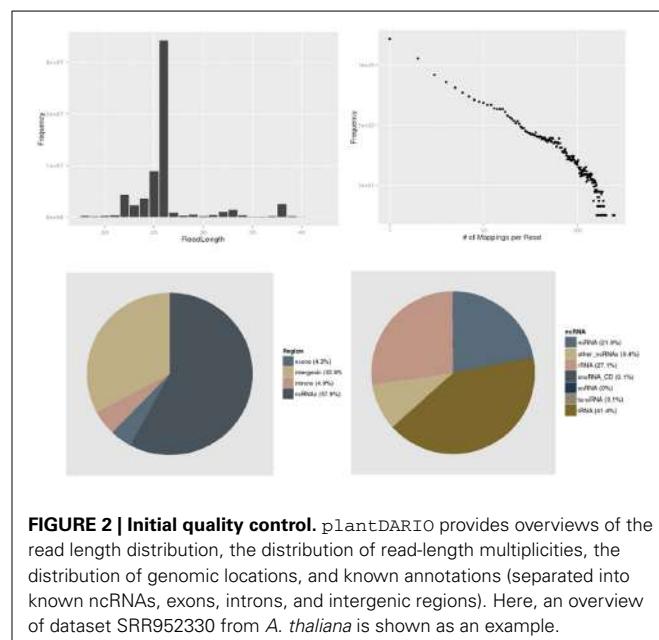
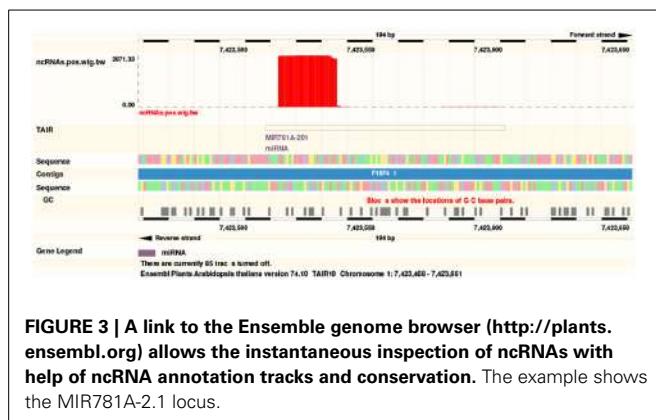


FIGURE 2 | Initial quality control. plantDARIO provides overviews of the read length distribution, the distribution of read-length multiplicities, the distribution of genomic locations, and known annotations (separated into known ncRNAs, exons, introns, and intergenic regions). Here, an overview of dataset SRR952330 from *A. thaliana* is shown as an example.

2011), tRNA annotations from tRNAscan-SE (Lowe and Eddy, 1997), ta-siRNA annotations from TAIR [ftp://ftp.arabidopsis.org](http://ftp.arabidopsis.org) and tasiRNADb <http://bioinfo.jit.edu.cn/tasiRNADatabase/> (Zhang et al., 2014), plant specific literature data (Barneche et al.,



2001; Brown et al., 2001; Dohm et al., 2014), as well as dedicated homology-based annotations for each individual genome. This internal annotation can be complemented by user-defined loci, which are then fully included in all downstream analyses. To handle multiple mappings, the number of reads for each sequence tag is divided by the number of its mapping loci, and this normalized expression value is assigned to each mapping locus.

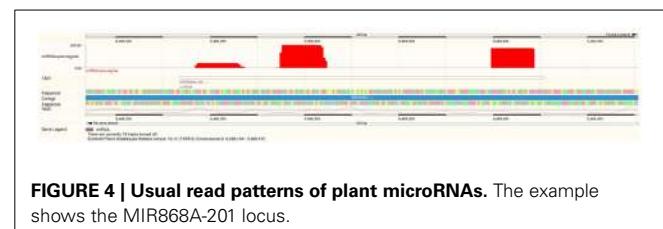
The web server generates a list of expressed ncRNAs, itemized by ncRNA classes. For each of them, a normalized expression value based on RPM (Reads per million) and the number of mapped reads (both in raw form and normalized for multiple mapping) is displayed. In addition a link to a genome browser is generated that allows the user to conveniently inspect the expression pattern at each individual locus (Figure 3). This can be helpful e.g., to distinguish between *bona fide* miRNAs from other RNA classes in case of misannotations (Langenberger et al., 2011), to inspect miRNA genes for the presence of offset RNAs (Langenberger et al., 2009; Shi et al., 2009), or to look for short reads generated from the antisense locus (Stark et al., 2008).

2.4. ANALYSIS OF UNANNOTATED LOCI

Mapped tags are merged to blocks and are aggregated to regions of blocks using blockbuster (Langenberger et al., 2009) with default parameters. Contrary to animals, the processing patterns of miRNAs are not very consistent in plants (Figure 4) so that patterns of mapped reads alone do not allow a sufficiently accurate classification. The same is true for snoRNAs. Hence the prediction of miRNAs and snoRNAs is assisted by the integration of novomir (Teune and Steger, 2010) and snoReport (Hertel et al., 2008) in plantDARIO. These tools are integrated as algorithms or scripts within the plantDARIO software. Both tools implement RNA folding and machine learning approaches to classify intervals of genomic sequences. We use blockbuster to identify accumulations of reads and then run the two tools on these loci.

2.5. ncRNA ANNOTATION IN SOLANUM LYCOPERSICUM

Non-coding RNAs have not been comprehensively annotated in many published genomes. This is also the case for *S. lycopersicum*, whereas most relevant annotation data were already available for the arabidopsis and sugar beet genomes. Hence, we produced an annotation track focussing on miRNAs, snoRNAs, and tRNAs for



the tomato genome roughly following the workflow employed for the annotation of the *B. vulgaris* genome (Dohm et al., 2014):

1. For miRNAs, plant miRNA pre-cursors were downloaded from miRBase and mapped against the genome using blast, employing a minimum alignment length of 60 nt and a sequence similarity of 80% as filter criteria. Overlapping matches were combined.
2. For snoRNAs, all plant snoRNAs were downloaded from the Rfam database and mapped against the genome with blast, employing a minimum alignment length of 70 nt and a sequence similarity of 80% as filter criteria. Overlapping matches were combined.
3. For tRNAs, tRNAscan (Lowe and Eddy, 1997) was run against the whole genome of *S. lycopersicum*.

The annotations can be downloaded from <http://plantdario.bioinf.uni-leipzig.de/annotations/>.

2.6. snRNA ANNOTATION IN SOLANUM LYCOPERSICUM AND ARABIDOPSIS THALIANA

For the *B. vulgaris* genome, snRNAs are already annotated and available along with other non-coding genes from the *B. vulgaris* resource (Dohm et al., 2014). For *A. thaliana* and *S. lycopersicum*, snRNA covariance models were downloaded from Rfam (<ftp://ftp.ebi.ac.uk/pub/databases/Rfam/>), and infernal (Nawrocki, 2014) was run against the respective genomes. For the purpose of providing a brief summary statistics, the spliceosomal RNAs U1, U2, U4, U5, U6, U11, U12, U4atac, and U6atac are grouped together with SRP RNA and RNase MRP RNA in the class “snRNAs.” They can be downloaded from the annotation URL given above.

2.7. GENOMES AND VISUALIZATION

plantDARIO references to the Ensembl genome browser (Hubbard et al., 2002) to visualize the read coverage at annotated loci and predictions as custom tracks for *A. thaliana*. This allows an interpretation of the user data in the context of information provided by the Gramene database (Youens-Clark et al., 2010), a resource for plant comparative genomics. For sugarbeet and tomato, we rely on the genome browser from the *B. vulgaris* resource (Dohm et al., 2014) and sol genomics network (SGN) (Tomato Genome Consortium, 2012), respectively, for visualization.

2.8. IMPLEMENTATION DETAILS

The technical details of plantDARIO parallel those of DARIO (Fasold et al., 2011). Web pages are created by python

scripts making use of the Mako template engine. Graphics are created using R and the graphics package `ggplot2` (Wickham, 2009). A queuing system is used to distribute analysis jobs.

3. RESULTS AND DISCUSSION

plantDARIO implements basic workflows for the analysis of small RNA-seq data. It allows the user to obtain a comprehensive overview starting after read mapping. To demonstrate the versatility of plantDARIO we re-analyzed publicly available small RNA-seq datasets from *Arabidopsis* SRR952330, (SRR167709 and SRR167710; Pélissier et al., 2011), sugarbeet (SRR868805) (Dohm et al., 2014), and tomato (SRR786984) (Weiberg et al., 2013). We used `segemehl` (Hoffmann et al., 2009) with default parameters to map the sequencing data to the respective reference genomes. Unlike many other mapping tools, `segemehl` has full support for multiple-mapping reads which is very important for small RNA-seq (Otto et al., 2014).

3.1. NEW miRNAs AND snoRNAs

In addition to more than 200 known miRNAs, we observed more than 100 expressed putative novel miRNAs in each of the datasets (Table 1). An example of a newly predicted miRNA is shown in Figure 5. It represents a perfect plant miRNA pattern as expected for sncRNAs processed by a plant DCL enzyme (Kurihara and Watanabe, 2004), resulting in one functional arm (proper read block in the figure) in this case. The irregular patterns found as little bumps in the structure are bulge loops or internal loops present in the pre-miRNA structure, which are usual, i.e., which are a thermodynamic feature of the RNA. Furthermore, the read pattern matches a stem-loop when traced back to a likely pre-microRNA, as shown in Figure 5.

Table 1 | Known and novel sncRNAs in four test datasets.

| Data | Species | miRNAs | | snoRNAs | |
|-----------|---------------|--------|-----|---------|-----|
| | | Known | New | Known | New |
| SRR167709 | <i>A. th.</i> | 276 | 121 | 78 | 348 |
| SRR167710 | <i>A. th.</i> | 236 | 139 | 71 | 268 |
| SRR786984 | <i>S. ly.</i> | 268 | 65 | 121 | 202 |
| SRR868805 | <i>B. vu.</i> | 197 | 41 | 60 | 22 |

For both microRNAs and snoRNAs, the number of expressed annotated sncRNA loci ("known") and the number of novel candidates ("new") is reported.

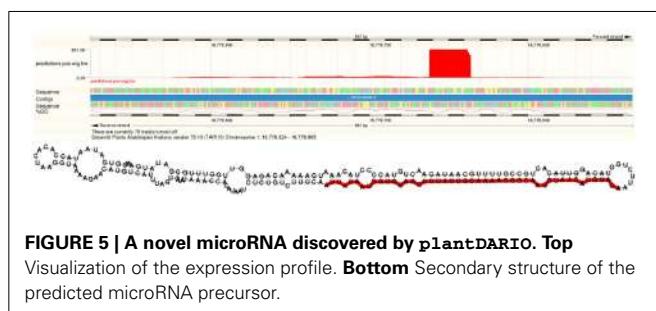


FIGURE 5 | A novel microRNA discovered by plantDARIO. **Top**

Visualization of the expression profile. **Bottom** Secondary structure of the predicted microRNA precursor.

For snoRNAs, we observed an even larger number of candidates. An example is detailed in Figure 6. The structure pattern shows a candidate snoRNA with typical C box and D box sequence patterns close to the ends. The middle region, presumably a loop, contains box C' and D' regions frequently found in box C/D snoRNAs.

3.2. DIFFERENTIAL EXPRESSION

In order to demonstrate that the output of plantDARIO is easy to use for downstream analyses, we compared small RNA expression for miRNA and snoRNA in the two *A. thaliana* datasets SRR167709 and SRR167710 (Pélissier et al., 2011) representing populations of small RNAs from *Arabidopsis* immature flowers of WT and drb2 mutants, respectively. The original study aimed at the antagonistic impact of dsRNA binding proteins DRB2 and DRB4 on polymerase dependent siRNA levels. Figure 7 shows that, overall, the miRNA expression levels correlate positively between the two datasets for both previously annotated and newly predicted miRNAs.

One of the miRNAs with extreme (> 8 fold) change in expression level is ath-MIR856. This miRNA, which is predominantly expressed in the floral organ (Meng et al., 2012), belongs to a set of miRNAs that are evolutionary transient within the genus

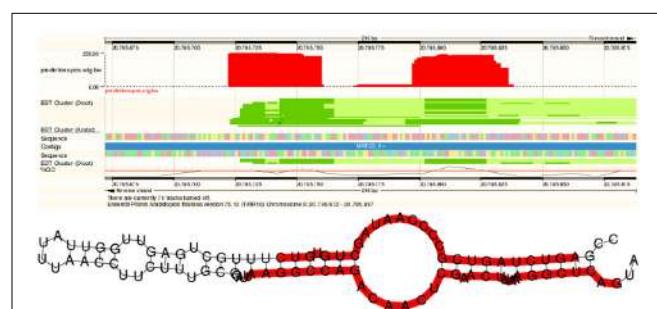


FIGURE 6 | A novel CD box snoRNA discovered by plantDARIO. **Top** Visualization of the expression profile. **Bottom** predicted secondary structure; the origin of the observed short reads is marked in red.

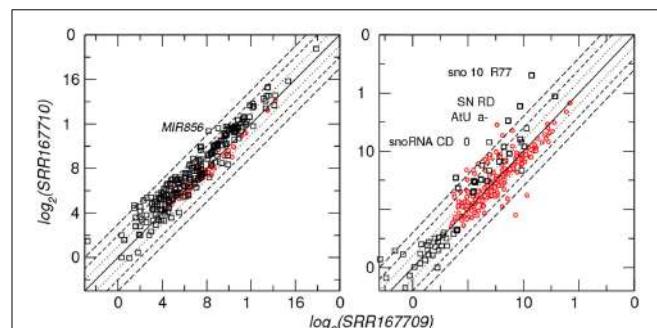


FIGURE 7 | Differential expression of microRNAs (left panel) and snoRNA-derived small RNAs (right panel) for two *A. thaliana* datasets. Diagonal lines indicate differences between 2^3 and 2^{-3} fold.

Black symbols indicate annotated microRNA and snoRNA loci, red dots refer to novel predictions. A few loci with extreme expression differences are labeled.

Arabidopsis (Ma et al., 2010; Shao et al., 2012) and shows an exceptional evolutionary behavior with relatively low levels of polymorphism but the highest level of divergence (de Meaux et al., 2008).

Surprisingly, we observe a much larger variability for the processing products of snoRNAs. The extreme case, snoZ102_R77, is a box C/D snoRNA belonging to the SNORD44 clan. Box C/D snoRNA_CD_230 (*Arabidopsis*, chr1:6697176-6697261) is related to snoR16 and snoR72 families according to a search in Rfam. All these snoRNAs have a primary function in ribosomal RNA processing (Brown et al., 2003). Interestingly, the examples with extreme differential expression belong to the box C/D class of snoRNAs that is not processed by Dicer but utilizes another, hitherto unknown, processing pathway at least in mammals (Langenberger et al., 2012).

4. CONCLUDING REMARKS

High-throughput sequencing has become the method of choice for the analysis of transcriptome data. For the special case of small RNA-seq data, web services provide a convenient means of conducting standard analyses. In this way the user can avoid the need to install, maintain, and update an array of individual tools. plantDARIO is such a service that, in contrast to comprehensive analysis environments like GALAXY (Goecks et al., 2010), provides a ready-to-use analysis workflow for small RNA-seq data. Together with pre-compiled sncRNA annotations this allows to inspect analysis results quickly after uploading the user data. In summary, plantDARIO provides the user with a valuable combination of annotation-based, standardized quantitative analysis and a simple facility for guided discoveries of novel small RNA loci.

The web service also provides the results in a bed format, which can easily be used for downstream analysis tasks such as the assessment of differential expression. Using publicly available small RNA-seq data for *A. thaliana* we noticed extreme differences in the levels of small RNAs processed from box C/D snoRNAs. Some of these sdRNAs are known to have a regulatory role in animals, so it might be of possible interest to further characterize small RNA processing from “house-keeping ncRNAs” in plants, and plantDARIO might be a convenient and versatile tool for this purpose.

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Mapping genes governing flower architecture and pollen development in a double mutant population of carrot

Holger Budahn^{1*}, Rafał Barański², Dariusz Grzebelus², Agnieszka Kiełkowska², Petra Straka³, Kai Metge³, Bettina Linke⁴ and Thomas Nothnagel¹

¹ Institute for Breeding Research on Horticultural Crops, Federal Research Centre for Cultivated Plants, Julius Kühn-Institute, Quedlinburg, Germany

² Department of Genetics, Plant Breeding and Seed Science, Faculty of Horticulture, University of Agriculture, Kraków, Poland

³ Institute for Biosafety in Plant Biotechnology, Federal Research Centre for Cultivated Plants, Julius Kühn-Institute, Quedlinburg, Germany

⁴ Department of Biology, Humboldt University, Berlin, Germany

Edited by:

Klaus Pillen,
Martin-Luther-University
Halle-Wittenberg, Germany

Reviewed by:

Daniel Pinedo, Universidad Nacional
Autónoma de México, Mexico
Matthew R. Willmann, University of
Pennsylvania, USA

***Correspondence:**

Holger Budahn, Institute for
Breeding Research on Horticultural
Crops, Julius Kühn-Institute, Federal
Research Centre for Cultivated
Plants, Erwin-Baur-Str. 27, D-06484
Quedlinburg, Germany
e-mail: holger.budahn@jki.bund.de

A linkage map of carrot (*Daucus carota* L.) was developed in order to study reproductive traits. The F₂ mapping population derived from an initial cross between a *yellow leaf* (yel) chlorophyll mutant and a *compressed lamina* (cola) mutant with unique flower defects of the sporophytic parts of male and female organs. The genetic map has a total length of 781 cM and included 285 loci. The length of the nine linkage groups (LGs) ranged between 65 and 145 cM. All LGs have been anchored to the reference map. The objective of this study was the generation of a well-saturated linkage map of *D. carota*. Mapping of the *cola*-locus associated with flower development and fertility was successfully demonstrated. Two MADS-box genes (*DcMADS3*, *DcMADS5*) with prominent roles in flowering and reproduction as well as three additional genes (*DcAOX2a*, *DcAOX2b*, *DcCHS2*) with further importance for male reproduction were assigned to different loci that did not co-segregate with the *cola*-locus.

Keywords: *Daucus carota*, linkage map, MADS-box genes, male gametogenesis, alternative oxidase, chalcone synthase

INTRODUCTION

Carrot is the second most economically valuable vegetable in the European Union and is also of worldwide significance. Increased production area and improved productivity highlight its economical importance in North America, Asia, and Europe (<http://faostat.fao.org/>). Over the last few decades, carrot consumption has continuously grown (Simon et al., 2009).

Daucus carota is a typical biannual diploid (2n = 2x = 18) outcrossing species with a relatively small genome estimated as 473 Mb. Carrot chromosomes are small but morphologically distinguishable (Schrader et al., 2003). Within the large family of Apiaceae, carrot is the best characterized species at the genetic and molecular level. Comprehensive research has been performed on the characterization of metabolite contents, morphological traits and resistances (for review see Bradeen and Simon, 2007; Simon et al., 2008). On the other hand, knowledge on genes controlling reproduction and flowering is yet limited. To facilitate genetic characterization by improved mapping studies, several tools have been made available in recent years. This includes a deep coverage BAC library (Cavagnaro et al., 2009), assignment of linkage groups (LGs) to chromosomes by fluorescence *in situ* hybridization (Iovene et al., 2011) and *de novo* assembly of the carrot transcriptome (Iorizzo et al., 2011) and opens advanced perspectives for carrot research and breeding.

Improved knowledge on genetic control mechanisms for plant reproduction traits such as flowering time, fertility and seed set is of essential importance for the breeding process, especially for the stabilization of seed yield under different environmental

conditions. One of the key steps is the determination of the basal floral architecture, i.e., the specification of sepals, petals, stamens, and carpels. Based on studies in model plants like *Arabidopsis* and *Antirrhinum*, the ABC model of flower development was proposed (Coen and Meyerowitz, 1991). The model was further extended by D- and E-class genes to include the advanced development of reproductive organs (Pelaz et al., 2000; Theissen, 2001). The majority of these genes belong to the MADS-box gene family of transcription factors (Schwarz-Sommer et al., 1990). MADS-box genes can be grouped into different clades with subfamily-specific functions in flower development (Becker and Theissen, 2003; Parenicova et al., 2003). Genes of these groups play a critical role in the determination of the identity of two neighboring flower whorls according to the ABCDE model. In the carrot, Linke et al. (2003) have identified five MADS-box genes (*DcMADS1* to 5) and assigned them to the previously defined groups SQUAMOSA, GLOBOSA, DEFICIENS, AGAMOUS, and SEPALLATA1.

Other classes of genes are involved in the development of functional pollen and are important for fertility/sterility. The alternative oxidase (AOX) is a mitochondrial ubiquinol:oxygen oxidoreductase (Affourtit et al., 2002). It is encoded by a small nuclear multigene family (*AOX1* and *AOX2*). For the gene *AOX2b* a striking effect on male and female gametogenesis was shown in soybean. Its regulation depends on the tissue and the developmental stage (Finnegan et al., 1997; Djajanegara et al., 2002). A significant effect on pollen abortion and pollen germination was measured (Chai et al., 2010). In the pistils of transgenic antisense

GmAOX2b soybean plants, a higher portion of immature-sized and non-fertile embryo sacs were observed. In addition, significant changes of the expression level for alternative oxidase in maize mitochondrial mutants were identified (Karpova et al., 2002).

Another enzyme with effects at particular stages of anther and pollen development is chalcone synthase (CHS), one of the key enzymes involved in the flavonoid biosynthesis pathway. Flavonoids are important for pollen development and plant fertility in several plant species (Dobritsa et al., 2010 and references therein). *CHS* has been reported as a potential sex-determination gene that is expressed in male cones of *Pinus* (Walden et al., 1999) and in male flower buds of *Silene* (Ageez et al., 2005). Significant effects of CHS on different types of nuclear male sterility were demonstrated for maize (Mo et al., 1992), petunia (Taylor and Jorgensen, 1992; van der Meer et al., 1992) and tobacco (Atanassov et al., 1998). In plants carrying the cytoplasmic male sterility trait (CMS), the deficiency of flavonoids caused by inhibition of *CHS* expression was strongly associated with pollen abortion (Yang et al., 2008). A role of *CHS* genes in the formation of the exine during microspore development has been reported in *Arabidopsis* (Dobritsa et al., 2010).

The first well-saturated map of all nine carrot LGs was generated by Just et al. (2007). Transposon display (td) markers were integrated into that map by Grzebelus et al. (2007) using *DcMaster*, a family of *PIF/Harbinger*-like transposable elements identified in *Daucus*. Simple sequence repeats (SSRs) have great advantages in comparison to other marker classes and were applied in research on carrot gene flow and genetic diversity (Rong et al., 2010; Barański et al., 2012). High reproducibility, abundance and a codominant inheritance make SSR markers particularly well suited for anchoring different maps of the same or closely related species. Niemann et al. (1997) have developed the first set of SSR markers for carrot and later assigned 19 SSR markers to a carrot linkage map (Niemann, 2001). Other sets of SSR markers were described by Umehara et al. (2005) and Cloutault et al. (2010). By far more SSR markers became available to anchor new *D. carota* linkage maps, when Cavagnaro et al. (2011) provided a set of 300 SSR markers for carrot and assigned 55 of them to all nine LGs. In parallel, Iorizzo et al. (2011) has described a set of SSR markers from transcribed sequences (eSSR). These sets of SSR markers were used by Alessandro et al. (2013) for mapping *Vrn1*, a gene controlling early flowering and *Rf1*, a restorer gene for the petaloid-type of CMS in carrot. Yıldız et al. (2013) reported the mapping of anthocyanin biosynthesis genes on five individual carrot LGs. Recently, a carrot map on the base of Diversity Array Technology (DArT) markers became available (Grzebelus et al., 2014).

Due to the high genetic diversity within the carrot with substantial allelic variability (Macko and Grzebelus, 2008; Grzebelus et al., 2014), an extended genetic map will be advantageous to allocate and improve genetic information and to facilitate the possibility to set up meta-analyses of favorable genetic traits (Brachi et al., 2011). The objective of the present study was the generation of a well-saturated linkage map of *D. carota* comprising markers linked to known and new loci associated with flower development and fertility.

MATERIALS AND METHODS

PLANT MATERIAL

The F₂ mapping population DM19 was developed from an initial cross of the homozygous recessive *yellow leaf* (*yel yel*) mutant as the maternal parent and the *compressed lamina* (*cola cola*) mutant as the pollen parent. The *yel*-mutant is easily distinguishable from the wild-type by the yellow leaf color due to chlorophyll deficiency (Nothnagel and Straka, 2003). The *cola*-mutant is characterized by compact small leaves. The flower architecture of *cola*-plants did not correspond to one of the major “homeotic” phenotypes as described by the ABCDE model indicating that the reproductive organs have acquired a correct identity. Instead, rather advanced stages of reproductive organ differentiation were impaired. The female organs revealed a slight, but unique difference of the ovary position that has changed from an inferior position (epigynous flower type) to a rather superior position (hypogynous flower type) as was shown in Figure 1C. Thus, the ovary of *cola*-mutant flowers was located above the insertion point of the perianth-ring. This trait is always associated with the trait “anther/locule defect” of the *cola*-type flowers, indicating an irregular structure of several theca and/or locules as was previously shown by histomorphological investigation (Nothnagel et al., 2005). For flower characterization of the mapping population we have used the trait “epigynous/hypogynous” because it is easy to characterize and do not require additional histological analyses. Hence, the *cola*-mutant flowers revealed defects of the sporophytic parts of both, male and female organs as a result of an impaired advanced differentiation. In contrast, the *yel*-parent of the mapping population revealed a wild-type phenotype for flower architecture.

Plants were grown in 16 cm plastic pots in a sand-humus mixture (v/v 3/1) in the greenhouse. To induce flowering, the plants were vernalized in a climatic chamber at 5°C for 12 weeks in the dark. Flowering plants were isolated and crossed manually to produce F₁ plants (*YEL yel COLA cola*). True hybrids expressed the wild-type phenotype and were self-pollinated to produce F₂ seeds. Leaf color and leaf shape of the F₂ plants were evaluated 30, 60, and 90 days after sowing. Bolting status of the F₂ plants was observed visually approximately each 5 days after vernalization and replantation in the glasshouse. The flower architecture was investigated using a stereoscopic microscope.

For the statistical analyses was used the software Systat 13 (Chicago, IL: Systat Software, Inc., 2009). Data of F₂-segregation were analyzed using the χ^2 -test to determine the goodness of fit for Mendelian inheritance (9:3:3:1 or 3:1, respectively).

ANALYSIS OF POLLEN VIABILITY

The pollen viability was determined by vital staining of microspore cells, using fluorescein diacetate (FDA) according to Heslop-Harrison and Heslop-Harrison (1970). From ten plants of the *cola*-mutant and ten plants of the wild-type, five protandrous flowers were collected and the anthers were immediately suspended in 0.5 ml of a solution containing 75 mg/ml sucrose and 1 mg/ml FDA (Serva, Heidelberg, Germany). Pollen viability was determined as ratio of pollen emitting strong fluorescence when subjected to UV light using Axioskop 2 (Zeiss, Oberkochen, Germany) equipped with a fluorescence filter set 44 [excitation BP 475/40 (455–495 nm)/emission BP 530/50 (505–555 nm)]. At

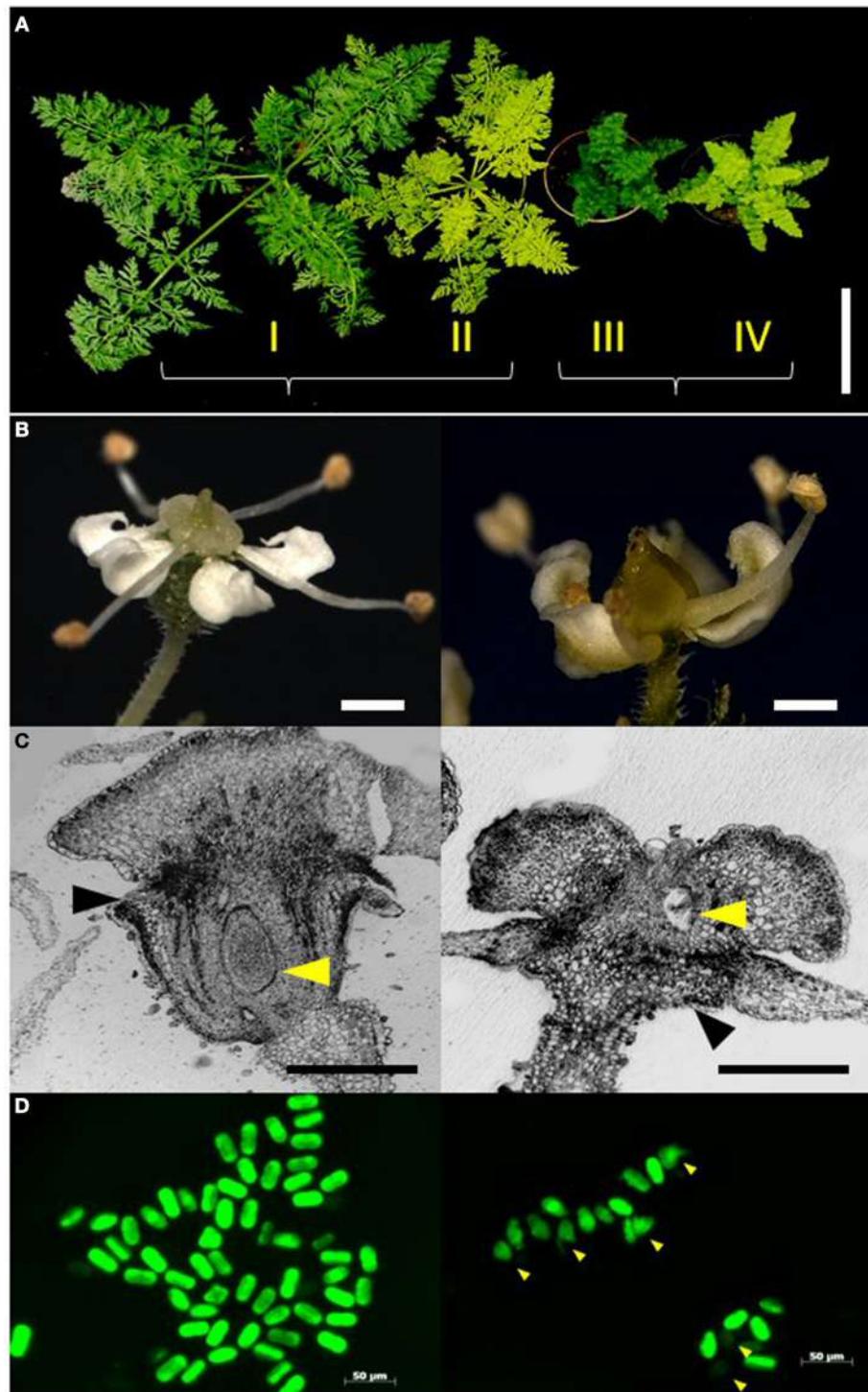


FIGURE 1 | Characterization of the phenotype classes. (A) Representative plants of the four phenotype classes I–IV in the F₂ segregating population DM19 obtained from a cross between mutants *yel* and *cola*. From left to right: wild-type plant with green and normal leaf structure (I); *yel*-mutant (II); *cola*-mutant (III); *yel/cola* double mutant (IV) (Bar = 10 cm). (B) Morphology of reproductive organs. Left: epigynous flower of wild-type. Right: hypogynous

flower of *cola*-mutant (Bars = 1.0 mm). (C) Histo-morphological sections of flowers. Left: wild-type. Right: *cola*-mutant. Black arrowheads: petals, yellow arrowheads: ovule (Bars = 0.5 mm). (D) FDA pollen staining. Fertile pollen grains revealed a intensive greenish stain, whereas sterile and undeveloped pollen grains were stained weakly; Left: wild-type, Right: *cola*-mutant, arrowheads indicate undeveloped microspores.

least 400 pollen grains per plant were classified. The mean values of pollen analysis data were compared by Student's *t*-test at a significance level $p < 0.05$.

DNA EXTRACTION, RAPD-, DOUBLE PRIMER (dp)RAPD- AND AFLP-ANALYSIS

Genomic DNA was extracted from 0.5 g of young leaf tissue from both parental lines and from 161 F₂ plants of the DM19 population, using the method of Porebski et al. (1997). RAPD-PCR reactions were performed in 10 μ l volume containing 1 x PCR buffer (InVitek, Berlin, Germany), 2.25 mM MgCl₂, 0.2 mM dNTPs, 0.2 μ M arbitrary decamer primer (Roth GmbH, Karlsruhe, Germany), 0.12 U InViTaq-polymerase (InVitek) and 8 ng total plant DNA. The thermocycler (PT 200; Bio-Rad, Hercules, CA, USA) was programmed as follows: initial denaturation at 94°C for 2 min; 45 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min and final extension at 72°C for 7 min. Amplification products were separated on 1% agarose gels using the 100 bp ladder (Invitrogen, Carlsbad, CA, USA) for size determination. The generation of dpRAPD markers followed the protocol described by Budahn et al. (2008). AFLP reactions were performed according to the instructions of the AFLP® analysis system I kit (Invitrogen, Carlsbad, CA, USA). The amplification products were separated at 50°C and 100 W for 3.5 h on 4% denaturating polyacrylamide gels (Sequigen GT; 38 \times 50 cm; Bio-Rad Laboratories, Hercules, CA, USA) followed by silver staining according to Bassam et al. (1991). Fragment sizes were estimated using the 123 bp DNA-ladder and the 1D-phoretix-Software 5.2 (Biostep, G, Nonlinear Dynamics Ltd., Newcastle, UK). Only unambiguous and polymorphic fragments were scored. The designation of the RAPD markers contained the used decamer primer sequence and, the AFLP markers the applied EcoRI- and MseI-primers added by the estimated fragment sizes.

SSR MARKERS

The original primers and amplification conditions were described by Cavagnaro et al. (2011). A universal fluorescent labeling strategy was used, as described by Schuelke (2000). The unlabeled forward primer extended at the 5' end by the 19 bp M13 sequence was combined with the original reverse primer and the fluorescently labeled M13 universal primer. The amplification products of two independent reactions with IRDye700 and IRDye800 labeled M13 primers were mixed. DNA fragments were separated on 6.5% polyacrylamide gels followed by fragment detection using a LI-COR 4300 automatic sequencer (LI-COR Biosciences, Lincoln, NE, USA). The fragment sizes were calculated by IRDye700 and IRDye800 labeled 50–350 or 50–700 bp size ladders, respectively. SSR markers of the DCM series (Niemann, 2001) were amplified, separated on the Sequigen GT system (Bio-Rad Laboratories, Hercules, CA, USA) and visualized by silver-staining. Primer sequences and annealing temperatures were summarized in Table S1.

td MARKERS

The primer sequences and amplification conditions have been described by Grzebelus et al. (2007). Ten primer combinations were selected for the analysis. The amplification products were

separated on 4.5% polyacrylamide gels and visualized by silver staining (Bassam et al., 1991).

GENE-SPECIFIC MARKERS

MADS-box genes of the carrot have been identified after generation of a cDNA library from wild-type carrot flowers (Linke et al., 2003). Additional sequences of genes with expected roles during reproductive organ differentiation and pollen development in the carrot were selected from NCBI database (complete list in Table S1; short list in Table 1). Gene-specific primers for PCR and mapping analyses were generated in the frame of earlier work (Linke et al., 2003; Campos et al., 2009; Cardoso et al., 2009; Grzebelus, pers. commun.). The deduced primer pairs have been tested for their specificity by expression analyses. In all cases, fragments of the expected sizes were obtained. Gene-specificity of the primers was further confirmed by the use of nested primers or additional primer combinations. Regarding different isoforms (AOX genes) and domain-derived sequence similarities (MADS-box genes), further analysis was performed by sequence-analyses [direct sequencing of PCR fragments obtained from cDNA (MADS-box genes)] or at least by internal PCR-tests with nested primers to ensure a clear differentiation between similar sequences. Flower-specific expression during different developmental stages was analyzed in wild-type carrot plants by RT-PCR or by *in situ* hybridization of mRNA (MADS-box genes, Linke et al., 2003; AOX genes, Campos et al., 2009).

The different primer pairs were tested for DNA polymorphisms in the *cola*- and the *yel*-parent and in selected progeny plants. PCR amplification products were separated on 1% agarose gels and visualized by ethidium bromide staining. Only primer pairs, providing clear polymorphisms, were used in the segregating F₂ population DM19. Primers and amplification conditions were summarized in Table 1. Primers for amplification of *DcMADS3* were deduced from NCBI accession number AJ271149. The forward primer binds at the nucleotide positions 114–132 within the MADS-domain. The reverse primer was deduced from the nucleotide positions 653 to 637 that encompassed the last ten amino acids of the coding sequence. For amplification of *DcMADS5*, the forward primer was deduced from the K-box region (LGTK-tag; positions 357–376). The reverse primer binds to the nucleotides 739–720 and was deduced from the PGWML-motif consisting of the five penultimate amino acids preceding the terminus of the predicted protein that reveal similarities within the SEPALLATA1-group of MADS-box genes. For both of the MADS-box genes, unique fragments were obtained that have been classified by PCR-sequencing previously (Linke et al., 2003). The forward primer for amplification of *DcAOX2a* was deduced from the nucleotide positions 539 to 559 of the mRNA-derived cDNA of the mitochondrial alternative oxidase 2a (accession number EU286575.2) in the ferritin-like-diiron-binding region. The reverse primer encompassed positions 1275–1252 and derived from the 3'-UTR region. Two alleles of *DcAOX2a* were known, the L-allele, consisting of 5263 bp (accession number GQ248714) and the S-allele consisting of 4977 bp (accession number GQ248713). Using the described primer set, an L-allele specific fragment of 1930 bp and a S-allele specific fragment of 1654 bp where intron 3 varied in size between 1226 and

Table 1 | Primer sequences and amplification conditions for *DcMADS3*, *DcMADS5*, *DcAOX2a*, *DcAOX2b*, and *DcCHS2*.

| Target | NCBI number | Marker type | Forward primer | Reverse primer | Annealing temperature | Fragments* detected in the <i>yel</i> —mutant | Fragments* detected in the <i>cola</i> —mutant |
|----------------|-------------|-------------|-------------------------------------------------------------------|--------------------------------------------------------------------|-----------------------|-----------------------------------------------|------------------------------------------------|
| <i>DcMADS3</i> | AJ271149 | EST | GTTGATGCTAAGGTTTCG GGGCACAAAGGGCTTGAGG TGCTGCATCTGAGTCTCTCC | GATCCTGCTCCGCATG AGAGCATCCACCCCTGGAATG CCAATCAATTCTACACAAACC | 54 | — 605 bp 1900 bp | 1500 bp 770 bp |
| <i>DcMADS5</i> | AJ271151 | CAPS** | EST | AGCTTGGTGACAGTTATAGG ATAGGCCATGTACTCGCAGAA | 50 | — — | — |
| <i>DcAOX2a</i> | EU286575 | EST | TGCATCGTCTCCCTTATTTC CTCAAGGAGAAGTTAGGGATG | 55 | — | 1400 bp | |
| <i>DcAOX2b</i> | EU286576 | EST | | 55 | 850 bp | 900 bp | |
| <i>DcCHS2</i> | D16255 | EST | | 56 | | | |

*Only polymorphic fragments were summarized. Fragments non-polymorphic between *yel*- and *cola*-parent were not mentioned.**The polymorphism was obtained after cleavage of the genomic DNA with *Alu*I to generate a CAPS (Cleaved Amplified Polymorphic Sequence) marker.

941 bp has been characterized (Cardoso et al., 2009). The same gene-specific fragments were also used for analyses of the present mapping population to include these pre-characterized polymorphisms into our yet more extended linkage map. As was shown by Cardoso et al. (2009), the complete gene sequence of *DcAOX2b*, including exons and introns, consisted of 1958 bp. The size of the introns have been assigned to 822 (intron 1), 91 (intron 2), and 85 (intron 3). The length of the deduced mRNA sequence was stated as 1267 bp. For the amplification of *DcAOX2b* we have used primers published by Campos et al. (2009), that covers the nucleotide positions 1–23 (forward primer) of the carrot mitochondrial alternative oxidase 2b (accession number EU286576). The reverse primer was deduced from positions 770 to 747. Forward and reverse primers designed for the amplification of *DcCHS2* were generated from an exon-intron-exon bridge, and were deduced from nucleotide positions 2583 to 2606 and 3484 to 3461 of the gDNA sequence D16255.1, respectively.

Non-polymorphic amplification products were digested with the restriction endonucleases *Alu*I, *Hae*III, *Rsa*I, *Taq*I, *Bsm*I, and *Hinf*I to identify recognition site polymorphisms and to develop CAPS markers by this way.

DATA SCORING, LINKAGE ANALYSIS, AND MAP CONSTRUCTION

The phenotypic and molecular data were formed as required for JoinMap version 4.0 (Van Ooijen, 2006). AFLP-, RAPD-, and dpRAPD-markers were scored in a dominant and SSR-markers in a co-dominant manner. The *p*-value was calculated after chi-square-test for all markers. Linked loci were grouped using a LOD thresholds from 2.0 to 10.0 in steps of 0.2 and recombination frequency lower than 0.4. The jump threshold was set to 5.0 and the third mapping round was carried out. The recombination frequencies were converted to mapping distances (in cM) using the Kosambi function.

RESULTS

PHENOTYPE SEGREGATION

A homozygous recessive *cola*-mutant plant was used to pollinate a homozygous recessive *yel*-mutant plant to generate the mapping population. All F₁ plants expressed the wild-type phenotype (green leaves and normal leaf shape). A total of 161 plants of the F₂ progeny DM19 segregated into four distinct phenotype classes (**Figure 1A**). The class I plants represent the wild-type of the cultivated carrot. The phenotype class II is highly similar to the wild-type, but the chlorophyll biosynthesis is dramatically delayed resulting in yellowish leaves (*yel*—mutant). Compressed above-ground plant tissues resulting in a semi-dwarf phenotype are the characteristics of the class III plants (*cola*—mutant). Plants of the phenotype class IV showed the characters of both mutants, semi-dwarf plants with yellowish leaves. The visual characters of the roots (size, shape, and color) were not influenced for the different phenotype classes. There was no significant deviation from Mendelian inheritance observed (ratio 9:3:3:1) for two independent loci (**Table 2**). After vernalization 121 F₂ plants (75%) flowered within a 100 day period. All plants of the phenotype classes I and II (*n* = 98) developed epigynous flowers, whereas all *cola*-mutant associated plants (phenotype class III and IV; *n* = 23) expressed hypogynous flowers (**Table 2**; **Figures 1B,C**).

Table 2 | Phenotypical segregation of flower traits in the F₂ progeny DM19.

| Phenotypic class | No. of plants ^a | Observed phenotype | Putative genotype | Bolting plants ^b | Flower development epigynous/hypogynous |
|------------------|----------------------------|------------------------|---------------------|-----------------------------|-----------------------------------------|
| I | 90 | Green / normal leaves | YEL. / COLA. | 77 (86%) | 77/0 |
| II | 32 | Yellow / normal leaves | yel yel / COLA. | 21 (66%) | 21/0 |
| III | 31 | Green / cola leaves | YEL. / cola cola | 19 (61%) | 0/17(2) ^c |
| IV | 8 | Yellow / cola leaves | yel yel / cola cola | 6 (75%) | 0/6 |

^aFit for Mendelian digenic inheritance χ^2 - (9:3:3:1, phenotype classes) = 0.56; $p = 0.91$.

^bFit for Mendelian monogenic inheritance χ^2 - (3:1, Bolting: non bolting) = 0.68; $p = 0.40$; Ratio of bolting plants in the cola classes (III+IV) was significantly lower (64%, $p = 0.001$) than in the WT (COLA) classes (I+II) with 80%.

This suggested a potential association to the cola mutant, but a verification by testing of a larger progeny is required;

^cTwo plants expressed partially both types of flowers.

Table 3 | Microspore characteristics of the *cola*-mutant in comparison to the wild-type.

| Flower type | n | Pollen viability ^a (%) Mean ± SD | S+U Pollen ^b (%) Mean ± SD | Size of viable microspores ^c | |
|----------------------------------|----|------------------------------------------------|------------------------------------------|-----------------------------------------|--------------|
| | | | | Length (μm) | |
| | | | | Mean ± SD | Mean ± SD |
| Wild-type (epigynous) | 10 | 68.67 ± 6.89 a | 1.36 ± 2.05 a | 32.4 ± 5.1 a | 16.2 ± 3.1 a |
| <i>cola</i> -mutant (hypogynous) | 10 | 49.88 ± 14.75 b | 9.16 ± 5.67 b | 28.3 ± 4.2 b | 15.5 ± 3.2 a |

n, tested plants, for each plant 400 pollen were evaluated.

^aUniformly green-yellow stained pollen.

^bNumber of small (S) and underdeveloped (U) pollen.

^cTwenty fertile microspores of each were measured. Different letters in a column indicate significant differences ($p < 0.05$; t-test).

Reduced pollen viability was observed in *cola*-mutant plants in comparison to the wild-type plants (49.9 and 68.7%, respectively). The proportion of small and undeveloped pollen grains was much higher and also the viable pollen grains were significantly smaller than those of the wild type flowers (Table 3; Figure 1D).

LINKAGE ANALYSIS

After a pre-screening experiment using the parental plants, 21 RAPD primers, 2 dRAPD, 28 AFLP, and 7 td primer combinations were chosen for the analysis of the segregating population. In total, 319 markers were polymorphic between the parents of DM19 and were used for map construction (Table 4). Nine major groups encompassing 285 markers were generated. The resulting carrot linkage map (Figure 2) had a total length of 781 cM. The number of markers per LG ranged from 18 to 45. The largest minor group, not connected with one of the nine major groups, contained five markers. LG-1 was the largest LG with 145 cM, while LG-7 was the shortest with 66 cM. The average distance between two neighboring markers was 2.7 cM.

ASSIGNMENT OF LINKAGE GROUPS TO THE REFERENCE MAP

The use of SSR and SCAR markers enabled the LGs of our carrot map to be assigned to the LGs of two reference maps. The original LG names were proposed by Iovene et al. (2011) and we assigned four markers (DcOR, Y2mark, Q1/800, and SSR-N6W93) anchored in their physical carrot map. The LG names were adopted by Cavagnaro et al. (2011) and we were able to

Table 4 | Summary of mapped markers for the nine linkage groups of the carrot map.

| Marker | AFLP | RAPD/ dRAPD | td | SSR | EST/ SCAR | Mapped traits | Total |
|-----------------------|------------|----------------|---------|----------|--------------|---------------|------------|
| Polymorphic mapped | 216 198 | 60 45 | 10 9 | 22 22 | 9 9 | 2 2 | 319 285 |
| LG-1 | 28 | 6 | 2 | 3 | 0 | YEL | 40 |
| LG-2 | 36 | 3 | 3 | 1 | 0 | 0 | 43 |
| LG-3 | 30 | 5 | 0 | 2 | 1 | 0 | 38 |
| LG-4 | 27 | 2 | 1 | 2 | 1 | COLA | 34 |
| LG-5 | 11 | 4 | 1 | 1 | 1 | 0 | 18 |
| LG-6 | 20 | 8 | 1 | 2 | 1 | 0 | 32 |
| LG-7 | 11 | 6 | 1 | 2 | 2 | 0 | 22 |
| LG-8 | 22 | 6 | 0 | 2 | 1 | 0 | 31 |
| LG-9 | 13 | 5 | 0 | 7 | 2 | 0 | 27 |

map 15 SSRs from LGs 1-9. Two additional SSR markers (gSSR91 and gSSR138) were allocated by Alessandro et al. (2013). The distribution of these 21 markers was generally consistent with the reference maps. Mapping of two or more markers in each LG allowed drawing of seven LGs in the orientation corresponding to that applied by the authors of the reference maps with short/long chromosome arms in the North/South orientation. The orientation of LG-2 and LG-5 remained ambiguous, as each of them was anchored only by one reference marker. Marker

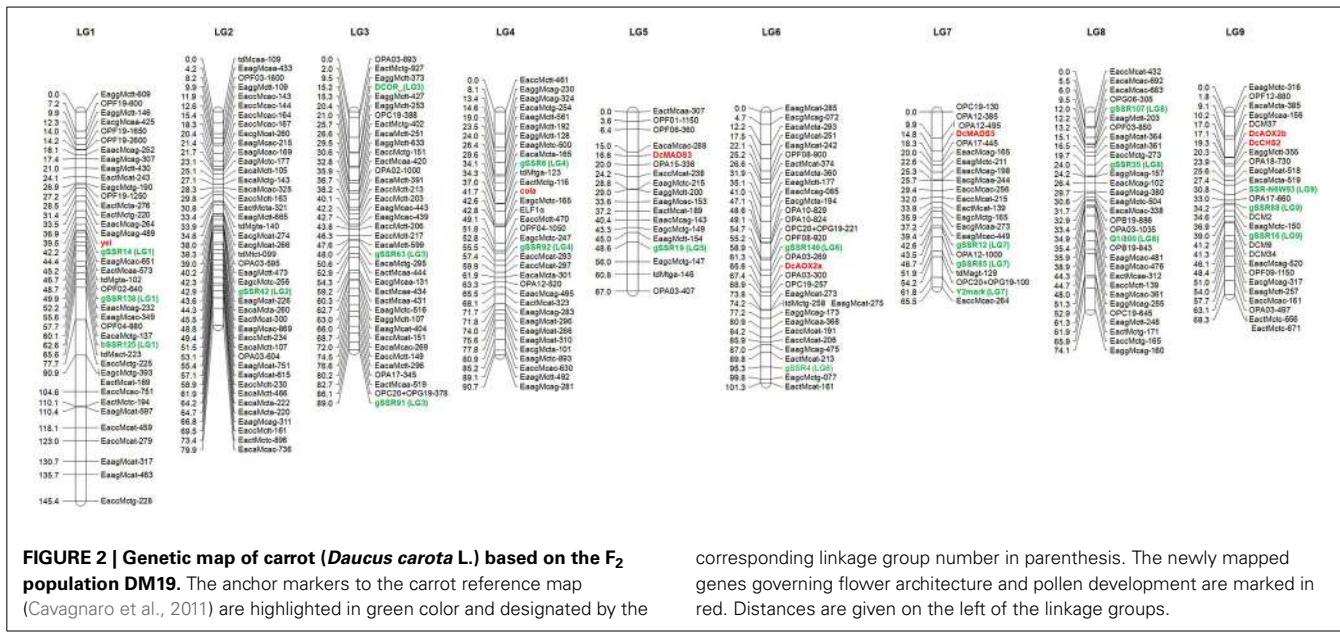


FIGURE 2 | Genetic map of carrot (*Daucus carota* L.) based on the F₂ population DM19. The anchor markers to the carrot reference map (Cavagnaro et al., 2011) are highlighted in green color and designated by the

gSSR138 was found to be located in our map between gSSR14 and bSSR125 and not distal from gSSR14 as shown by Alessandro et al. (2013).

MAPPING GENES RESPONSIBLE FOR FLOWERING TRAITS

The *COLA*-locus was mapped successfully on LG-4 and the *YEL*-locus on LG-1 of the carrot map. To analyze, whether the flower-specific *cola*-mutant reveal any relationship to one of the flower-specific genes, known to play a prominent role for reproductive organ differentiation and pollen development (Gene-Specific Markers and **Table 1**), our aim was their preliminary assignment to appropriate LGs. Therefore, we have searched for polymorphisms between the parents of our mapping population. Although phenotypic segregation and our genetic data (compare to PHENOTYPE SEGREGATION) did not favor any association, we have included the data obtained. We have identified sequence polymorphisms for *DcMADS3* and *DcMADS5* as well as *DcAOX2a*, *DcAOX2b*, and *DcCHS2* (**Table 1**) and mapped them to search for co-localization with the *COLA*-gene.

All of the analyzed gene-fragments revealed a different distribution than the *COLA*-locus, which indicated that there were no associations to the flower-specific defect that has been described here. *DcMADS3* belonging to the B-class of homeotic genes was assigned to LG-5 and *DcMADS5*, a predicted member of the SEPALLATA-group of MADS-box genes, was mapped after cleavage of the amplification product with *AluI* to LG-7. For *DcAOX2a* a polymorphism was found concerning presence/absence of the fragment specific for the L-allele which was used successfully to assign it to LG-6. In our mapping analyses the polymorphism associated with *DcAOX2b* was assigned to LG-9 indicating a different localization than the *DcAOX2a* sequence.

For the *CHS* gene, the length of the obtained fragment polymorphisms corresponded with the predicted size deduced from the genomic sequence of *CHS2* in the *cola*-parent (900 bp),

corresponding linkage group number in parenthesis. The newly mapped genes governing flower architecture and pollen development are marked in red. Distances are given on the left of the linkage groups.

whereas the fragment-length was slightly shortened in the *yel*-mutant (850 bp).

Despite of this tentative assignment, future work demands a more detailed analysis. Fine-mapping is still required to assign precisely the allelic state and yet undetected potential differences of intron-sizes, like InDels of smaller sizes that could not have been matched in this initial map. For example, a tool for subfamily grouping of the large *AOX* family has been developed, where classification (or re-classification) of *AOX*-subfamily members by specific sequence-features was facilitated (Costa et al., 2014). In summary, all of the five proven genes did not co-segregate with the *cola*-associated flower defects we mentioned in this publication.

DISCUSSION

To date, a complete carrot genome sequence was not yet available, thus well-saturated genetic linkage maps are of essential relevance for successful carrot breeding and breeding research. Cavagnaro et al. (2011) have developed a linkage map containing 55 SSR markers, offering an important tool for further integration of molecular markers and gene-specific sequences of carrot. Currently, mapping of genes governing traits of economical relevance (e.g., flavors, volatiles and bioactive compounds) in individual well-characterized populations segregating for gene-specific markers is under investigation (Nothnagel, pers. commun.). However, SSR markers offer a useful tool to integrate the results into a consensus map. The total length of the carrot map presented in this work was 781 cM, compared to 669 cM of the map shown by Alessandro et al. (2013), Just et al. (2007), and Cavagnaro et al. (2011) earlier published separated maternal and paternal maps with a total length between 1050 and 1273 cM. The marker density of our map was nearly as high as that presented by Alessandro et al. (2013) and the largest gap was 14 cM. Using SSRs as anchor markers, the LGs of our carrot map matched exactly to the LGs of the reference maps. For seven of the nine LGs at least two anchor markers were allocated

enabling us to determine the orientation of the LGs in comparison to the reference maps. Thus, we showed that 21 markers included in the reference maps were suitable for linkage analysis performed on the DM19 population. The carrot populations used for map generation by Cavagnaro et al. (2011), Alessandro et al. (2013) and our group were unrelated and had different pedigrees. Therefore the defined marker set is of fundamental importance for independent mapping projects, as it facilitates the creation of a consensus map.

The *cola*-mutation revealed a compact leaf-habit and a semi-dwarf phenotype that is associated to a reduced cell elongation. Moreover, flowering traits were also affected including an impaired transition to flowering, and abnormal flower architecture with partial male sterility (Nothnagel et al., 2005, this study). The phenotype was inherited in a monogenic recessive fashion causing hypogynous flowers in homozygous state which could be clearly distinguished from epigynous flowers of the wild-type. Additionally, the distorted anther development resulted in lower pollen viability probably due to an impaired development of the sporophytic tissue that surrounded the pollen chambers. The *COLA*-locus was mapped on LG-4.

Although phenotype classification and genetic segregation did not favor the hypothesis, that the *COLA*-locus is related to one of the obtained polymorphisms derived from flower-specific sequences, we have included several of the obtained polymorphisms into our map regarding future aims to extend analyses on flower- and reproductive genes in the carrot. The initiation of flowering and the patterning of floral primordia into discrete domains that give rise to different types of floral organs have been well investigated in model plants. Flower development depends on a complex gene regulation network (Immink et al., 2010; Liu and Mara, 2010). Most of the participating genes encode for transcription factors involved in the regulation of gene expression in a strictly hierarchical manner. A vast majority of the central regulating genes belong to the MADS-box family. Carrot MADS-box genes *DcMADS3* and *DcMADS5* belong to the B-class and E-class MADS-box genes specifying the identity of stamens and hence, the development of anthers and pollen. *DcMADS3* likely plays a similar role in *Daucus* as shown for *Arabidopsis* and *Antirrhinum*. This was supported by the fact that *DcMADS3* was down-regulated in homeotic flowers of the carpeloid CMS type of carrot where stamens were completely replaced by carpels (Linke et al., 2003). For *DcMADS5*, a significant sequence similarity to SEPALLATA1 group and a continuous expression throughout flower development supported the hypothesis that the *SEP1* gene was required from early stages of floral development onwards to mediate activities especially of the B- and C-organ identity genes (Flanagan and Ma, 1994; Pelaz et al., 2000; Honma and Goto, 2001). In the present work, the two carrot MADS-box sequences *DcMADS3* and *DcMADS5* were assigned to LG-5 and LG-7, respectively.

Campos et al. (2009) have shown differential expression of *DcAOX1* and *DcAOX2* genes at early stages of floral organ formation. As reported by Cardoso et al. (2009), *AOX2a* was successfully analyzed in a preliminary mapping approach of the DM19 population. In the present work, obtained polymorphisms for *AOX2a* and *AOX2b* were assigned to the LGs LG-6 and LG-9, respectively.

Taylor and Jorgensen (1992) demonstrated that the development of the male gametophyte essentially required flavonoid biosynthesis. Hence, CHS deficient plants (with so-called white pollen) were self-incompatible. Pollen germination on the stigmata could be restored when a small amount of kaempherol was provided (Mo et al., 1992). In this study, a *CHS* sequence of *D. carota* was assigned to LG-9.

In conclusion, a well-saturated map of carrot was developed. The *cola*-locus, associated to advanced defects of male and female organ differentiation was associated to LG-4 and revealed no cosegregation with several markers for genes involved in flowering and reproduction. Hence, six loci significant for these processes were mapped to five LGs. Considering two other genes, *Vrn1*, responsible for early flowering habit and *Rf1* restoring petaloid CMS, have been assigned by Alessandro et al. (2013) to LG-2 and LG-9, respectively, it can be assumed that eight loci associated with flower architecture and reproduction are dispersed to six out of nine carrot chromosomes. Indeed, fine-mapping of the identified loci will be required, especially regarding the growing number of subgroup members within the selected gene-families that are not yet completely available in the database. Several tools for structural analyses of high diversity of gene families, like the compositions of the *AOX* genes might facilitate a detailed analysis also in non-model plants (Costa et al., 2014).

Finally, the use of anchor markers that exclusively derived from the American gene pool, enables the generation of the first integrated carrot map based on markers of both, the European and American carrot gene pool. This opens advanced options for the establishment of a comprehensive consensus map by co-operative activities between the research groups working on carrot genetics. Furthermore, the addition of gene-derived markers to the present map may provide a good starting point for comparative mapping in other Apiaceae species like *Apium*, *Petroselinum*, *Carum*, or *Foeniculum* for which *Daucus* can serve as well-characterized representative. This might facilitate the future research on genes associated with traits important for both, food and non-food sector (Barańska et al., 2005; Ulrich et al., 2011) and allows the identification of genes with general interest for Apiaceae species.

AUTHOR CONTRIBUTIONS

Holger Budahn: SSR analysis and map construction. Rafał Barański: AFLP and SSR analysis. Dariusz Grzebelus: td analysis. Agnieszka Kiełkowska: RAPD and AFLP analysis. Petra Straka: RAPD and AFLP analysis. Kai Metge: RAPD and AFLP analysis. Bettina Linke: Primer design MADS-box genes, PCR-analyses of parental plants of the mapping population, substantial contributions to conception and design of the manuscript. Thomas Nothnagel: Generation of the mapping population, flower morphology, histological analysis, substantial contributions to conception and design of the manuscript.

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SUPPLEMENTARY MATERIAL

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