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ORIGINAL PAPER

Diversification of three *APETALA1/FRUITFULL*-like genes in wheat

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Abstract The genomes of grass family species have three paralogs of *APETALA1/FRUITFULL* (*AP1/FUL*)-like genes (*FUL1*, *FUL2* and *FUL3*) that are derived from the *FUL* lineage. In this study, we focus on the different roles of the wheat *AP1/FUL*-like genes, *WFUL1* (identical to *VRN1*), *WFUL2* and *WFUL3*, during the transition from vegetative to reproductive growth. Sequence analysis indicated that there was a high level of variability in the amino acid sequence of the C-domain among three *WFUL* genes. Expression analyses using the spring wheat cultivar Chinese Spring indicated that *WFUL1/VRN1* was expressed in leaves as well as spike primordia of non-vernalized plants at the vegetative stage just before phase transition, while *WFUL2* and *WFUL3* were not expressed in leaves. This result indicates that *WFUL1/VRN1* performs a distinct role in leaves before phase transition. In young spikes, *WFUL1/VRN1* and *WFUL3* were expressed in all developing floral organs, whereas *WFUL2* expression was restricted in the floral organs to the lemma and palea. Furthermore, yeast two-hybrid and three-hybrid analyses revealed that WFUL2, but not WFUL1/VRN1 or WFUL3, interacted with class B and class E proteins. These results suggest that *WFUL2* of wheat has class A functions in specifying the identities of floral meristems and outer floral organs (lemma and palea) through collaboration with class B and class E genes.

Keywords *APETALA1/FRUITFULL* · Flowering · Class A function · Wheat (*Triticum aestivum*)

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Introduction

Flowering is a phase transition from vegetative to reproductive growth in higher plants, and is controlled by vernalization, photoperiod, and autonomous genetic pathways (Wellmer and Riechmann 2010). In temperate cereals such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), heading-time associated with the timing of flowering is an important character because of its influence on adaptability to different environmental conditions. Vernalization requirement is concerned with the sensitivity of the plant to cold temperature for accelerating spike primordium formation, and is mainly determined by the major genes *VRN1*, *VRN2* and *VRN3* in temperate cereals (Distelfeld et al. 2009). Despite the fact that they have the same gene symbol, *VRN1* and *VRN2* of temperate cereals are not related to *VRN1* and *VRN2* of *Arabidopsis*. Yan et al. (2003) isolated *VRN1* in diploid einkorn wheat *T. monococcum* using a map-based method, and showed that *VRN1* has a high sequence similarity to *APETALA1* (*AP1*)/*FRUITFULL* (*FUL*) of *Arabidopsis*. Subsequently, *VRN1* of hexaploid wheat was identified as *WAP1* (*wheat AP1*) (Murai et al. 2003; Trevaskis et al. 2003) or *TaVRT-1* (*Triticum aestivum vegetative to reproductive transition-1*) (Danyluk et al. 2003). The *VRN2* locus consists of two similar genes (*ZCCT* genes) that encode proteins with a putative zinc finger and a CCT domain and act as a flowering repressor (Yan et al. 2004). There are no clear homologs

of *VRN2* in the *Arabidopsis* genome. *VRN3* has high sequence similarity to *FLOWERING LOCUS T* (*FT*) of *Arabidopsis* (Yan et al. 2006). Therefore, *VRN3* is also called *FT1* in barley (Hemming et al. 2008) and *WFT* (*wheat FT*) in wheat (Shimada et al. 2009). *Arabidopsis FT*, which encodes a protein similar to animal Raf kinase inhibitor-like protein, functions as a flowering promoter (Kardailsky et al. 1999; Kobayashi et al. 1999). The FT protein acts as a systemic signaling molecule from leaf to apex (Corbesier et al. 2007; Jaeger and Wigge 2007; Mathieu et al. 2007). At the apex, the FT protein interacts with the bZIP transcription factor FD to activate the floral meristem identity genes *AP1* and *LEAFY* (*LFY*) (Abe et al. 2005; Wigge et al. 2005). Expression analyses and a transgenesis study indicated that wheat *VRN3* has a similar function as *Arabidopsis FT* (Yan et al. 2006; Shimada et al. 2009).

In wheat and barley, *VRN2* and *VRN3* are preferentially expressed in leaves (Yan et al. 2004, 2006; Sasani et al. 2009), while *VRN1* is expressed in both leaves and shoot apical meristems (SAMs) (Yan et al. 2003; Sasani et al. 2009). These expression patterns suggest that the genes might cooperate in the up-regulation of florigenic VRN3 proteins in leaves. VRN3 proteins are transported from the leaves to the SAMs, where they interact with *VRN1* to induce flowering (Wellmer and Riechmann 2010; Trevaskis 2010). The wheat functional homologue of *Arabidopsis* FD, TaFDL2 protein, can interact with the VRN3 protein and bind in vitro with the promoter region of *VRN1* (Li and Dubcovsky 2008), supporting the idea that the VRN3 protein interacts with *VRN1* in SAMs. In *Arabidopsis*, *AP1* has two (class A) functions in SAMs: the first is the specification of floral meristem identity, and the second is the specification of sepal and petal identities (Bowmann et al. 1993). Transgenesis, mutation and expression studies suggest that *VRN1* functions in the specification of floral meristem identity in wheat SAMs (Yan et al. 2003; Murai et al. 2003; Loukoianov et al. 2005; Shitsukawa et al. 2007a; Adam et al. 2007). However, it is unclear whether *VRN1* has the second class A function, that is, specification of floral organ identity.

Arabidopsis has two genes, *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*) that are closely related to *AP1* (Ferrandiz et al. 2000). *CAL* has some redundant functions with *AP1* in specifying floral meristem identity, but *CAL* is unable to substitute for *AP1* in specifying sepal and petal identity. The different roles of *AP1* and *CAL* during floral meristem formation and floral organ development are associated with differences in the amino acid sequences of the AP1 and CAL proteins in the K-domain (Alvarez-Buylla et al. 2006). Although expression of *AP1* is restricted to SAMs, *FUL* is expressed in leaves and SAMs. *FUL* is known to function in fruit dehiscence (Ferrandiz et al. 2000). More recently, it has been reported that *FUL* both specifies floral meristems and plays a key role in determining the herbaceous growth form in *Arabidopsis* (Melzer et al. 2008).

The grass family genome has three paralogs of *AP1/FUL*-like genes, namely, *FUL1* (corresponding to *VRN1* in temperate cereals), *FUL2* and *FUL3*, which are all derived from the *FUL* lineage (Litt and Irish 2003). Expression analyses in oat and einkorn wheat have shown that both *FUL1* and *FUL2* are expressed in leaves and SAMs (Preston and Kellogg 2008). Up-regulation of *FUL1* occurs significantly later in SAMs than in leaves of vernalized plants, suggesting that *FUL1* may perform discrete roles in leaves and SAMs. *FUL2* expression also increases in SAMs following the attainment of flowering initiation, suggesting that *FUL2* functions redundantly with *FUL1* in SAMs for transition to flowering. Although subfunctionalization of *FUL2* was hypothesized to specify floral organ identity (Preston and Kellogg 2007), it is still unclear whether *FUL1* and *FUL2* have distinct roles in SAMs.

In this study, we focus on the functional diversification in three paralogs of *AP1/FUL*-like genes, *WFUL1* (which is identical to *VRN1*), *WFUL2* and *WFUL3*, in the spring bread wheat cultivar Chinese Spring (CS). CS wheat shows early flowering without vernalization, although vernalization does moderately accelerate flowering time. Expression analyses in non-vernalized CS plants indicated that *WFUL1/VRN1* performs a distinct role in leaves before phase transition, probably the up-regulation of *VRN3*. In situ expression analyses, yeast two- and three-hybrid analyses, and transgenic study using *Arabidopsis* system indicated that *WFUL2* has the class A functions of specifying the identities of the floral meristem and outer floral organs (lemma and palea) through collaboration with class B and class E genes.

Materials and methods

Plant materials

Bread wheat (*Triticum aestivum*, $2n = 6x = 42$, genome constitution AABBDD) cv. Chinese Spring (CS) was used for cDNA cloning and expression analyses of *WFUL1/VRN1*, *WFUL2* and *WFUL3*. CS is a spring wheat cultivar and is known to carry the vernalization-insensitive (spring habit) gene, *VRN-D1*, and the photoperiod-insensitive gene, *Ppd* (McIntosh et al. 1998). CS plants flower without vernalization, although vernalization does moderately accelerate flowering time. *Arabidopsis thaliana* ecotype Columbia was used for the experiments on ectopic expression of *WFUL2*.

Phylogenetic analysis

Multiple amino acid sequence alignment was carried out using the program CLUSTAL W (Thompson et al. 1994) with a BLOSUM matrix (gap open penalty, 10; gap extension penalty, 0.2; gap distance, 8), and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). Support values for nodes on the tree were estimated with 1,000 bootstrap replicates (Felsenstein 1985). The programs used here were provided by the DNA database of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/search/clustalw-e.html). The accession numbers of *WFUL1/TaMADS11/VRN1*, *WFUL2/WM8* and *WFUL3/WM7* are AB007504, AM502871 and AM502870, respectively. The accession numbers of other genes in the phylogenetic tree are as follows; *HvMADS5/BM5* (AJ249144), *HvMADS8/BM8* (AJ249146), *HvMADS3/BM3* (AJ249143), *OsMADS14* (AF058697), *OsMADS15* (AF058698), *OsMADS18* (AF091458), *LtMADS1* (AF035378), *LtMADS2* (AF035379), *AsFUL1a* (DQ792965), *AsFUL2* (DQ792967), *ZmMDS4* (AJ430641) and *ZAP1* (L46400).

Growth conditions

The RT-PCR expression analyses were carried out using spikes at various growth stages and floral organs of CS wheat plants grown in the experimental field. The real-time PCR analysis used non-vernalized CS plants at the 3-leaf and 5-leaf stages maintained in a growth chamber under long-day conditions (16 h light/8 h dark, light intensity 100 μE m^{-2} s^{-1}) at 20°C. The CS plants at the 3-leaf stage were in the vegetative growth phase. The CS plants at the 5-leaf stage were also in vegetative growth phase, but just before phase transition. Phase transition from vegetative to reproductive growth usually occurs between the 5-leaf and 6-leaf stages in plants kept under long-day conditions in a growth chamber. Transgenic *Arabidopsis* plants were maintained in a growth chamber under short-day conditions (10 h light/14 h dark, light intensity 100 μE m^{-2} s^{-1}) at 22/20°C.

RT-PCR analysis

Total RNAs were isolated from spikes of CS plants at various developmental stages (less than 3, 3–10, 10–15 and 15–25 mm in length, and from flag leaf unfolding to heading stages) using ISOGEN (Nippon-gene, Japan); they were also isolated from floral organs (glume, lemma, palea, stamen and pistil) at the reproductive heading stage. Spikes and floral organs were obtained from three or more plants, and total RNA was isolated from each combined sample. Ten or more plants were needed to obtain sufficient tissue for RNA isolation from young spikes of 10 mm or less in length. DNase-digested total RNA (5 μg) was reverse-transcribed using an oligo-dT primer and first-strand cDNA was obtained using a first-strand synthesis RT-PCR kit (GE Healthcare Biosciences). The following PCR primers were designed using the appropriate gene sequences: for *WFUL1/VRN1*, WFUL1-L (5′-GGAGAGGTCACTGCAGGAGGA-3′) and WFUL1-R (5′-GCCGCTGGATGAATGCTG-3′); for *WFUL2*, WFUL2-L (5′-CAACCCCAAGCCCAGACCA-3′) and WFUL2-R (5′-GGGCAAGCAAACATAACCATAGCAG-3′); for *WFUL3*, WFUL3-L (5′-TCGCGCTCATCGTCTTCTC-3′) and WFUL3-R (5′-TTCTTTTGTGGTGAGTGGGTCC-3′). Annealing was carried out at 65°C for these primer sets. As a control, a fragment from the wheat actin gene (*ACTIN*) was amplified using the primers actin361-L (5′-TATGCCAGCGGTCGAACAAC-3′) and actin361-R (5′-GAACAGCACCTCAGGGCAC-3′) at an annealing temperature of 58°C. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed. Each PCR assay was performed twice with either 27 or 30 cycles. Invariably, the band intensity was lower in the PCR with the smaller number of cycles, indicating that this PCR must still have been in the exponential range of amplification.

Real-time PCR analysis

Real-time PCR analyses were performed using a LightCycler 2.0 (Roche Diagnostics GmbH) with the same gene-specific primer sets for *WFUL1/VRN1*, *WFUL2*, and *WFUL3* as those used in RT-PCR analysis. Total RNAs were isolated from young spikes and leaves of non-vernalized CS plants at the 3-leaf and 5-leaf stages, and the cDNAs were used in the analysis. Shoot apices (SAMs) were collected 1 h before the beginning of the dark period (during light period). The leaves were sampled 1 h before and 1 h after the beginning of the dark period (during light and dark periods, respectively) because it has been reported that *VRN1* shows a diurnal expression pattern (Shimada et al. 2009). SAMs and leaves were sampled from a minimum of ten plants in the growth chamber. Transcript yields were quantified by SYBR Green fluorescence labeling and comparison with *ACTIN* amplified using the primer set actin361-L and actin361-R. The template cDNAs were amplified with Brilliant SYBR Green QPCR Master Mix using the same primer sets for *WFUL1/VRN1*, *WFUL2* and *WFUL3* as in the RT-PCR experiment; an annealing temperature of 65°C was used. Data were analyzed using Stratagene MXPRO ver. 3.0 software (Agilent Technologies).

In situ hybridization analysis

In situ hybridization was performed using the method described previously (Shitsukawa et al. 2006). SAMs at the

vegetative stage, spike primordial, and young spikes at different developmental stages (from just before the double-ridge stage to the late floral organ differentiation stage) were sampled from CS plants and fixed with FAA solution (3.7% paraformaldehyde, 5% acetic acid, 50% EtOH) at 4°C overnight. The fixed tissues were dehydrated and embedded in Paraplast Plus (Oxford Labware, USA). The tissues were cut into 20-μm sections and dried overnight. Hybridization was carried out overnight at 52°C. DIG-labeled RNA probes were synthesized by in vitro transcription using a DIG RNA labeling kit (Roche Diagnostics GmbH) from plasmids containing *WFUL1/VRN1*, *WFUL2* or *WFUL3* cDNA. After hybridization, the sections were washed twice with 0.5× SSC at 52°C. Immunological detection of the hybridized probe was performed as described by Hama et al. (2004). As controls for specificity, consecutive sections were hybridized with sense and antisense probes of the same region of the *WFUL* genes.

Scanning electron microscope (SEM)

SEM analysis was performed using the method described previously (Shitsukawa et al. 2009). A low-vacuum scanning electron microscope (SEM) (S-3000 N, Hitachi Co. Ltd. Japan) was used to observe the morphological features of the inflorescence. SAMs and young spikes were chilled to −15°C on a cooled stage and examined under low-vacuum conditions (30 Pa) and an accelerating voltage of 15 or 20 kV.

Yeast two- and three-hybrid assays

Yeast two- and three-hybrid assays were performed using the methods described previously (Shitsukawa et al. 2007b). For the yeast two-hybrid assay, the Gal4 Two-Hybrid Phagemid Vector Kit (Stratagene) was used to investigate protein–protein interactions among wheat MADS-box genes. The vector pBD-Gal4 was used to clone the entire ORF sequence of wheat class B MADS-box genes (*WAP3*, *WPI-1*, *WPI-2*) (Murai et al. 1998; Hama et al. 2004), class E genes (*WSEP*, *WLHS1*) (Shitsukawa et al. 2007b) and *WFUL* genes (*WFUL1/VRN1*, *WFUL2*, *WFUL3*) into *Eco*RI and *Sal*I sites. The entire ORF sequences of *WFUL* genes were cloned into the *Eco*RI and *Sal*I sites of pAD-GAL4-2.1. Ternary complex formation was studied with a pBridge vector (CLONTECH), which expresses a DNA-binding domain fusion and an additional protein. The complete *WAP3* coding sequence was cloned into MCS1 to generate a hybrid protein that contains the sequences for the GAL4 DNA-binding domain; *WPI-1* or *WPI-2* was cloned into MCS2 to generate a third protein, respectively. All constructs were sequenced and then transformed into the yeast strain *YRG2*, which has *His3* and *LacZ* reporter genes, using the *S. cerevisiae* Direct Transformation Kit (Wako, Japan). Double transformants were grown on selective medium and tested by histidine prototrophy.

Transgenic study using Arabidopsis system

WFUL2 cDNA was inserted into the binary vector pIG121 with the cauliflower mosaic virus (CaMV) 35S promoter (P_{35S}) for overexpression in *Arabidopsis*. The binary vector was used to transform *Agrobacterium tumefaciens* GV3101. *Arabidopsis thaliana* ecotype Columbia (Col) plants were then transformed using the floral-dip method (Clough and Bent 1998). T_1 seeds were placed onto MS agar plates with 50 μg ml^{-1} kanamycin as the selection agent. Agar plates were placed at 4°C for 3 days to break dormancy, and then at 20°C in growth chambers under short-day conditions (10 h light/14 h dark) with a light intensity of 100 μE m^{-2} s^{-1}. T_1 seedlings at the 3-leaf stage were transplanted into soil and kept under the same temperature and light regime. As a control, *GUS* (beta-glucuronidase) cDNA was introduced into *Arabidopsis* plants using the same method. Flowering time was determined by counting the total number of rosette leaves on the main shoot at the bolting stage in three transgenic plants of each type, i.e., P_{35S}::*WFUL2* and P_{35S}::*GUS*.

Results

Sequence analysis of three *APETALA1/FRUITFULL*-like genes in wheat

A wide range of grass family members possess three copies of *AP1/FUL*-like genes, *FUL1*, *FUL2* and *FUL3*, that derive from two gene duplication events (Preston and Kellogg 2006). In wheat, *VRN1* is anticipated to be a *FUL1* paralog (*WFUL1*) based on sequence similarity and expression analysis (Preston and Kellogg 2006). To identify other wheat *FUL* genes, we screened approximately 900,000 EST (expressed sequence tag) contigs, developed by the Japanese Wheat Genomics Consortium (Ogihara et al. 2003), using a BLASTN search with *VRN1* query. This search identified two EST contigs, whyd7c05 and whflp23, as candidate *AP1/FUL*-like genes in wheat. Sequence analysis indicated that whyd7c05 is identical with *WM8* (accession no. AM502871) and whflp23 is identical with *WM7* (accession no. AM502870), which were reported by Paolacci et al. (2007).

To determine the relationship between wheat *AP1/FUL*-like genes and other grass *FUL* genes, a phylogenetic tree was constructed using amino acid sequences (Fig. 1). The phylogenetic analysis indicated that the grass *FUL* genes could be separated into three subclades, the *FUL1*, *FUL2*

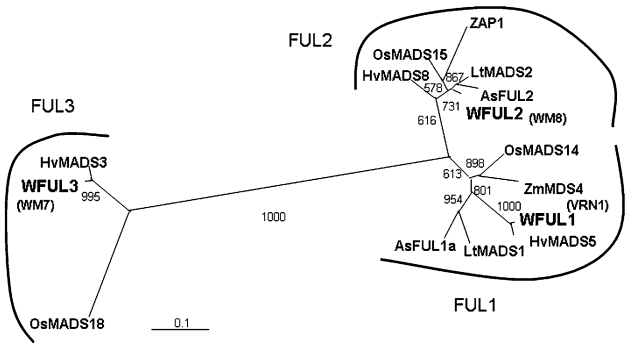

Fig. 1 Phylogenetic tree of *AP1/FUL*-like genes in monocots. The phylogenetic tree was constructed by the neighbor-joining method using deduced amino acid sequences. The *numbers* at the node show bootstrap values after 1,000 replicates. *WFUL1*, *WFUL2* and *WFUL3* are identical to the previously reported *VRN1*, *WM8* and *WM7*, respectively. Accession numbers of genes are given in the "Materials and methods"

and *FUL3* subclades, in agreement with a previous report (Preston and Kellogg 2006). The EST contig whyd7c05 (*WM8*) fell into the *FUL2* subclade and whflp23 (*WM7*) was a member of the *FUL3* subclade. This suggests that *WM8* and *WM7* are orthologous to grass *FUL2* and *FUL3* genes, respectively. In this study, therefore, we rename *WM8* as *wheat FUL2* (*WFUL2*) and *WM7* as *wheat FUL3* (*WFUL3*) in recognition of the nature of the genes. Furthermore, *VRN1* was classified into the *FUL1* subclade and is also called *WFUL1* in this report.

Multiple amino acid sequence alignments of WFUL1/VRN1, WFUL2 and WFUL3 showed that they had high amino acid sequence similarity in their MADS-box domains, and moderate similarity in their K-box domains (Fig. 2). There was a high level of variability in the C regions of the WFUL proteins; these various differences are illustrated in Fig. 2.

Expression levels of *FUL* genes in SAMs and leaves of non-vernalized spring wheat

The expression levels of the *WFUL* genes in SAMs and leaves were compared by real-time PCR analyses using specific primer sets for *WFUL1/VRN1*, *WFUL2*, and *WFUL3*. cDNAs were obtained from SAMs and leaves at the 3-leaf and 5-leaf stages of non-vernalized CS plants (Fig. 3). At the 3-leaf stage, the SAM is a vegetative meristem (Fig. 3a), while at the 5-leaf stage, it has formed an elongated vegetative meristem in preparation for transition into an inflorescence meristem (Fig. 3b). This indicates that

Fig. 2 Alignment of the deduced amino acid sequences of the three *WFUL* genes. Sequences were obtained from the DDBJ database and aligned using CLUSTAL W. Amino acid residues that are identical in all sequences are indicated by *asterisks*, and those that are similar are marked by *dots*. A *dash* indicates a gap inserted to maximize alignment. The MADS-domain, K-domain and C-domain are indicated. Accession numbers of genes are given in the "Materials and methods"

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                                                MADS-domain
                                    ═══════════════════════════════════════════
WFUL1 (VRN1)    MGRGKVQLKRIENKINRQVTFSKRRSGLLKKAHEISVLCDAEVGLIIFSTKGKLYEFST-
WFUL2           MGRGKVQLKRIENKINRQVTFSKRRNGLLKKAHEISVLCDAEVAVIVFSPKGKLYEHAT-
WFUL3           MGRGPVQLRRIENKINRQVTFSKRRSGLLKKAHEISVLCDAEVALIVFSTKGKLYEYSSQ
                **** ***:***************.*****************.:*:**.******.::
                                                                          .....
WFUL1 (VRN1)    ESCMDKILERYERYSYAEKVLVSSESEIQGNWCHEYRKLKAKVETIQKCQKHLMGEDLES
WFUL2           DSSMDKILERYERYSYAEKALISAESESEGNWCHEYRKLKAKIETIQKCHKHLMGEDLDS
WFUL3           DSSMDVILERYQRYSFEERAVLDPSIGNQANWGDEYGSLKIKLDALQKSQRQLLGEQLDP
                :*.** *****:***: *:.::...   :.** .** .** *:::**.:::*:**:*:.
                                  K-domain
                ..................═══════════.- - - - - - - - -.
WFUL1 (VRN1)    LNLKELQQLEQQLESSLKHIRSRKNQLMHESISELQKKERSLQEENKVLQKELVEKQKAQ
WFUL2           LNLKELQQLEQQLESSLKHIRSRKSHLMMESISELQKKERSLQEENKALRKELVERQKAA
WFUL3           LTTKELQQLEQQLDSSLKHIRSRKNQLLFESISELQKKEKSLKDQNGVLQKHLVETEKEK
                *. **********:**********.:*: **********:**:::* .*:*.*** :*
                                                C-domain
                - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -.
WFUL1 (VRN1)    AAQQ----------------------DQTQPQTSSSSSSFMMRD----APPAAATSIHP
WFUL2           ASRQQQQQQQQQQMQWEHQAQTTHTHTQNQPQAQTSSSSSSFMRRDQQAHAPQQNVCSYPP
WFUL3           NNVLSNIHHQEQLN-----GATNINHQEQLNGATTSSPSPTPATAQDS-MATPNIGPYQS
                                    :* :  *:**.*.         .     .  .
                - - - - - - - - - - - - - - - - - -
WFUL1 (VRN1)    AAAGERAGDAAVQP--QAPPRTG-LPLWMVSHING-
WFUL2           VTMGGEAAAAAAAPGQQAQLRIGGLPPWMLSHLNA-
WFUL3           RESGGGNPEPQPSP---AQANNSNLPPWMLSTISNR
                   *     .   *   *  . . ** **:* :.
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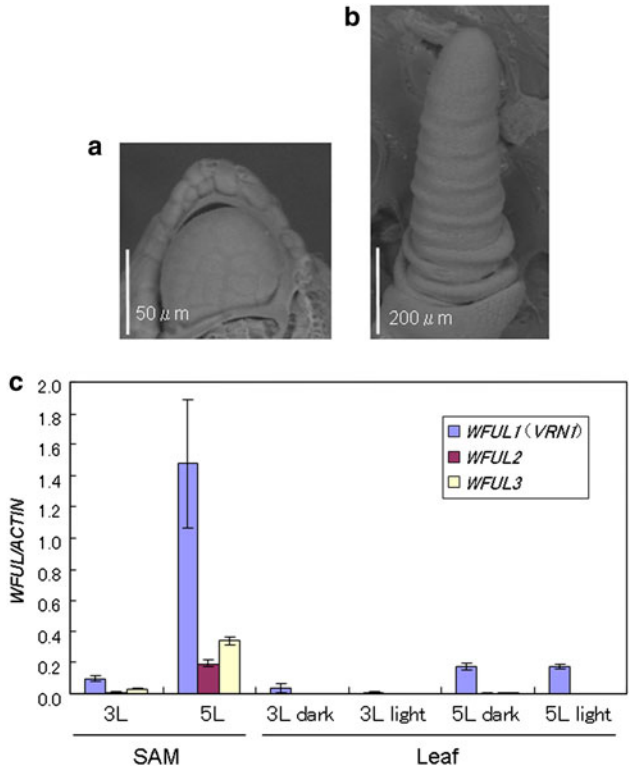

Fig. 3 Scanning electron microscope (SEM) images of SAMs of spring wheat cv. Chinese Spring (CS) at the early (**a**) and late (**b**) stages of the vegetative phase, and expression levels of *WFUL* genes in SAMs and leaves of CS by real-time PCR analysis (**c**). **a** SEM image of vegetative SAM at the 3-leaf stage in non-vernalized CS. **b** SEM image of elongated vegetative SAM at the 5-leaf stage just before phase transition in non-vernalized CS. **c** Real-time PCR analysis of SAMs (at light period) and leaves (at light and dark periods) in non-vernalized CS grown under long-day conditions. The *ACTIN* gene was used as the internal control for calculating the relative levels of *WFUL* genes. Each point represents the average of two replicates and the error bars indicate the range

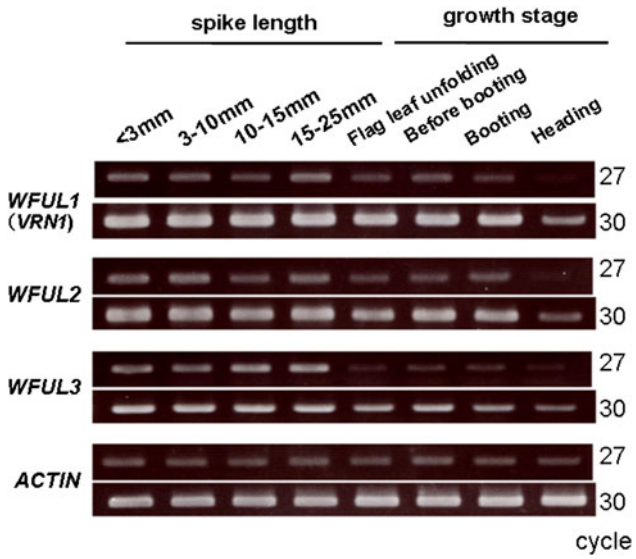

Fig. 4 RT-PCR analysis of the expression patterns of *WFUL* genes. Expression patterns in various stages of spike development in CS wheat grown in the experimental field. The young spikes (<3–25 mm in length) cover the stages of ear differentiation, spikelet differentiation, floret differentiation and floral organ development. Each PCR assay was performed twice with different numbers (*27* and *30*) of amplification cycles. *ACTIN* was used as the internal control

the CS plants at the 5-leaf stage were just before phase transition. The expression levels of *WFUL* genes in SAMs were increased at the 5-leaf stage compared to the 3-leaf stage; at the 5-leaf stage, *WFUL1/VRN1* expression was much higher than that of *WFUL2* or *WFUL3* (Fig. 3c). This result suggests that *WFUL* genes, especially *WFUL1/VRN1*, are required in SAMs for phase transition. In leaves, only *WFUL1/VRN1* was up-regulated at the 5-leaf stage during both light and dark periods, suggesting that it may also have a role in leaves during phase transition. However, the expression level of *WFUL1/VRN1* was considerably lower in leaves than in young spikes at the 5-leaf stage (Fig. 3c).

Expression patterns of *WFUL* genes in young spikes

The expression patterns of the *WFUL* genes were analyzed by RT-PCR using gene-specific primer sets at various stages of spike development (Fig. 4). The three *WFUL* genes showed a similar expression pattern with abundant transcripts in young spikes (<3–25 mm) of CS plants; this developmental sample covers the stages of spike differentiation, spikelet differentiation, floret differentiation and floral organ development. Expression of all three genes decreased after development of the floral organs. The results of this analysis suggest that the three *WFUL* genes function in the development of young spikes. To further examine the possible roles of these genes, we performed an expression analysis in various floret organs at the heading stage (Fig. 5). *FUL1/VRN1* and *FUL3* were expressed all organs tested including stamens and pistils, but we were unable to detect any *FUL2* transcripts in stamens or pistils under the PCR conditions used for the analysis (at the lower PCR cycle). By contrast to its expression pattern in stamens and pistils, *WFUL2* was highly expressed in the outer floral organs, i.e., the lemma and palea.

In situ localization of *WFUL* gene transcripts in young spikes

In situ hybridization analyses were performed to determine the distributions of *WFUL* transcripts in SAMs and during spike development in CS plants (Fig. 6). Expression of *FUL* genes was identified at low levels in SAMs before the transition from the vegetative to the reproductive phase (Fig. 6a, f, k). At the spikelet primordia initiation stage, transcripts of *WFUL1/VRN1* and *WFUL2* were detected throughout the whole of the young spikes, whereas *WFUL3* was expressed only in spikelet primordia (Fig. 6b, g, l).

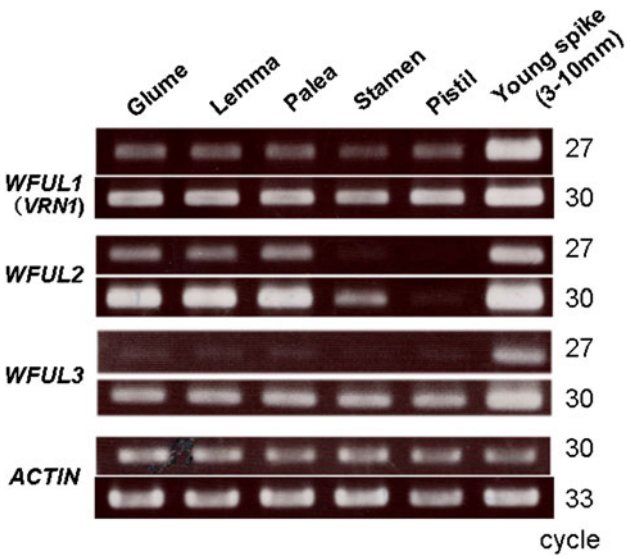

Fig. 5 RT-PCR analysis of the expression patterns of *WFUL* genes. Expression patterns in various organs of the floret at the heading stage together with young spikes (3–10 mm in length) in CS wheat grown in the experimental field. Each PCR assay was performed twice with different cycle numbers (*27* and *30*) of amplification cycles. *ACTIN* was used as the internal control

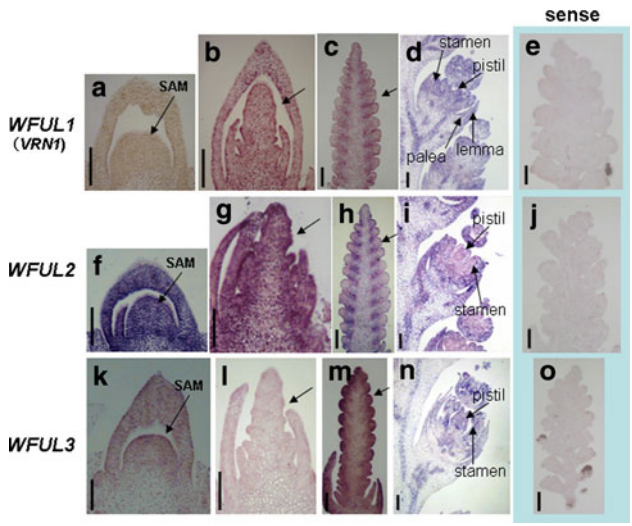

Fig. 6 RNA in situ hybridization analysis of *WFUL1* (**a–e**), *WFUL2* (**f–j**) and *WFUL3* (k–o) in CS wheat. **a**, **f**, **k** Longitudinal sections of the shoot apical meristem (SAM) at the vegetative stage. **b**, **g**, **l** Spike meristems just before the double-ridge stage. *Arrows* indicate a spikelet meristems initiation site. **c**, **h**, **m** Young spikes at the spikelet differentiation stage. *Arrows* indicate a spikelet primordium. **d, i**, **n** Spikelets with developing florets at the late floral organ differentiation stage. **e**, **j**, **o** Young spikes at the spikelet differentiation stage analyzed with the sense probe. *Scale bars* 200 μm

Expression of *WFUL3* in the spikelet primordia continued during spikelet development (Fig. 6m). The expression patterns of *WFUL1/VRN1* and *WFUL2* were similar in young spikes at the spikelet development stage: for both genes, transcripts were restricted to the basal part of the spikelet primordia (Fig. 6c, h). Each wheat floret contains a pistil, stamens and lodicules, and is enclosed by leaf-like structures, the lemma and palea (Murai et al. 2002). *WFUL1/VRN1* was expressed all of these organs at the floret development stage, but there was no indication of *WFUL2* expression in stamens and pistils (Fig. 6d, i); these expression patterns are consistent with the results of the RT-PCR analysis described above of spikes at the heading stage (Fig. 5). *WFUL3* expression signals were also found in all floral organs, but weaker than those of *WFUL1/VRN1* and *WFUL2* (Fig. 6n), consistent with the results of the RT-PCR analysis (Fig. 5). The present in situ expression analyses suggest that, in addition to their redundant function, the three *WFUL* genes have some specific functions in the developing spikes.

Interaction between *WFUL* proteins and other MADS-box proteins

Protein–protein interactions among MADS-box proteins are central to the ABCDE model of flower formation (Kaufmann et al. 2005). Yeast two- or three-hybrid systems were used to investigate interactions between the WFUL proteins and wheat class B and E MADS-box proteins (Fig. 7). Wheat class B genes include one *APETALA3* (*AP3*) ortholog, *WAP3*, and two *PISTILLATA* (*PI*) orthologs, *WPI-1* and *WPI-2* (Hama et al. 2004). In a previous study, we also identified two class E genes, *Wheat SEPALLATA* (*WSEP*) and *Wheat LEAFY HULL STERILE 1* (*WLHS1*) (Shitsukawa et al. 2007b). The WFUL2 proteins interacted with WAP3 and WPI (class B proteins), and with WSEP and WLHS1 (class E proteins). In comparison, WFUL1/VRN1 and WFUL3 interacted only with WSEP. The interaction between WFUL2 and class B and E proteins indicates that *WFUL2* is associated with specification of floral organ identity with class B and E MADS-box genes.

Ectopic expression analysis of *WFUL2* using an *Arabidopsis* transgenic system

To gain further insight into the class A function of *WFUL2*, constructs containing *WFUL2* cDNA driven by the CaMV *35S* promoter (P_{35S}), were transformed into *Arabidopsis* (Fig. 8). The effect of *WFUL2* ectopic expression on flowering in *Arabidopsis* was investigated under short-day conditions. Under these conditions, wild-type *Arabidopsis* normally shows delayed flowering. However, *Arabidopsis* plants transformed with P_{35S}::*WFUL2* exhibited an early flowering phenotype under flowering suppressive short-day conditions, in contrast to control P_{35S}::*GUS* plants (Fig. 8a). The average rosette leaf number in P_{35S}::*WFUL2* plants was significantly lower than in control P_{35S}::*GUS* plants

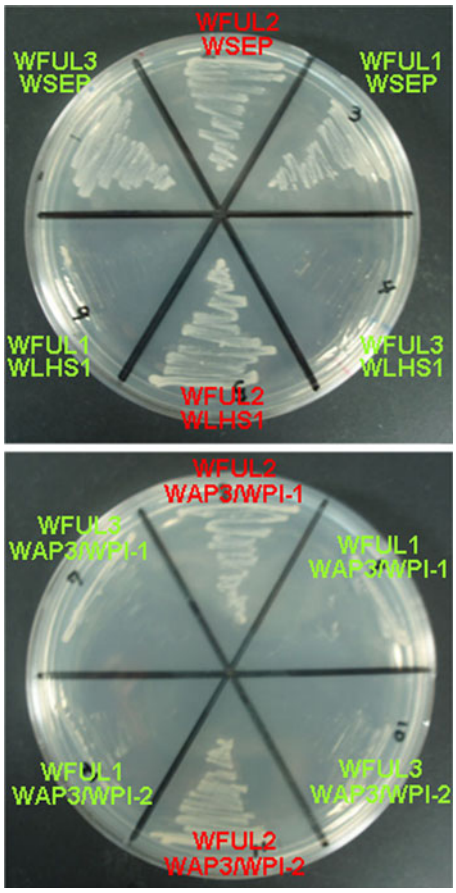

Fig. 7 Yeast two-hybrid and three-hybrid assays showing protein–protein interactions among the three *WFUL* genes and other class B (*WAP3*, *WPI-1*, *WPI-2*) and E (*WSEP*, *WLHS1*) MADS-box genes

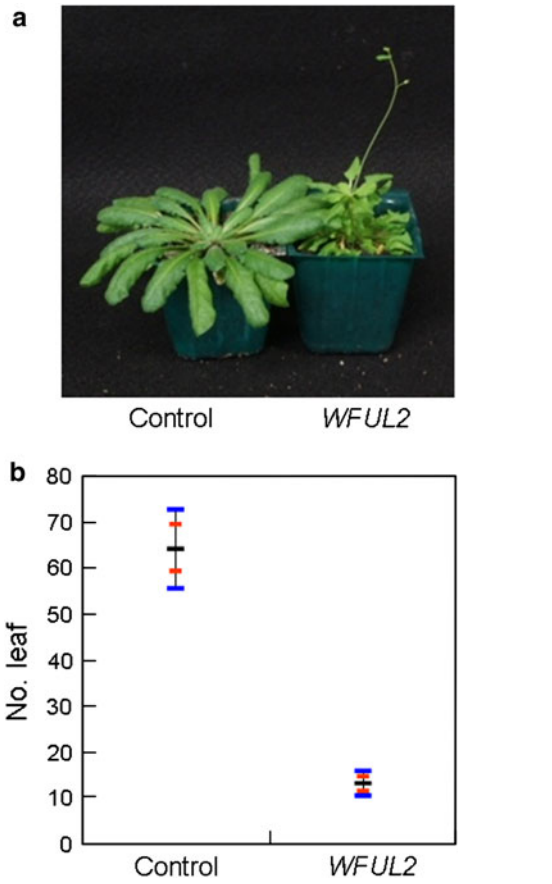

Fig. 8 Phenotypic analysis of transgenic *Arabidopsis* plants grown under short-day conditions. **a** Phenotype of a typical P_{35S}*::WFUL2* transgenic plant compared with a control P_{35S}*::GUS* transgenic plant. **b** Comparison of rosette leaf production among transgenic plants. Average, standard error and standard deviation are indicated by *black*, *red* and *blue* bars, respectively

(Fig. 8b). These results indicate that *WFUL2* functions as a flowering activator in *Arabidopsis* plants, probably due to specifying floral meristem identity. Transformants with ectopic expression of *WFUL2* genes were fertile and showed no morphological changes in any organ (data not shown).

Discussion

WFUL1/VRN1 functions in acquisition of flowering competency in leaves

Diploid species of Poaceae possess three copies of *APETALA1* (*AP1*)/*FRUITFULL* (*FUL*)-like genes derived from two duplication events in the *FUL* lineage (Litt and Irish 2003). The first duplication occurred around the base of the monocots, giving rise to the *FUL3* clade, and the second happened somewhat later and produced the *FUL1* and *FUL2* clades (Litt and Irish 2003). In this study, we compared the expression patterns of three wheat *AP1/FUL*-like genes, which we named *WFUL1*, *WFUL2* and *WFUL3* (Figs. 1, 2). *WFUL1* is identical to *VRN1,* which has been shown to control flowering in response to vernalization. Mutant plants with a deletion that encompasses the *VRN1* gene cannot transit to the reproductive phase (Shitsukawa et al. 2007a). Similarly, reduction of *VRN1* expression by co-suppression or RNAi delays flowering (Murai et al. 2003; Loukoianov et al. 2005). These findings indicate that *VRN1* is essential to flowering in wheat. In the non-vernalized plants, *WFUL1/VRN1* transcripts were present at low basal levels during the vegetative growth phase; however, expression increased in leaves after prolonged cold treatment (Murai et al. 2003; Danyluk et al. 2003). Similar changes in expression patterns in response to vernalization have been reported for barley *VRN1* (Trevaskis et al. 2003; Sasani et al. 2009). In barley, up-regulation of *VRN1* by vernalization seems to be associated with the chromatin state within the first intron and/or promoter region of *VRN1* (Oliver et al. 2009). Contrary to *WFUL1/VRN1*, the expression of *WFUL2* is not affected by cold treatment in diploid

wheat and barley (Preston and Kellogg 2008; Sasani et al. 2009), suggesting that *WFUL2* is not regulated by the vernalization pathway in leaves.

In this study, we observed that expression of *WFUL1/VRN1* increased during the growth of the spike primordia and leaves of CS plants (Fig. 3). CS has recessive alleles with homoeology to *VRN1* in the A and B genomes (*vrn-A1* and *vrn-B1*, respectively), and a dominant allele in the D genome (*VRN-D1*) (McIntosh et al. 1998). As a consequence of the latter, the plants can flower without vernalization, although vernalization treatment does moderately accelerate flowering time. In the leaves of non-vernalized CS plants, expression of *WFUL2* and *WFUL3* occurred at a low level, whereas transcripts of *WFUL1/VRN1* increased during the growth from the 3-leaf stage to the 5-leaf stage (Fig. 3). Non-vernalized CS plants transit into reproductive growth phase between the 5-leaf stage and the 6-leaf stage under long-day conditions. Overall, we conclude that *WFUL1/VRN1*, but not *WFUL2* or *WFUL3*, has a distinct function in phase transition in leaves.

The mechanism of flowering, that is the transition from the vegetative to reproductive growth phases, can be divided into two steps: first the establishment of flowering competency; and second the determination of floral meristems (Preston and Kellogg 2008). The first step entails acquisition of systemic signaling to induce flowering competency. A number of previous studies have shown that the flowering competency signal is first induced in leaves, and is transferred to SAMs by a mobile florigen (the FT protein in *Arabidopsis*) (Wellmer and Riechmann 2010). Once flowering competency is established, the specification of floral meristem identity can be induced in SAMs. The present results, together with those of earlier studies (Preston and Kellogg 2008; Shimada et al. 2009), indicate that of the three *WFUL* genes, it is *WFUL1/VRN1* that provides flowering competency (Fig. 9).

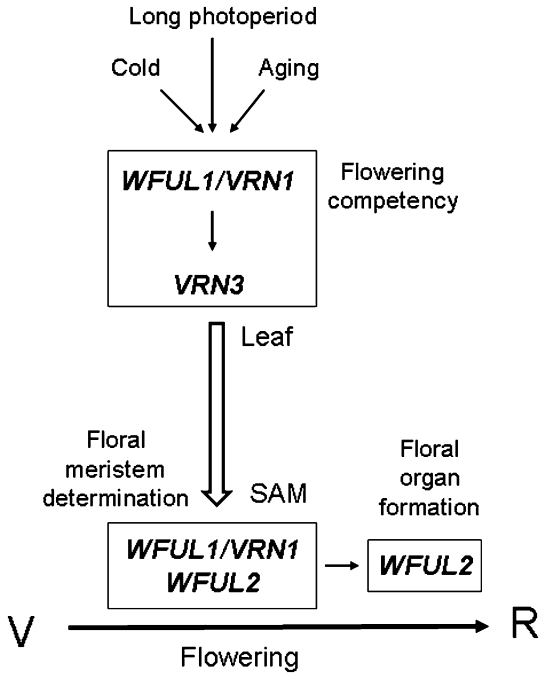

Fig. 9 A model for the genetic network that is suggested to regulate flowering in temperate cereals. Environmental signals such as cold and long photoperiod, and internal signals such as aging, are cues of flowering, and *WFUL1/VRN1* functions in acquisition of flowering competency in leaves. *WFUL1/VRN1* is upstream of *VRN3* and directly or/and indirectly activates *VRN3* expression in leaves (Shimada et al. 2009; Trevaskis 2010). VRN3 protein is postulated to be the mobile florigen that transits from leaves to SAMs. In SAMs, VRN3 up-regulates *WFUL/VRN1* expression, thereby inducing floral meristem determination and promoting phase transition from vegetative (*V*) to reproductive (*R*) growth. *WFUL2* has a redundant function in the specification of floral meristem identity, a class A function, with *WFUL1/VRN1*. *WFUL2* also has a role in another class A function, specification of outer floral organ identity

WFUL1/VRN1 functions in specifying floral meristem identity in SAMs together with *WFUL2*

In vernalization-sensitive temperate cereals, such as wheat, barley and oat, prolonged cold exposure induces expression of *FUL1* in SAMs as well as in leaves (Yan et al. 2003; Preston and Kellogg 2008; Sasani et al. 2009). The up-regulation of *FUL1* occurs significantly later in SAMs than in leaves of vernalized oat plants (Preston and Kellogg 2008), indicating that *FUL1* performs distinct roles in SAMs and leaves. In non-vernalized CS wheat plants, the *WFUL* genes were up-regulated during growth, and a high level of expression was observed in SAMs of 5-leaf stage plants just before phase transition (Fig. 3). Since *WFUL1/VRN1* seems to play a role in specifying floral meristem identity (Yan et al. 2003; Murai et al. 2003; Loukoianov et al. 2005; Shitsukawa et al. 2007a; Adam et al. 2007), then it is possible that flowering competency is transmitted from the leaves to SAMs via a mobile florigen. Although there is no direct biochemical proof for this hypothesis, transgenic and expression studies indicated that the VRN3 protein is a florigen in temperate cereals and is transported from the leaves to SAMs (Yan et al. 2006; Hemming et al. 2008; Shimada et al. 2009). The wheat ortholog of *FD*, *TaFDL2*, is expressed in wheat SAMs as well as in leaves together with *WFUL1/VRN1*, and the TaFDL2 protein can interact with the VRN3 protein and bind in vitro with the promoter region of *WFUL1VRN1* (Li and Dubcovsky 2008). It is possible that the VRN3-TaFDL2-WFUL1/VRN1 complex functions in SAMs, specifying floral meristem identity.

In SAMs, spike primordia, and young spikes, the transcript levels of *WFUL2* and *WFUL3* also increased during the growth stage (Figs. 3, 4), suggesting that *WFUL2* and *WFUL3* have some functions in floral meristem development. In oat and diploid wheat, it has been reported that *FUL2* expression is increased in SAMs following attainment

of flowering competency (Preston and Kellogg 2008), suggesting that *FUL2* functions redundantly with *FUL1* for transition to flowering in SAMs. The present study showed that overexpression of *WFUL2* in transgenic *Arabidopsis* plants caused early flowering (Fig. 8), supporting the idea that *WFUL2* functions in specifying floral meristem identity. In the SAMs of rice plants, both *OsMADS14* (a *FUL1* gene) and *OsMADS15* (a *FUL2* gene) act downstream of *RICE FT1* (*RFT1*) in floral induction (Komiya et al. 2009). Furthermore, it has been shown that *OsMADS15* is up-regulated by the rice FT homologue (Hd3a) and rice FD homologue (OsFD1) through interaction with the 14-3-3 protein, leading to specification of the floral meristem identity (Taoka et al. 2011). These observations, together with the present results, suggest that *FUL2* genes have redundant functions with *FUL1* genes for specifying floral meristem identity.

WFUL2 has class A functions in wheat

In situ analysis indicated that *WFUL3* was expressed in the spikelet primordia, and also throughout the spikelet meristem that forms alternately along the floret meristems (Fig. 6). In contrast, the transcripts of *WFUL1/VRN1* and *WFUL2* were restricted to the basal part of spikelet meristems. In developing florets, *WFUL3* was expressed in all floral organs, but transcripts were present at a lower level than those of *WFUL1/VRN1* (Figs. 5, 6). A lower level of expression of *FUL3* compared to *FUL1* has been also reported in the developing florets of barley (Schmitz et al. 2000). These observations suggest that *WFUL3* plays a different role in the development of young spikes to that of *WFUL1/VRN1* and *WFUL2*.

In young spikes, expression of *WFUL2* was greatly reduced in stamens and could not be detected in pistils, whereas *WFUL1/VRN1* and *WFUL3* were expressed in all floral organs (Figs. 5, 6), suggesting that *WFUL2* has a different function in the outer floral organs (lemma and palea) compared to the inner floral organs (stamen and pistil). Similar patterns of expression of *FUL1* and *FUL2* genes have been reported in *Lolium temulentum* (Gocal et al. 2001). The transcripts of *LtMADS1* (a *FUL1* gene) are present in all floral organs, while the expression of *LtMADS2* (a *FUL2* gene) is reduced in the stamens and pistils. In rice (*Oryza sativa*), *OsMADS14* (a *FUL1* gene) is expressed in all floral organs, whereas *OsMADS15* (a *FUL2* gene) is expressed only in the outer bracts (Pelucchi et al. 2002). Furthermore, the maize *FUL2* gene *ZAP1* is not expressed in stamens and pistils (Mena et al. 1996). Taken together, the evidence indicates that *FUL2* has a specific role in the development of florets in grass species. In situ analysis of the barley *FUL* genes, *HvMADS5/BM5* (a *FUL1* gene), *HvMADS8/BM8* (a *FUL2* gene), and *HvMADS3/BM3* (a *FUL3* gene), showed that all three are expressed in all floral organ primordia (Schmitz et al. 2000). The reason for this apparent difference in *FUL2*-specific expression in young spikes of barley is unclear.

The yeast two- and three-hybrid analysis performed here demonstrated that WFUL2 interacted with class B and class E proteins (Fig. 8). In combination with the expression analyses, this result suggests that *WFUL2* specifies the identity of the outer floral organs in the wheat floret. In rice, both FUL1 and FUL2 proteins (OsMADS14 and OsMADS15, respectively) interact with a class E protein (OsMADS1/LHS1) (Lim et al. 2000), suggesting that the diversification of function between FUL1 and FUL2 detected in wheat has not occurred in rice. In this study, *Arabidopsis* transformants with ectopic expression of *WFUL2* genes showed no morphological changes in any floral organ. Possibly, this lack of effect was due to a requirement for the monocot-specific class E gene, *WLHS1* by *WFUL2* for initiation of outer floral organ formation (Shitsukawa et al. 2007b).

In *Arabidopsis*, *AP1* is a class A gene that specifies the floral meristem identity and outer floral organ (sepal and petal) identity together with class B and class E genes (Mandel et al. 1992), thus it has class A functions. The present study showed that *WFUL2* in wheat has a class A function in specifying the identity of the floral meristem together with *WFUL1/VRN1*; it also has another class A function in specifying the identity of the outer floral organs (lemma and palea) in combination with class B and class E MADS-box genes (Fig. 9). Hexaploid wheat has three homoeologous genes located on homoeologous A, B and D chromosomes. Additional experiments are necessary to distinguish the functional difference and/or similarity in flowering among three *WFUL2* homoeologous genes.

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