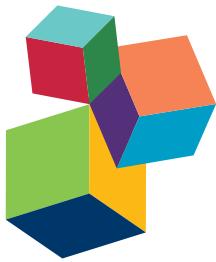


RECENT ADVANCES OF EPIGENETICS IN CROP BIOTECHNOLOGY

EDITED BY: Clelia De-la-Peña, Raúl Alvarez-Venegas and
Christopher Cullis

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RECENT ADVANCES OF EPIGENETICS IN CROP BIOTECHNOLOGY

Topic Editors:

Clelia De-la-Peña, Centro de Investigación Científica de Yucatán, Mexico

Raúl Alvarez-Venegas, Centro de Investigación y de Estudios Avanzados del IPN, Mexico

Christopher Cullis, Case Western Reserve University, USA

Epigenetics is a new field that explains gene expression at the chromatin structure and organization level. Three principal epigenetic mechanisms are known and hundreds of combinations among them can develop different phenotypic characteristics. DNA methylation, histone modifications and small RNAs have been identified, and their functions are being studied in order to understand the mechanisms of interaction and regulation among the different biological processes in plants.

Although, fundamental epigenetic mechanisms in crop plants are beginning to be elucidated, the comprehension of the different epigenetic mechanisms, by which plant gene regulation and phenotype are modified, is a major topic to develop in the near future in order to increase crop productivity. Thus, the importance of epigenetics in improving crop productivity is undoubtedly growing.

Current research on epigenetics suggest that DNA methylation, histone modifications and small RNAs are involved in almost every aspect of plant life including agronomically important traits such as flowering time, fruit development, responses to environmental factors, defense response and plant growth.

The aim of this Research Topic is to explore the recent advances concerning the role of epigenetics in crop biotechnology, as well as to enhance and promote interactions among high quality researchers from different disciplines such as genetics, cell biology, pathology, microbiology, and evolutionary biology in order to join forces and decipher the epigenetic mechanisms in crop productivity.

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Editorial: Recent Advances of Epigenetics in Crop Biotechnology

Raúl Álvarez-Venegas^{1*} and Clelia De-la-Peña²

¹ Unidad Irapuato, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Guanajuato, México,

² Centro de Investigación Científica de Yucatán, Unidad de Biotecnología, Mérida, México

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The Editorial on the Research Topic

Recent Advances of Epigenetics in Crop Biotechnology

Ever since the first Agricultural Revolution, humans have domesticated hundreds of plant species and it is considered that the evolution of crop plants took place as human behavioral ecology changed from food gathering to farming. As a result of wild species domestication, the selection of populations with desirable alleles, the breeding of high yielding genotypes, the ease of farming and quality, and many technological advances have allowed crop production to increase and become better adapted to environmental changes (Bennett et al., 2013). During the last decades, modern breeding methods, as well as novel research, development, and new technologies have improved considerably agriculture production worldwide. This has been achieved thanks to the enhancement of agronomic traits such as, increased abiotic/biotic stress tolerance, reduced toxicity, bigger seed size, increased yield, superior nutritional quality, delayed ripening, better post-harvest quality, etc. (Meyer and Purugganan, 2013). However, due to an increasing human population, nowadays arable soil is becoming less available and the climate change problem is a worldwide emergency.

Plants are indispensable in our life because they supply us with oxygen, food, and medicines. Therefore, it is important to study and examine the mechanisms that plants have evolved to adapt to diverse environments, and in particular how crop species deal with different types of biotic and abiotic stress. In addition, it is essential to understand how the epigenetic component regulates plant gene expression and the plant phenotype, and we must focus on how the epigenome works as a powerful source of diversity for important agronomical traits and on how its exploitation, in crop improvement programs, would be of benefit to our modern society.

It is now known that epigenetic modifications control gene expression by modulating the access of regulatory complexes to the genome. Furthermore, current research on epigenetic mechanisms indicate that DNA methylation, histone posttranslational modifications and small non-coding RNAs are involved in almost every aspect of plant life including agronomically important traits such as flowering time, fruit development, responses to environmental factors, and plant immunity. Even though the basic epigenetic mechanisms in crop biotechnology are starting to be uncovered, soon they will be extensively employed for crop improvement and to increase crop productivity in challenge environments. This research Topic includes an excellent combination of Mini Reviews, Reviews, Original Research Articles, and Methods focused on the role of epigenetics in crop biotechnology, and provides up-to-date information on epigenetics in crop plants during *in vitro* culture, abiotic and biotic stresses, and gene silencing.

One of the biotechnological tools for crop improvement has been the use of plant *in vitro* culture and their effects on epigenetic mechanisms. For instance, in their Original Research article Barraza et al. show that down-regulation of the *PvTRX1h* gene (which codes for a histone lysine methyltransferase, HKMTase) is accompanied by an altered concentration of distinct plant hormones in common bean embryogenic calli. Specifically, “*PvTRX1h* regulates the expression

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Edited and reviewed by:

James Lloyd,

Stellenbosch University, South Africa

*Correspondence:

Raúl Álvarez-Venegas

ralvarez@ira.cinvestav.mx

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of genes involved in auxin biosynthesis, and embryogenic calli, in which *PvTRX1h* is down-regulated, are able to differentiate into and overproduce somatic embryos." Also, down-regulation of *PvTRX1h* increase transcript abundance of *PvASHH2h*, a gene coding for a second HKMTase, and point out that epigenetic changes such as histone methylation have an active role in the regulation of plant hormone biosynthesis in common bean calli. Specific topics on the role of chromatin modifications in plant somatic embryogenesis (SE) are discussed by De-la-Peña et al. and provide interesting new insights into the field. In their Review they highlight recent discoveries on the mechanisms of epigenetic regulation in SE that could help to increase plant productivity and improve agronomical breeding practices. On the other hand, Kitimu et al. analyzed epigenetic changes during propagation by meristem culture and by field cuttings in cassava (*Manihot esculenta*) cultivars. They identify candidate epimarks that distinguish between field cutting and meristem culture samples. This will certainly help in the identification of specific methylation signatures associated to *in vitro* propagation and in the optimization of *in vitro* meristem propagation protocols and in the diagnosis of the origin of clonal stocks. Chávez-Hernández et al. report on the miRNA abundance and their target gene expression in response to light exposure and hormone depletion during maize SE. They find that most of the miRNA examined increase upon hormone depletion, regardless of photoperiod absence/presence, whereas expression of miRNA target genes is effectively regulated by the photoperiod exposure. Furthermore, "stress-related miRNA targets show greater differences between cultivars than development-related targets, with a miRNA/target inverse relationship more frequently observed in darkness than light." Such results will help to understand and manipulate the plant regeneration process in crops like maize. Also, the effect of light was studied in the Original Research article of Liu et al. who show that in rice the enhancer of zeste [E(z)] genes *SDG711* and *SDG718*, are involved in the regulation of key flowering genes and imply that "Polycomb Repressive Complex2 (PRC2)-mediated epigenetic repression of gene expression is involved in the accurate photoperiod control of rice flowering." Although miRNAs have been analyzed mainly by Northern blots, Rosas-Cárdenas et al. show that tissue-printing hybridization is very useful for detection and localization of miRNAs in fruits of crop plants.

Crop agriculture has two major problems, which are deficiency in nutrient and in water supply, and different authors address these challenges. Firstly, in their Review, Paul et al. highlight the role of miRNAs in macro- or micro-nutrient deficiencies in plants, and how miRNA-mediated regulation of nutrient transporters and other metabolic enzymes could be used in future biotechnological research. Bocchini et al. investigate the effect of iron deficiency, in barley plants, on plant growth and using gene silencing and the changes in the DNA methylation status caused by Fe deprivation, amongst other traits. They find a clear effect of Fe starvation on the level of DNA methylation/demethylation of the barley genome. This kind of research will certainly help to elucidate "how the plants modulate gene expression to cope with nutrients fluctuations," considering that such modifications could be transmitted to progeny. On the other hand, Su et al. find, in peanut

(*Arachis hypogaea*), an RPD3/HDA1-like superfamily histone deacetylase (HDAC), termed AhHDA1, which is seemingly involved in the epigenetic regulation of stress resistance genes in response to osmotic stress and ABA treatment. Accordingly, studies on the molecular mechanisms of drought resistance are necessary and could be used to generate new crop varieties for agriculture in water-limiting conditions. Rodríguez López and Wilkinson review current knowledge on epigenetic states (in particular DNA methylation) and responses of crop plants to specific characteristics of the growing environment (epigenetic fingerprinting) that could be used for the improvement of crop production and quality. More specific topics on genotype × environment interactions that may be beneficial for long-term improvement of crop performance are addressed by King. In his Review, King addresses the biophysical and thermodynamic properties of DNA, histones and nucleosomes, and explores the consequences of thermal and ionic variation on the biophysical behavior of epigenetic marks and how these contribute to maintenance of chromatin integrity and gene regulation in the plant nucleus. Loza-Muller et al. describe how the *Brassica oleracea* fibrillarin methyltransferase is capable to methylate nucleolar histone H2A while bound to the rDNA and carry out its methylation in the rDNA promoter. But not only abiotic conditions affect epigenetics and therefore plant behavior, also biotic challenges are an important topic of study. In their Review Article, Ding and Wang draw attention to the molecular mechanisms of histone modifications and chromatin remodeling that contributes to plant immunity against pathogens. Also, Hohn put emphasis in his Mini Review on the RNA-based silencing suppression mechanisms in plant pararetroviruses. In Meyer et al. the authors analyze the effects of the ectopic overexpression of the *Arabidopsis Enhancer of RNAi* (ERI) gene and the link between plant growth and siRNAs. In their Mini Review Rajeevkumar et al. examine the field of epigenetic silencing in transgenic plant systems.

The Research Topic presented here is significant because it is expected to increase and strengthen the information needed to develop, in the near future, novel approaches to manipulate and selectively activate, and/or inhibit gene expression, proteins and metabolic pathways to counter plant pathogens, to treat important diseases and to increase crop productivity. New approaches of the type presented here and the advancement of new technologies will certainly increase our knowledge of currently known epigenetic factors and chromatin modifications and will facilitate the understanding of their roles in, for example, host-pathogen interactions and crop productivity.

AUTHOR CONTRIBUTIONS

RA and CD provided the idea of the work. RA and CD critically reviewed the manuscript. RA wrote the paper. All authors read and approved the final manuscript.

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The *Phaseolus vulgaris* *PvTRX1h* gene regulates plant hormone biosynthesis in embryogenic callus from common bean

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Edited by:

Soren K. Rasmussen,
University of Copenhagen, Denmark

Reviewed by:

Fumihiro Sato,
Kyoto University, Japan

Eva Zazimalova,

Institute of Experimental Botany of the
Academy of Sciences of the Czech
Republic, Czech Republic

***Correspondence:**

Raúl Álvarez-Venegas,
Departamento de Ingeniería Genética,
Centro de Investigación y de Estudios
Avanzados del Instituto Politécnico
Nacional Unidad-Irapuato, Km. 9.6
Libramiento Norte, Carretera
Irapuato-León, C.P. 36821, Irapuato,
México
ralvarez@ira.cinvestav.mx

†Present Address:

Francisco Luna-Martínez,
Universidad Politécnica de
Guanajuato, Juan Alonso Cortazar,
México

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Aarón Barraza, José L. Cabrera-Ponce, Roberto Gamboa-Becerra,
Francisco Luna-Martínez †, Robert Winkler and Raúl Álvarez-Venegas *

Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Irapuato, Guanajuato, México

Common bean is the most important grain legume in the human diet. Bean improvement efforts have been focused on classical breeding techniques because bean is recalcitrant to both somatic embryogenesis and *in vitro* regeneration. This study was undertaken to better understand the process of somatic embryogenesis in the common bean. We focused on the mechanisms by which somatic embryogenesis in plants is regulated and the interaction of these mechanisms with plant hormones. Specifically, we examined the role of the gene *PvTRX1h*, an ortholog of a major known histone lysine methyltransferase in plants, in somatic embryo generation. Given the problems with regeneration and transformation, we chose to develop and use regeneration-competent callus that could be successively transformed. Embryogenic calli of common bean were generated and transformed with the *PvTRX1h*RNAi construction to down-regulate, by RNA interference, expression of the *PvTRX1h* gene. Plant hormone content was measured by mass spectrometry and gene expression was assessed by q-PCR. Detailed histological analysis was performed on selected transgenic embryogenic calli. It was determined that down-regulation of *PvTRX1h* gene was accompanied by altered concentrations of plant hormones in the calli. *PvTRX1h* regulated the expression of genes involved in auxin biosynthesis and embryogenic calli in which *PvTRX1h* was down-regulated were capable of differentiation into somatic embryos. Also, down-regulation of *PvTRX1h* showed increased transcript abundance of a gene coding for a second histone lysine methyltransferase, *PvASHH2h*. Accordingly, the *PvTRX1h* gene is involved in the synthesis of plant hormones in common bean callus. These results shed light on the crosstalk among histone methyltransferases and plant hormone signaling and on gene regulation during somatic embryo generation.

Keywords: *Phaseolus vulgaris*, plant hormone, histone methyltransferase, auxin, callus, somatic embryo

Introduction

Epigenetic phenomena affect the structure and organization of chromatin and through this mechanism can influence gene expression by modulating the access of regulatory proteins and protein complexes to the genome. Epigenetic mechanisms appear to be involved in almost every aspect of plant life, from embryo development to plant immunity. The former is, in part, apparent

as epigenetic mechanisms modulate embryogenic capacity of plant cells in culture (Rival et al., 2013).

Plant hormones are another important regulator of development. They are directly involved in seed germination, tissue and organ differentiation and development, flowering, fruiting, tropisms, and responses to biotic and abiotic stress (Santner and Estelle, 2009). Plant hormones are a structurally unrelated collection of small molecules that act at low concentrations. The main plant hormones and hormone classes are auxins, cytokinins, gibberellins (GA), abscisic acid (ABA), ethylene, brassinosteroids (BR), jasmonic acid (JA), salicylic acid (SA), nitric oxide, and strigolactones.

From this brief review, it should be apparent that both of these areas—epigenetic regulation of the chromatin and plant signaling—are critically important in all aspects of plant development. It is also easy to envision that the two processes interact. However, studies of this interaction are scarce. Little is known about the effect of plant hormones on plant chromatin structure and the reverse is also true: little is known about the role of chromatin modifications or chromatin modifiers on plant hormone biosynthesis.

Chromatin structure is regulated in part by modification of histones. Through this modification, chromatin function and, therefore, gene regulation are affected. One can regard the higher order of chromatin and nucleosome structure as the final regulatory point in plant hormone signaling pathways for regulation of transcription factors and subsequent gene expression.

At least some effects of plant hormones on gene expression have been shown to depend on specific chromatin modifications, which include histone variants and histone modifications. For example, the expression and accumulation of a “drought-inducible” H1 histone variant (H1) from three tomato species was induced by ABA alone, that is, its induction was unrelated to water deficit (Kahn et al., 1993; Wei and O’Connell, 1996). Other examples come from studies with Arabidopsis. An increase in acetylated histone H3 lysine 14 (H3K14), histone H4 lysine 5 (H4K5), and in tri-methylated H3K4 accompanied ABA activation of the *phaseolin* (*phas*) promoter in transgenic leaves (Ng et al., 2006). Expression of *WRKY70*, a gene that is antagonistically regulated by the SA- and JA-signaling pathways (Li et al., 2004), is controlled by the *ARABIDOPSIS TRITHORAX 1* (*ATX1*) gene, an Arabidopsis homolog of the Drosophila *trithorax*, which activates *WRKY70* expression by establishment of the trimethylation pattern of histone H3 lysine 4 (H3K4me3) of its nucleosomes (Alvarez-Venegas et al., 2007). In rice, down-regulation of *SDG725*, which encodes a histone H3K36 methyltransferase, causes phenotypic defects similar to those described for some brassinosteroid (BR) mutants (Sui et al., 2012), suggesting that *SDG725* depletion results in down-regulation of genes known to be involved in BR signaling, namely, *D11* (*OsDWARF11*), *BU1* (*OsBRASSINOSTEROID UPREGULATED 1*), and *BRI1* (*OsBRASSINOSTEROID INSENSITIVE 1*). Apparently, *SDG725*-mediated H3K36 methylation regulates BR-related gene expression (Sui et al., 2012). These examples support the assumption that chromatin

modification is an important regulator of hormone action on gene expression.

Grain legumes are agronomically important and the common bean (*Phaseolus vulgaris* L.) is the most important grain legume in the human diet (Food and Agricultural Organization of the United Nations, 2014). Grain legumes are recalcitrant to *in vitro* regeneration and *Phaseolus vulgaris* is particularly recalcitrant to *in vitro* induction of somatic embryogenesis and regeneration. Consequently, stable genetic transformation is hard to achieve for this organism, although *P. vulgaris* composite plants, with wild-type (WT) shoots and transgenic hairy roots (derived from *Agrobacterium rhizogenes*-mediated genetic transformation), have been successfully developed (Estrada-Navarrete et al., 2007). However, complete plant genetic transformation of *P. vulgaris* is still elusive. We report here that the creation and establishment of regeneration-competent callus and its transformation are an important first step in the establishment of a stable system of genetic transformation (and possible regeneration) in *P. vulgaris*, necessary for the elucidation of gene function in this important plant. Specifically, we have employed this method to study epigenetic mechanisms that regulate somatic embryogenesis in common bean.

In this study, we were able to generate transgenic embryogenic calli of *P. vulgaris* by particle gun bombardment (Cabrera-Ponce et al., 2015). In these calli, we investigated the role of the gene ortholog to the *Drosophila trithorax* gene, the *Phaseolus vulgaris* *Trithorax 1* gene homolog, named *PvTRX1h* (Quiceno-Rico et al., 2012), in somatic embryo development and plant hormone synthesis. When *PvTRX1h* was downregulated by RNA interference (RNAi), pro-embryogenic calli differentiated and formed somatic embryos in abundance and with diverse phenotypes, plant hormone concentrations were altered in ways consistent with the phenotype of the embryos, and *PvTRX1h* downregulation affected the expression of genes involved in auxin biosynthesis. Particularly, our study highlights a clear role of *PvTRX1h* in the regulation of somatic embryogenesis and plant hormone synthesis in plant cells.

We hope that the implemented technology for the generation of transgenic somatic embryos with the potential to regenerate whole transgenic common bean plants will prove useful in the continued development of *P. vulgaris* as a model crop plant and will increase productivity of this important food source.

Materials and Methods

Plant Material

Phaseolus vulgaris L. cultivar “Negro Querétaro” was used in this study.

Vector Construction

Construction of the *PvTRX1h*RiA silencing vector, driving the expression of an antisense sequence from the *PvTRX1h* gene (GenBank locus #JF262910; Phytozome #Phvul.008G018500), under control of the ectopic CaMV35S promoter, was created as follows: a 602bp PCR fragment of the *PvTRX1h* cDNA was amplified by using gene-specific forward and reverse primers (PvRX1RiF 5'-tctagagcaaaggcatccacataaagg-3';

PvRX1RiR 5'- ggatccgaaacaatggagaatcag-3'; underlined sequences correspond to artificially introduced *Xba*I and *Bam*HI restriction sites, respectively). Next, the pFGC5941 binary vector was digested with *Xba*I and *Bam*HI and the backbone was purified and ligated to the *PvTRX1h* PCR product with the T4 DNA ligase at the *Xba*I and *Bam*HI sites of the pFGC5941 plasmid to generate the PvTRX1hRiA silencing vector, which was used to down-regulate, by RNA interference (RNAi), *PvTRX1h* gene expression.

Culture Media

Osmotic treatment medium: a modified MS medium was used as the basal media and supplemented with Murashige and Skoog micro and macronutrients, 12% (w/v) sucrose (0.368 M), 10 mg/L of 6-Benzylaminopurine (BAP), 40 mg/L adenine free-base, and 2.5 g/L gelrite (Malik and Saxena, 1992; Cabrera-Ponce et al., 2015).

Embryo Induction Medium (EIM): a modified MS medium was used as the basal media and supplemented with Murashige and Skoog micro and macronutrients, 6% (w/v) glucose, 10 mg/L of 6-Benzylaminopurine (BAP), 40 mg/L adenine free base, and 2.5 g/L gelrite (Malik and Saxena, 1992; Cabrera-Ponce et al., 2015).

Regeneration Medium (RM): Murashige and Skoog micro and macronutrients, 0.1 mg/L of kinetin, and 0.4 mg/L of N⁶-(Δ²-isopentenyl)adenine (2iP), and 2.5 g/L gelrite (Cabrera-Ponce et al., 2015).

Zygotic Embryo Dissection, Osmotic Treatment, and Callus Induction

Embryonic axes containing the cotyledonary and apical dome were cultivated for 48 h under osmotic stress in osmotic treatment medium in a growth chamber with a light/dark cycle of 16 h/8 h, a photon flux density of 50 μmol m⁻² s⁻¹ provided by fluorescent lamps and one 60-W incandescent bulb, and maintained at 26°C (Cabrera-Ponce et al., 2015). Next, the embryonic axes were transferred to EIM media, kept in a growth chamber under the conditions described above, and used for embryo induction. The first pro-embryogenic mass was obtained 4 weeks after the osmotic shock, mainly from the cotyledonary zone. Pro-embryogenic callus were dissected and transferred to fresh EIM for propagation every 4 weeks (Cabrera-Ponce et al., 2015).

Callus Transformation and Selection

Microcarriers for particle gun bombardment were prepared with 100 ng of plasmid DNA. A Bio-Rad PDS-100/He particle delivery system was used (Cabrera-Ponce et al., 1997, 2015). An equimolar mixture of plasmid PvTRX1hRiA and pWRG1515 (Christou et al., 1991) was precipitated onto tungsten microprojectiles of 1.0 μm diameter and delivered onto early globular-stage pro-embryogenic callus that had been sub-cultured for 2–3 months. Ten Petri dishes, each containing 16–20 calli for a total of 2 grams of fresh weight, were bombarded. Control calli were transformed with the pWRG1515 vector only. pWRG1515 contains the *uidA* reporter gene (*gusA*) and the *hptII* gene that confers resistance to hygromycin. Transformed callus were selected in EIM plates

containing 50 mg/L hygromycin. After bombardment, calli were sub-cultured every 2 weeks for 3 months in fresh EIM plates containing hygromycin. The transgenic clones maintained their embryogenic capacity, while the construct used in this experiment was stably expressed in the embryogenic callus.

cDNA Synthesis and qRT-PCR Analysis

TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) was used to isolate RNA from transgenic callus, control untransformed callus and callus transformed with the empty vector. For qRT-PCR analysis, RNA was treated with DNaseI (Invitrogen, Carlsbad, CA, U.S.A.) to remove genomic DNA. The absence of DNA was confirmed by performing PCR (40 cycles, similar to the real-time PCR program) on the DNaseI-treated RNA using Taq-DNA polymerase. A StepOne® Real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) was used for real-time PCR quantifications. qRT-PCR was performed according to the standard SuperScript® II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, U.S.A.) with the Maxima® SYBR Green/ROX qPCR Master Mix (2x) protocol (Thermo Scientific, Waltham, MA, U.S.A.). A “no DNA” template control was used in each analysis. The results presented are from three independent ($n = 3$) biological replicates (each with eight transgenic calli), and statistical significance was determined with an unpaired two-tailed Student's *t*-test. Each biological replicate was tested by triplicate and data were normalized to the *Actin11* (*PvActin11*) reference gene (*PvActin11F* 5'-tgcatacgttgtatgagg-3', and *PvActin11R* 5'-agcattgggttaaggagg-3' (Borges et al., 2012), and to the elongation factor 1-α (*PvEF1α*) reference gene (*PvEF1aF* 5'-ggcattggcatgtcgactctgg-3', and *PvEF1aR* 5'-gcaccaggcatacttgaatgacc-3') (Barraza et al., 2013). The method used to analyze the data from real time PCR experiments corresponds to the relative quantification method, or $2^{-\Delta\Delta CT}$ method, where the $\Delta\Delta CT$ value = $((CT_{1\text{Target}} - CT_{1\text{Reference}}) - (CT_{0\text{Target}} - CT_{0\text{Reference}}))$ (Livak and Schmittgen, 2001). The mean C_T values for both the target and internal reference genes were determined and the fold change in the target gene normalized to *PvActin11* and *PvEF1α* and relative to the expression in the control sample. A list of the auxins (IAA), cytokinins (2iP and zeatin), brassinosteroids (epibrassinolide) and abscisic acid (ABA) biosynthetic pathway genes analyzed by q-PCR is shown on **Supplementary Table ST1** and a list of the primers used is in **Supplementary Table ST2**. Amplification of the CaMV35S fragment was performed under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C/20 s, 54°C/30 s, and 72°C/40 s.

Frequency of Embryogenesis

To determine the frequency of embryogenesis the callus were transferred to fresh EIM for propagation. After 3 weeks, observations on frequency (%) of embryos were recorded. Thirty calli were analyzed for each of the different transgenic clones, as well as for the control callus (non-transformed calli and calli transformed with the empty vector). The number of somatic embryos present per pro-embryogenic mass (PEM) was registered in each clone. Data were analyzed with an unpaired two-tailed Student's *t*-test to compare sample means.

Histology and Optical Microscopy

Calli (six per experiment) were embedded in Paraplast after being fixed in 100 mL FAA solution (90 mL 70% ethanol, 5 mL 37% formaldehyde, 5 mL glacial acetic acid), and subjected to a short dehydration ethanol series. Sections of 10 μm were prepared with a microtome (Leica Ultracut R, Vienna, Austria). Samples for optical microscopy were stained with Peryodic acid-Leucobasic Fuchsin-Aniline blue black (Schneider, 1981), analyzed with a light microscope (Motic BA300, Xiamen, China), and photographed with a digital camera (Motic M1000, Xiamen, China).

Profile of phytohormones By UPLC-ESI-MS

Calli were collected, weighed (200 mg), frozen and ground in liquid nitrogen. Then, the ground tissue was resuspended in 1 mL of methanol:isopropanol:glacial acetic acid (80:19:1), incubated for 48 h at room temperature in darkness and then centrifuged at 10,000 rpm for 15 min, the supernatant was collected and filtered with a 0.22 μm PTFE membrane.

The sample extracts were analyzed using an Ultra Performance Liquid Chromatography Electrospray Mass Spectrometry (UPLC-ESI-MS) system Accela LCQ Fleet Ion trap, Thermo Finnigan, San Jose, CA, USA. The compound mixture was separated on a Hypersil Gold C18 column (50 \times 2.1 mm, 1.9 μm particle size). Ten micro-liters of sample were injected. The mobile phase consisted of H₂O with 0.1% (v/v) formic acid (solvent A) and solvent B was methanol with 0.1% (v/v) formic acid. The column oven temperature was maintained at 35°C, the flow rate was 400 $\mu\text{L}/\text{min}$. The solvent gradient program for free IAA was as follows: 5% B, 0–1 min; 5–95% B, 1–8.9 min; 95% B–95% A, 8.9–9 min; and finally, column re-equilibration for 4 min (9–13 min) with 95% A; while for 2iP, ABA and zeatin the gradient was: 5% B, 0–1 min; 5–60% B, 1–4.9 min; 60% B–95% A, 4.9–5 min; and finally, column re-equilibration for 4 min (5–9 min) with 95% A.

Spectra were acquired in SIM (selected ion monitoring) mode focusing on protonated forms [M + H]: m/z 176 (IAA), m/z 204 (2iP), m/z 220 (zeatin) and m/z 265 (ABA) with a m/z width of 10, operating in positive mode. The scan time was 500 ms (3 micro-scans).

The ESI source parameters were set as follows: capillary temperature 300°C; capillary voltage 35 V; spray voltage 4.8 kV; tube lens 80 V; nitrogen sheath gas 45 arbitrary units (AU); auxiliary gas 10 AU.

Histone Isolation and Western Blots

Histones from calli were isolated as previously described (De-La-Peña et al., 2012). In brief, histones were isolated from 1.2 g of tissue from WT and 3-week-old transgenic calli using sulphuric acid extraction of nuclei, followed by acetone precipitation according to established protocols (Jackson et al., 2004). Five micrograms of isolated histones per sample were used for Western blots. The proteins were run on a MINI-PROTEAN SFX, 12% gel (Bio-Rad #456-8043) and transferred to a nitrocellulose membrane (0.45 μm ; 24 h, 100 mA constant current, 4°C). The membrane was blocked with 0.5% Tween in Tris-buffered saline (TBS), and re-probed with various antibodies

(Merck Millipore, Billerica, MA, USA) as follows: anti-trimethyl-histone H3 [Lys-4] (cat. # 17-614), and anti-trimethyl-histone H3 [Lys-36] (cat. #17-10032). Trimethylated (H3me3/H3) levels were measured and compared to histones isolated from the different transgenic clones and from control calli. The amount of histone H3 for each sample was determined from Western blots using antibodies specific to non-methylated H3 (cat. # 04-928). The Clarity Western ECL Substrate kit from Bio-Rad (cat. # 170-5060), was used to develop the signal (according to the manufacturer's instructions). Both the gel and the membrane were analyzed and documented with the ImageLab software on a Bio-Rad Chemi Doc XR+ imaging system (www.bio-rad.com). Images were recorded every 1.0 s. Signals from bands obtained with methylation-specific antibodies were normalized against the respective histone H3 amounts (measured as the signal intensities of Western blot bands obtained with anti-histone H3 antibodies). Data from four independent measurements ($n = 4$) consistently gave the same results.

Results

Induction, Cellular Differentiation, and Somatic Embryogenesis in *Phaseolus vulgaris* Callus

To acquire somatic embryos, we employed a recently developed protocol designed for this purpose (Cabrera-Ponce et al., 2015). Common bean embryonic axes from zygotic embryos were cultivated in the osmotic-shock treatment media specified in the protocol (Figure 1A). After incubation, the embryonic axes were transferred to the embryo induction media (EIM). The first pro-embryogenic mass (PEM) was obtained 4 weeks after the osmotic shock, mainly from the cotyledonary zone (Figure 1B), and the pro-embryogenic callus were dissected and transferred to fresh EIM for propagation every 4 weeks.

Callus Transformation and Selection

The *PvTRX1h* gene of common bean is an ortholog of a major histone lysine methyltransferase and a focus of this study. As a first step, we created the *PvTRX1h*RiA construction, driving the expression of an antisense sequence from the *PvTRX1h* gene. This was used for callus transformation (Supplementary Figure SF1), with the intention that *PvTRX1h* would be down-regulated, by RNA interference (RNAi), in the transformed callus. Transformed calli were sub-cultured in fresh EIM containing hygromycin every 2 weeks for 3 months,

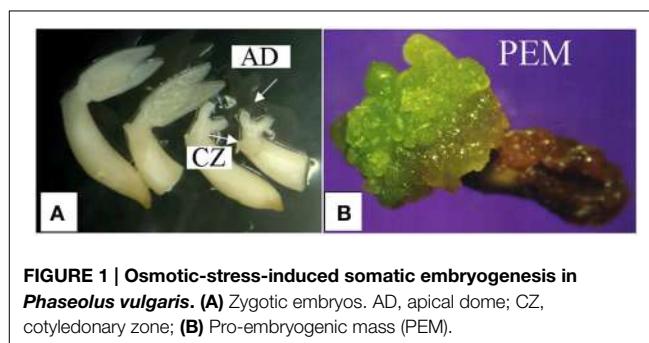


FIGURE 1 | Osmotic-stress-induced somatic embryogenesis in *Phaseolus vulgaris*. **(A)** Zygotic embryos. AD, apical dome; CZ, cotyledonary zone; **(B)** Pro-embryogenic mass (PEM).

until non-bombarded embryogenic callus stopped growing and eventually died. Thus, we monitored stable hygromycin-resistance calli with embryogenic capability for six successive generations following transformation (**Figure 2**).

After six generations, we had a total of 13 transgenic clones (**Figure 2D**). However, further maintenance of embryogenic calli for nine of the transgenic clones was not possible. They died after the sixth generation. Thus, 4 out of 13 resistant calli continue dividing for long-term propagation and later characterization (**Figure 3**). The transgenic calli had quite variable phenotypes and were classified into subgroups based on similarity of their phenotype. According to a recent classification (Ikeuchi et al., 2013), calli with no visible organ regeneration were denoted as friable or compact callus (**Figure 3A**) and calli that exhibited some degree of organ regeneration were denoted rooty, shooty, or embryonic callus, based upon the type of organs that developed (**Figures 3B–L**). Clone 8 developed both embryonic and shooty macroscopic structures (**Figures 3B–E**), clone 10 developed embryonic and rooty macroscopic structures (**Figures 3F–H**), clone 11 did not develop any organs, (**Figures 3I,J**), and clone 12 developed embryonic macroscopic structures (**Figures 3K,L**). Based on these phenotypes, transgenic clones 8, 10, and 12 were selected for further experiments. Clone 11 was not further analyzed because it had no visible organ regeneration.

Down-regulation of *PvTRX1h* Gene in Transgenic Callus Cultured *in vitro*

We next wished to verify and quantify down-regulation of *PvTRX1h* gene expression in the transgenic clones.

We performed qRT-PCR analysis in 3-week-old calli (**Figures 4A–D**). Transgenic clones 8 and 10 had a 2.4-fold down-regulation and clone 12 had a 3-fold down-regulation of the *PvTRX1h* transcript levels compared to the control, un-transformed calli (and when normalized with both reference genes, *PvActin11* and *PvEF1α*; **Figure 4E**). The transgenic nature of the calli was further confirmed by performing PCR amplification of the CaMV35S promoter (*PvTRX1hRiA* construction) from genomic DNA of transgenic callus clones 8, 10, and 12 (**Figure 4F**), as well as qRT-PCR to test for expression of the *hptII* gene (**Figure 4G**). This confirmed the transgenic nature of the calli, in agreement with their hygromycin selection or resistance.

Effects of Changes in Plant Hormones Content on Callus and Shoot Formation

The course of acquisition of embryogenic competence by somatic cells involves reprogramming of gene expression patterns in addition to changes in the morphology, physiology, and metabolism of plant cells. Endogenous hormone levels are major factors influencing somatic embryo induction (Fehér et al., 2003).

Thus, in order to study the effect of *PvTRX1h* down-regulation on plant hormone synthesis during somatic embryo formation, we analyzed the concentration of five different plant hormones in the three previously selected transgenic callus clones when they were three 3 s old. The hormones analyzed were indole-3-acetic acid in its free form (or IAA, an auxin), zeatin and N⁶-(Δ²-isopentenyl)-adenine (or 2iP; the latter two are cytokinins), ABA (an isoprenoid), and epibrassinolide (a brassinosteroid).

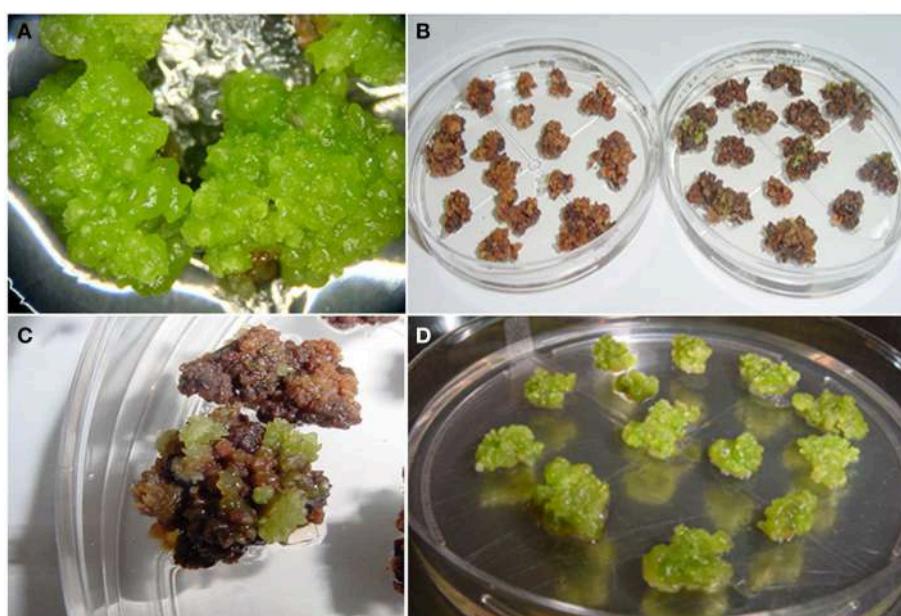


FIGURE 2 | Transformation and selection of *P. vulgaris* calli. (A)

Target embryogenic calli for particle gun bombardment transformation. Bombardment was performed on early globular-stage pro-embryogenic callus that had been sub-cultured for 3 months. Petri dishes containing 16–20 calli were bombarded. **(B)** Hygromycin resistant calli.

Transformed callus were selected in EIM plates containing 50 mg/L hygromycin. **(C)** Hygromycin resistant calli, close-up. **(D)** Propagation of hygromycin resistant callus. After bombardment, calli were sub-cultured every 2 weeks for 3 months in fresh EIM plates containing hygromycin.



FIGURE 3 | Phenotypes of pro-embryogenic callus and transgenic embryo formation in 6-week-old common bean. (A) Control pro-embryogenic callus (friable compact callus). **(B–L)** Transgenic callus transformed with the *PvTRX1h*-RiA silencing vector:

(B–E) Transgenic Clone 8 (embryonic and shooty callus). **(F–H)** Transgenic Clone 10 (embryonic and rooty callus). **(I,J)** Transgenic Clone 11 (friable callus). **(K,L)** Transgenic Clone 12 (embryonic callus).

Transgenic calli were cultured on auxin-free EIM medium supplemented with BAP, an inducer of somatic embryogenesis. Next, the plant hormone concentration was determined in the different calli by UPLC-ESI-MS. For IAA the LOD was 20.45 pmol/g fresh weight (FW) and the limit of quantification, LOQ, was 22.69 pmol/g FW; for ABA the LOD was 6.09 pmol/g FW and the LOQ was 10.27 pmol/g FW; for Zeatin the LOD was 22.69 pmol/g FW and the LOQ was 22.69 pmol/g FW; and for 2iP the LOD was 17.51 pmol/g FW and the LOQ was 18.64 pmol/g FW (Supplementary Figure SF2).

Compared to the control callus, where the concentration was below the LOD, the IAA concentration in all the transgenic clones was greater than the control callus, ranging from 47 to 92 pmol per gram of fresh weight (Figure 5A). The concentrations of the cytokinins (zeatin and 2iP) are shown in Figures 5B,C. In clones 8 and 10, zeatin concentration increased 232% and 196% compared to the control, respectively, while 2iP increased 76.9% and 83% over the control, respectively. The lowest zeatin concentration was detected in clone 12 (Figure 5B). In this clone, zeatin remained unchanged and 2iP increased as much as 63%, compared to the control. The ABA concentration of clones 10 and 12, as well as the control, were below the LOQ, while there was an increase in ABA concentration in clone 8 (11.8 pmol per gram of fresh weight), as compared to the control (Figure 5D). The epibrassinolide concentration, for all clones, was below the LOD (data not shown).

Down-regulation of *PvTRX1h* in Embryonic Callus Effects on Auxin Synthesis

Given that the concentration of IAA in its free form increased in all clones in which the *PvTRX1h* gene was down-regulated,

we analyzed the transcript levels of some of the genes coding for enzymes directly involved in IAA synthesis. The ones chosen represent the main pathways of tryptophan (Trp)-dependent IAA synthesis (Figures 6, 7; Supplementary Tables ST1, ST2). Specifically, we determined the transcript levels of the gene orthologs to the *Arabidopsis* *WEI2* (*WEAK ETHYLENE INSENSITIVE2/ANTHRANILATE SYNTHASE alpha 1*, *WEI2/ASA1*) (Stepanova et al., 2005); *WEI7* (*ANTHRANILATE SYNTHASE beta 1*, *WEI7/ASB1*) (Stepanova et al., 2005); *TAA1* (*TRYPTOPHAN AMINOTRANSFERASE 1*) (Stepanova et al., 2008); *AMI1* (*INDOLE-3-ACETAMIDE HYDROLASE 1*) (Pollmann et al., 2003); *CYP79B2* (*CYTOCHROME P450 MONOOXYGENASE CYP79B2*); *CYP79B3* (*CYTOCHROME P450 MONOOXYGENASE CYP79B3*) (Hull et al., 2000; Zhao et al., 2002); *NIT1* (*NITRILASE 1*) (Normanly et al., 1997); and *YUC1* and *YUC6* (*YUCCA1* and *YUCCA6* flavin-containing monooxygenases) (Stepanova et al., 2011; Mano and Nemoto, 2012; Dai et al., 2013) (see Figure 6 for a scheme of IAA synthesis).

We determined the expression levels of these auxin-synthesis-related genes in 3-week-old callus. Compared to the control callus (and when normalized with both reference genes, *PvActin11* and *PvEF1α*), the transcripts of *PvWEI2*, *PvWEI7*, and *PvCYP79B3* all increased in clones 8 and 10 (at least doubled, by about 50%, and at least tripled, respectively), but was unchanged in clone 12. In contrast, the transcript level of *CYP79B2* increased by 2.6-fold in clone 12, but was unchanged in the other clones (Figure 7C). The expression levels of *TAA1*, *NIT1*, and *AMI1* increased in all transgenic callus clones compared to the control (Figures 7E–G). Also, transcripts levels of *YUC1* decreased in transgenic clones 8 and 12, but was unchanged in clone 10

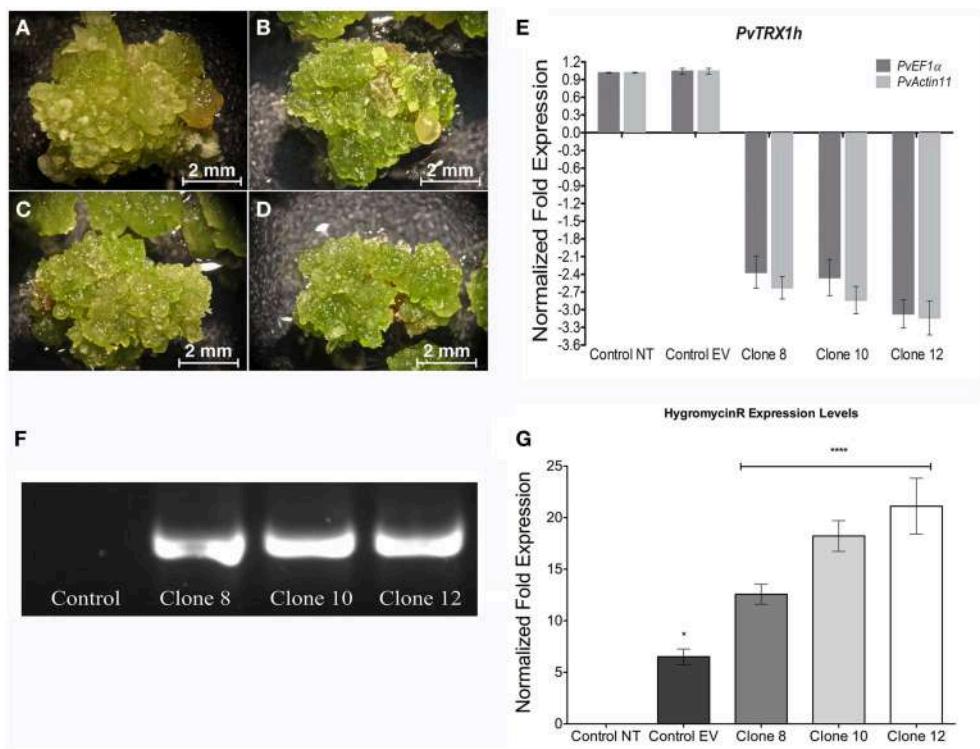


FIGURE 4 | Phenotypes of 3-week-old transgenic embryogenic calli and down-regulation of *PvTRX1h* in transgenic calli. **(A)** Control embryogenic callus. **(B)** Transgenic clone 8. **(C)** Transgenic clone 10. **(D)** Transgenic clone 12. **(E)** qRT-PCR analysis of *PvTRX1h* down-regulation in the transgenic clones. Each biological replicate was tested by triplicate and data were normalized to the *Actin11* (*PvActin11*) and to the Elongation Factor 1- α (*PvEF1 α*)

reference genes. Data represent mean \pm SD ($n = 3$ independent experiments) and were analyzed with an unpaired two-tailed Student's *t*-test (* $P < 0.05$, *** $P < 0.0001$). **(F)** PCR amplification of the CaMV35S promoter from genomic DNA to show the transgenic nature of the calli. **(G)** q-RT-PCR analysis of the *hptll* gene, to show the transgenic nature of the calli. Abbreviations: NT, non-transformed callus; EV, callus transformed with empty vector.

(Figure 7H). However, YUC6 transcript levels increased from about 2- to 4.8-fold in clones 10 and 12 compared to the control, respectively (Figure 7I); but was unchanged in clone 8.

Down-regulation of *PvTRX1h* Affects the Expression of *PvASHH2h*

The concentration of the cytokinins (zeatin and 2iP) and ABA (clone 8) was altered in the transgenic calli compared to the transformed controls (Figure 5), prompting us to measure the expression level of *PvASHH2h*, the gene orthologous to the *Arabidopsis thaliana* ABSENT, SMALL or HOMEOSTATIC DISCS 1 HOMOLOG 2 gene (*ASH1 HOMOLOG 2* or *ASHH2*) in *P. vulgaris*. *ASHH2* is a major H3K36 histone lysine methyltransferase (HKMT) in *Arabidopsis* (Xu et al., 2008). This gene has also been shown to be involved in the induction of the jasmonate/ethylene pathway genes (Berr et al., 2010), in the regulation of carotenoid biosynthesis and carotenoid-derived hormones (Cazzonelli et al., 2009), and in the regulation of the expression of BR-related genes (Wang et al., 2014). Expression of *PvASHH2h* increased in clones 8, 10 and 12, as much as 35, 76, and 96% (Figure 7K).

Next, we determined the transcript levels in the transgenic calli of some genes involved in the synthesis of

cytokinins (zeatin and 2iP), ABA, and BR (Figure 8 and Supplementary Table ST1). Specifically, we determined the transcript levels of the gene orthologs to the *Arabidopsis* *IP1* (ADENYLATE ISOPENTENYLTRANSFERASE 1), *CYP735A1* (CYTOCHROME P450, FAMILY 735, SUBFAMILY A, POLYPEPTIDE 1), *NCED3* (9-CIS-EPOXYCAROTENOID DIOXYGENASE 3), *ABA2* (XANTHOXIN DEHYDROGENASE), *AAO3* (ABSCISIC ALDEHYDE OXIDASE 3), *DET2/DWARF6* (STEROID REDUCTASE DET2/DWARF6), *BR6OX1* (BRASSINOSTEROID-6-OXIDASE 1), *BR6OX2.1* (BRASSINOSTEROID-6-OXIDASE 2 ISOFORM 1), *BR6OX2.2* (BRASSINOSTEROID-6-OXIDASE 2 ISOFORM 2).

Compared to the control callus (and when normalized with both reference genes, *PvActin11* and *PvEF1 α*), the expression of *PvIP1* and *PvCYP735A1*, two genes involved in cytokinin biosynthesis, increased in all transgenic calli, in a straight correlation with the increased concentrations of the cytokinins (zeatin and 2iP) (Figures 8A,B). In contrast, the expression levels of *PvABA2*, *PvAAO3*, and *PvNCED3*, all involved in ABA biosynthesis, showed dissimilar patterns of expression (Figures 8C–E). *PvABA2* increased by about 0.2-fold in all clones, *PvNCED3* decreased 30% in clone 8, but was unchanged in the other clones, and *PvAAO3* increased by 37-fold in clone 12.

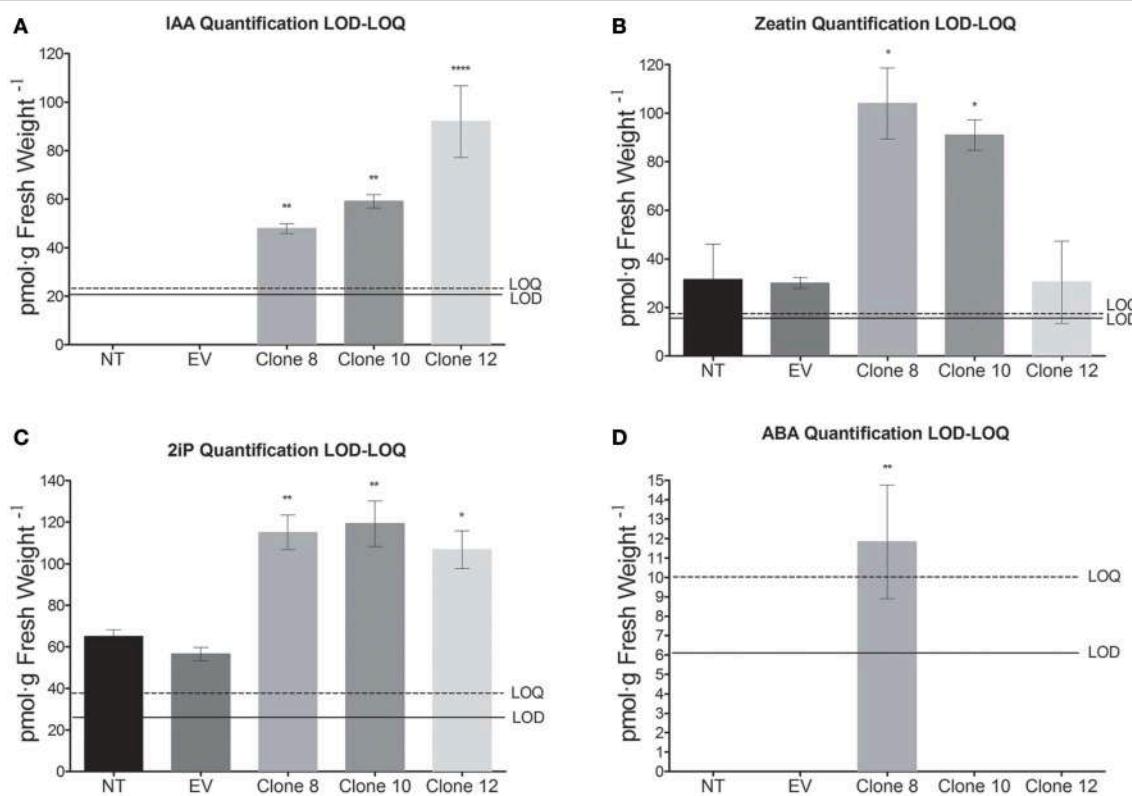


FIGURE 5 | Plant hormone content determined by UPLC-ESI-MS in 3-week-old *P. vulgaris* transgenic callus clones. **(A)** IAA, **(B)** zeatin, **(C)** 2iP, **(D)** ABA. Data represent mean \pm SD ($n = 3$ independent experiments) and were analyzed with an unpaired two-tailed Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). For IAA the limit of detection, LOD, was 20.45 pmol/g fresh weight (FW) and the

limit of quantification, LOQ, was 22.69 pmol/g FW; for ABA the LOD was 6.09 pmol/g FW and the LOQ was 10.27 pmol/g FW; for Zeatin the LOD was 22.69 pmol/g FW and the LOQ was 22.69 pmol/g FW; and for 2iP the LOD was 17.51 pmol/g FW and the LOQ was 18.64 pmol/g FW. Abbreviations: NT, non-transformed callus; EV, callus transformed with empty vector.

Expression of four genes involved in BR biosynthesis (*BR6OX1*, *BR6OX2.1*, *BR6OX2.2*, and *DET2/DWARF6*) increased in all transgenic calli (except for *BR6OX2.2* in clone 12), indicative of an up-regulation of BR biosynthesis.

Changes in Histone H3K4 Trimethylation Patterns as a Result of PvTRX1h Down-regulation

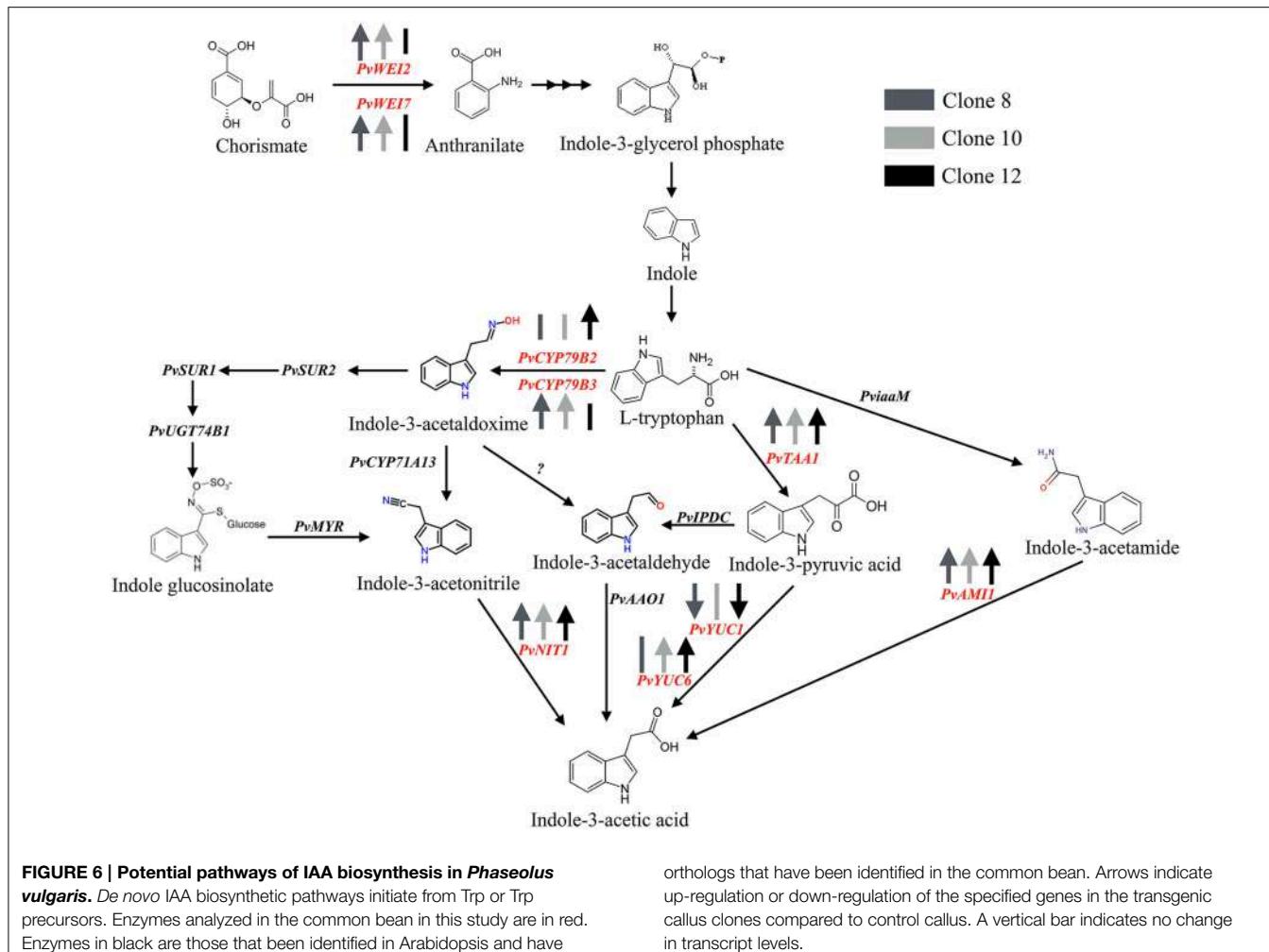
We next wished to examine the effect of down-regulation of *PvTRX1h* on the global patterns of histone methylation. Three-week-old calli were analyzed by Western blots using antibodies against the H3K4 trimethylated isoform (H3K4me3; a histone mark catalyzed by the orthologous gene *ATX1*), as well as for the H3K36me3 mark (a histone mark catalyzed by orthologous gene *ASHH2*).

We observed changes in global H3K4me3 methylation patterns in the different transgenic clones that are related to *PvTRX1h* down-regulation, to the *PvASHH2h* transcript levels, and to the plant hormone concentration. In clones 8 and 10, with a 2.4-fold down-regulation of the *PvTRX1h* gene, the global H3K4me3 mark decreased (22 and 10%, respectively; Figure 9), but *PvASHH2h* expression increased (35 and 76%, respectively), accompanied by an increase in of IAA (in its free form) and

cytokinins compared to the control. In transgenic clone 12, with 3-fold down-regulation of *PvTRX1h*, the global H3K4me3 mark increased 6%, *PvASHH2h* expression increased 96.7%, and IAA and 2iP increased compared to the control. However, even though *PvASHH2h* expression increased, the traceable changes in the global H3K36me3 methylation patterns are not statistically significant (Figure 9). This could be due to, for example, the level of methylation (mono-, di-, tri-) imparted and associated with the transcription of active euchromatin, as well as the transcriptional repression associated with H3K36 methylation, through modulating H3K36 (mono-, di-, tri-) methylation levels (Wagner and Carpenter, 2012).

Morphology and Histology of Transgenic Embryonic Callus

The next endeavor was to characterize the developmental stages of common-bean somatic embryos and the histology of the various structures and tissues at the stages (shown in Figure 10). For this purpose, and taking into account the inherent variation of transgene expression in the different transformants, clone 8 was chosen because it evidenced several developmental stages of somatic embryos in the same embryogenic callus (Figures 3,



10, 11). This clone also had an interesting phenotype, with embryonic, shooty, and rooty macroscopic structures and was the most prolific of the clones with respect to somatic embryogenesis (**Figure 12**). That is, 3 weeks after the callus were transferred to fresh EIM, clone 8 had 7.2 somatic embryos present per proembryogenic mass (PEM), clone 10 had 6.6 and clone 12 had 6.8 somatic embryos present per PEM, respectively. Whereas the non-transformed calli and those calli transformed with the empty vector had 3.8 and 3.9 somatic embryos present per PEM.

Histological examination of clone 8 (ontogeny of somatic embryogenesis; **Figure 11**), revealed that early-stage calli were not masses of unorganized cells, but had highly organized early-stage structures, specifically: globular (**Figures 11A,B,E**), heart (**Figures 11C,F,G**), intermediate, or torpedo early stages (**Figures 10D,H**), reminiscent of dicotyledonous embryos (**Figures 11J,K**). The globular, heart, and torpedo embryos of this clone and the WT's were of similar size. As many as 15 somatic embryos per 10- μm section of callus could be discerned under the microscope (**Figure 11**).

Figure 11 shows an embryo at late globular stage with a suspensor-like structure, in which some rows of cells appear

to support a connection between the globular embryo and the parental tissue (**Figures 11A,B,E**). According to Williams and Maheswaran (1986), embryos attached to parental tissue by a suspensor-like structure may originate from a single cell.

The embryos progress was monitored. They developed normally from globular to heart-shaped, torpedo, then cotyledonary stages. Typical structures of WT mature embryo stages (late torpedo to cotyledonary stage) were apparent: cotyledons, apical meristems, procambium tissue, shoot primordia, and root axes (**Figures 11J-L**). In addition, we observed *de novo* meristemoid structures and unipolar adventitious shoots that emerged from the parental tissue (**Figure 11L**). Some abnormal histo-differentiated embryos were observed. For example, fused embryos developed into what looked like fasciated-like cotyledonary embryonic structures (**Figure 11M**) that seemed to result from fusion of early-stage globular embryos (Dos Santos et al., 2006).

At least for clone 8, the decrease in global H3K4me3, the small increase in the *PvASHH2h* expression, and the increase in the concentration of all four hormones tested may indicate that the level of down regulation of *PvTRX1h*, associated with such changes, are favorable conditions to initiate callus differentiation.

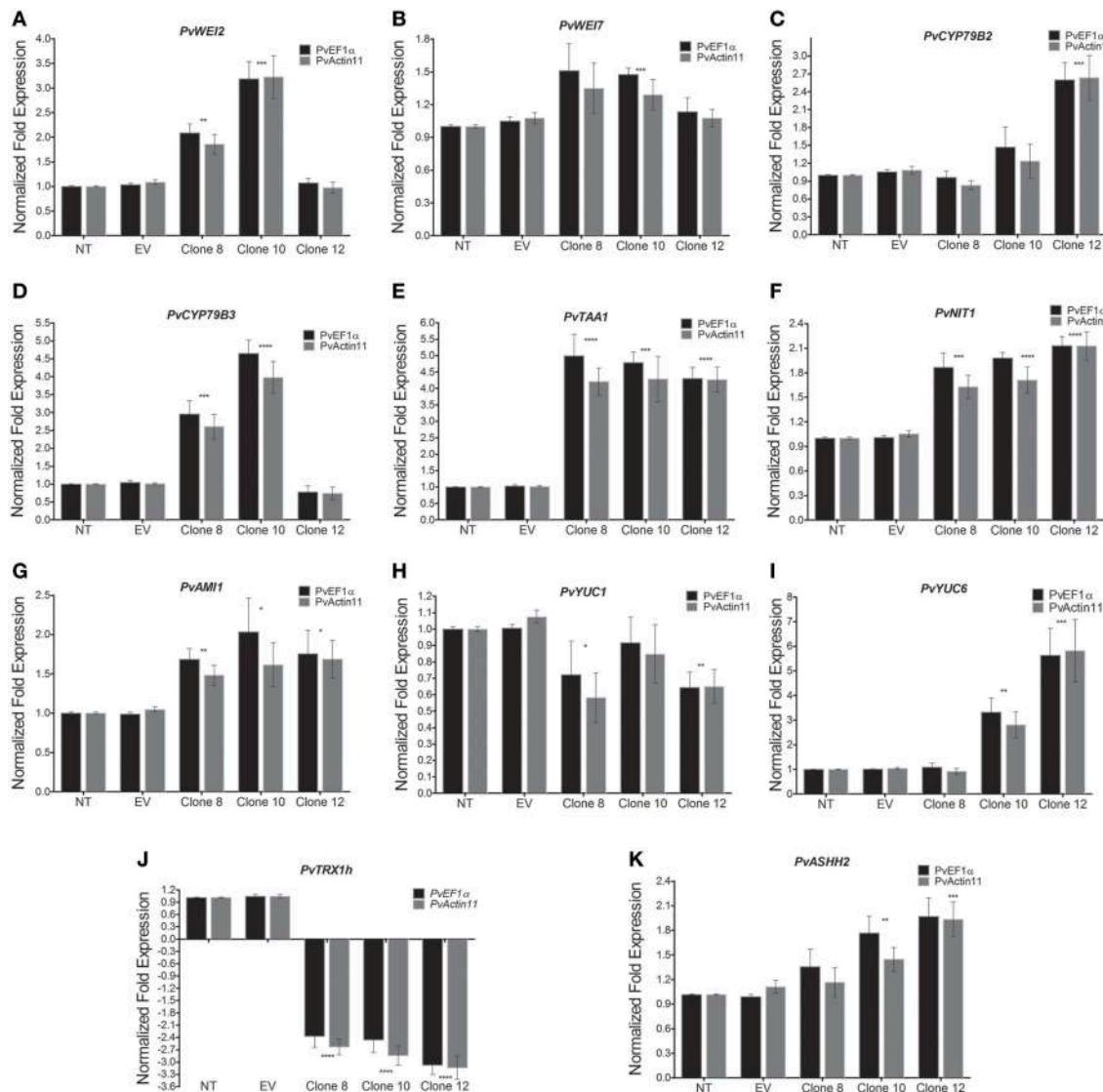


FIGURE 7 | Transcript levels of some genes involved in IAA biosynthesis as determined by qRT-PCR in 3-week-old callus. (A) *PvWEI2* (ANTHRANILATE SYNTHASE alpha 1, ASA1), (B) *PvWEI7* (ANTHRANILATE SYNTHASE beta 1, ASB1), (C) *PvCYP79B2* (CYTOCHROME P450 MONOOXYGENASE CYP79B2), (D) *PvCYP79B3* (CYTOCHROME P450 MONOOXYGENASE CYP79B3), (E) *PvTAA1* (TRYPTOPHAN AMINOTRANSFERASE 1), (F) *PvNIT1* (NITRILASE 1), (G) *PvAM1*

(INDOLE-3-ACETAMIDE HYDROLASE 1), (H) *PvYUC1* (YUCCA1 flavin monooxygenase), (I) *PvYUC6* (YUCCA6 flavin monooxygenase) (J) *PvTRX1h*, (K) *PvASHH2* (*P. vulgaris* absent, small or homeotic discs 1 homolog 2). Data represent mean \pm SD ($n = 3$ independent experiments) and were analyzed with an unpaired two-tailed Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Abbreviations: NT, non-transformed callus; EV, callus transformed with empty vector.

Discussion

The goals of this study were 2-fold: first, we have endeavored to develop regeneration-competent callus in common bean and second, we have studied the regulation of somatic embryogenesis in this crop plant, choosing to focus on epigenetic regulation. Common bean is an important food and feed crop worldwide and there are ongoing, major efforts to improve it. These efforts, at present, are mainly limited to conventional breeding practices, as this crop is recalcitrant to both induction of somatic embryogenesis and transformation, and is difficult to regenerate.

Consequently, stable genetic transformation is hard to achieve for this organism. Thus, the development of regeneration-competent callus and its successful transformation would be a valuable first step toward establishing an efficient plant regeneration system and genetic transformation in *P. vulgaris*. Although regeneration-competent callus have been obtained from pedicels of two genotypes of *P. vulgaris* (Mohamed et al., 1993) and, through a similar approach, regeneration was achieved in tepary bean (*P. acutifolius*) (Dillen et al., 1996), the protocol is considerably less reproducible and efficient than reported (Zambre et al., 1998).

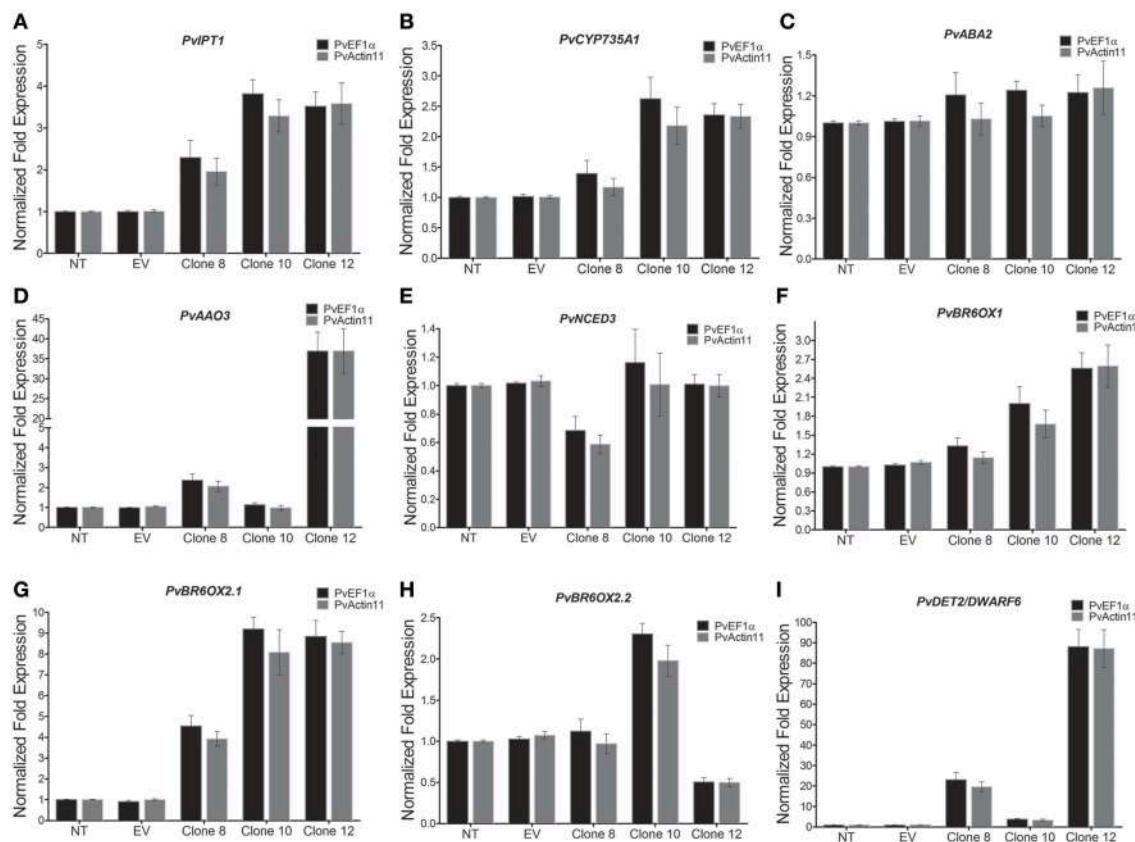


FIGURE 8 | Transcript levels of some genes involved in zeatin, 2iP, ABA, and BR biosynthesis as determined by qRT-PCR in 3-week-old callus. (A) *PvIPT1* (ADENYLATE ISOPENTENYLTRANSFERASE 1), (B) *PvCYP735A1* (CYTOCHROME P450, FAMILY 735, SUBFAMILY A, POLYPEPTIDE 1), (C) *ABA2* (XANTHOXIN DEHYDROGENASE), (D) *AAO3* (ABSCISIC ALDEHYDE OXIDASE 3), (E) *NCED3*, (F) *PvBR6OX1* (9-CIS-EPOXYCAROTENOID DIOXYGENASE 3), (G) *PvBR6OX2.1*

(BRASSINOSTEROID-6-OXIDASE 1), (G) *BR6OX2.1* (BRASSINOSTEROID-6-OXIDASE 2 ISOFORM 1), (H) *BR6OX2.2* (BRASSINOSTEROID-6-OXIDASE 2 ISOFORM 2), (I) *DET2/DWARF6* (STEROID REDUCTASE *DET2/DWARF6*). Data represent mean \pm SD ($n = 3$ independent experiments) and were analyzed with an unpaired two-tailed Student's *t*-test. Abbreviations: NT, non-transformed callus; EV, callus transformed with empty vector.

The potential for somatic embryogenesis varies with plant species and among genotypes within a species (Deo et al., 2010) and, therefore, would seem to be related to gene expression and not to the absence of the relevant genes. One possible mechanism of regulation, chromatin remodeling, has two major roles during the early stages of somatic embryogenesis. Differentiation (associated with the first phase of chromatin decondensation) requires unfolding of the supercoiled chromatin structure, allowing expression of genes previously inactivated by heterochromatinization. Subsequent chromatin remodeling results in the specific activation of a set of genes required for embryonic development (Fehér et al., 2003; Solís-Ramos et al., 2012).

We consider that the phenotypic variation observed in our transgenic lines, which ranged from highly embryogenic to minimally embryogenic or recalcitrant, was due to differential expression of *PvTRX1h*, specifically, down-regulation, in association with the changes in plant hormone concentration reported here, although the known variation of transgene expression in transformants should not be discounted.

Furthermore, the alterations in the expression of the genes analyzed here, specifically, the genes coding for enzymes in the plant hormones biosynthetic pathways, *PvASHH2h*, and the many other unidentified genes important at various stages of somatic embryogenesis, are presumably an indirect result of *PvTRX1h* down-regulation, since the orthologs to *PvTRX1h* are involved in the establishment of the trimethylation pattern of histone H3 lysine 4 (H3K4me3), a mark related to gene activation.

Since the pioneering work by Skoog and Miller (1957), it has been understood that the balance between auxins and cytokinins is reflected in the state of cell differentiation and dedifferentiation. A high auxin to cytokinin ratio induces root regeneration, whereas a low ratio promotes shoot induction, suggestive of auxin-cytokinin crosstalk during *in vitro* organogenesis, although the molecular mechanism of such interaction in the *in vitro* meristem formation is unknown (Su et al., 2011). The observation that the somatic embryos of the different clones in this study had a variety of developmental stages leads us to speculate that down-regulation of *PvTRX1h*

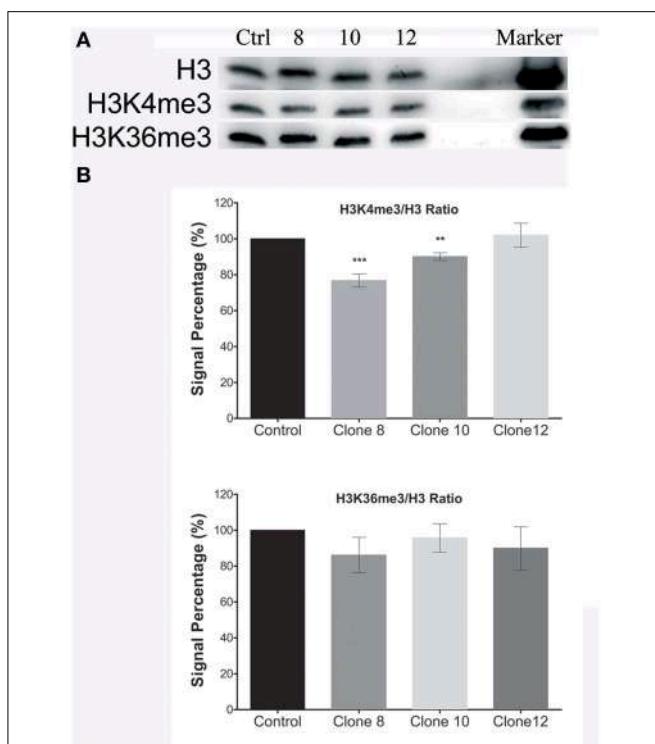


FIGURE 9 | Histone H3 methylation patterns tested by using Western blot analyses with specific antibodies generated toward each modified histone H3. (A) Western blot analysis shows the amount of histone methylation in the transgenic clones. **(B)** Bar graphs showing quantification of the histone methylation (H3K4me3 and H3K36me3) in the transgenic clones. Total histones extracted from 3-week-old non-transformed (control) and transgenic calli samples were probed with antibodies specific for tri-methylated K4/H3 and K36/H3 in Western blots. Subsequent to the hybridization, membranes were stripped off and reprobed with antibodies specific for non-modified histone H3. The levels of histone H3-tail methylation, defined as the ratio of mK/H3-to-H3 intensity signals, were taken as 100%. Data represent mean \pm SD ($n = 4$ independent experiments) and were analyzed with an unpaired two-tailed Student's *t*-test (** $P < 0.01$, *** $P < 0.001$).

in the transgenic callus influenced the concentrations of plant hormones, particularly auxins (free IAA) and cytokinins.

This speculation was directly supported with plant hormone measurements in the *PvTRX1h*-RNAi transgenic calli and the non-transformed callus. The IAA content of all three transgenic calli clones was greater than the control line and we hypothesize that the changes in plant hormones concentration, particularly the auxin to cytokinin ratio and the increase in free IAA content, caused the developmental effects observed, most striking in clone 8 (Figures 3, 10). We stress the importance of auxin partly because of its well-known effects on nearly every aspect of plant growth and development (Woodward and Bartel, 2005) as well as our finding that IAA, in its free form, was the only plant hormone that could be quantified in excess in all clones tested.

The quantification of the plant hormones analyzed leads us to hypothesize that there was crosstalk between the different hormones, especially between IAA and cytokinins. Also, this crosstalk appears to be a result of the different degrees of

PvTRX1h gene down-regulation in the respective calli (Figure 4) and may be related to the different phenotypes observed (Figures 3, 10). However, the molecular mechanism of such hormone interaction in calli remains unknown.

The increase of free IAA content in all the *PvTRX1h*-RNAi clones led us to analyze the expression patterns of the genes involved in its biosynthesis (Figures 6, 7). Two major pathways for IAA biosynthesis have been proposed: the Trp-dependent and Trp-independent. The Trp-independent path branches from the L-Trp biosynthetic pathway at steps involving indole or indole-3-glycerol phosphate, although the pathway has not been completely elucidated. On the other hand, the Trp-dependent pathways have been carefully characterized and we analyzed the transcript levels of some genes involved in these pathways.

Four main paths for the Trp-dependent IAA syntheses have been described: the indole-3-acetaldoxime (IAOx), tryptamine (TAM), indole-3-acetamide (IAM), and the indole-3-pyruvic acid (IPA) pathways (Stepanova et al., 2005; Su et al., 2011) (Figure 7). *WEI2* and *WEI7* code for the alpha- and beta-subunits, respectively, of a rate-limiting enzyme for Trp biosynthesis, anthranilate synthase (Stepanova et al., 2005), which catalyzes the conversion of chorismate to anthranilate. Transcriptional induction of *WEI2* and *WEI7* should, therefore, enhance auxin biosynthesis, as proposed by Stepanova (Stepanova et al., 2005), and as we have shown here for the transgenic calli. The two P450 monooxygenases, CYP79B2 and CYP79B3, oxidize Trp into indole-3-acetaldoxime (IAOx) *in vitro*. We suggest then, that the differential and specific expression levels of these two genes in the different transgenic clones reflect the redundancy within IAA biosynthetic pathways, as they might compensate each other during IAOx synthesis (Zhao et al., 2002). Whereas the YUCCA (YUC1 and YUC6) flavin-containing monooxygenases (FMOs) catalyze a rate-limiting step in auxin biosynthesis; they convert indole-3-pyruvate (IPA) to indole-3-acetate (IAA).

WEI2, *WEI7*, and *CYP79B3* were up-regulated in clones 8 and 10, while clones 10 and 12 showed specific up-regulation of *CYP79B2* (Figure 7). The *TAA1*, *AMI1*, and *NIT1* genes were up-regulated in all three clones. *YUC6* was up-regulated in clones 10 and 12, while *YUC1* was down-regulated in all clones. From these results, we hypothesize that *PvTRX1h* has an indirect effect on the synthesis of IAA. It appears that *PvTRX1h*, by regulating chromatin structure in developmental transitions, is able to regulate the expression of genes involved in IAA biosynthesis, most likely through the activation or repression of an unknown intermediary effector. Altogether, the changes in the transcript levels for the genes involved in IAA biosynthesis, as shown on Figures 6, 7, led to an overproduction of free IAA in the transgenic clones.

We were also interested in the expression levels of *PvASHH2h*, an ortholog to a major histone lysine methyltransferase in *Arabidopsis* able to methylate lysine 4 and 36 of histone H3 (H3K4 and H3K36; Xu et al., 2008). Like *PvTRX1h*, this gene is implicated in plant hormone biosynthesis. We observed an inverse correlation between the up-regulation of this gene and the down-regulation of *PvTRX1h* (Figures 7H,I). Transgenic callus clone 12 had the greatest down-regulation of *PvTRX1h*



FIGURE 10 | Differentiation of embryogenic calli from 10-week-old transgenic clone 8, cultured on regeneration media. **(A–F)**: callus No. 1, **(G)**: callus No. 2, **(H)**: callus No. 3, **(I–K)**: calli No. 4 and 5.

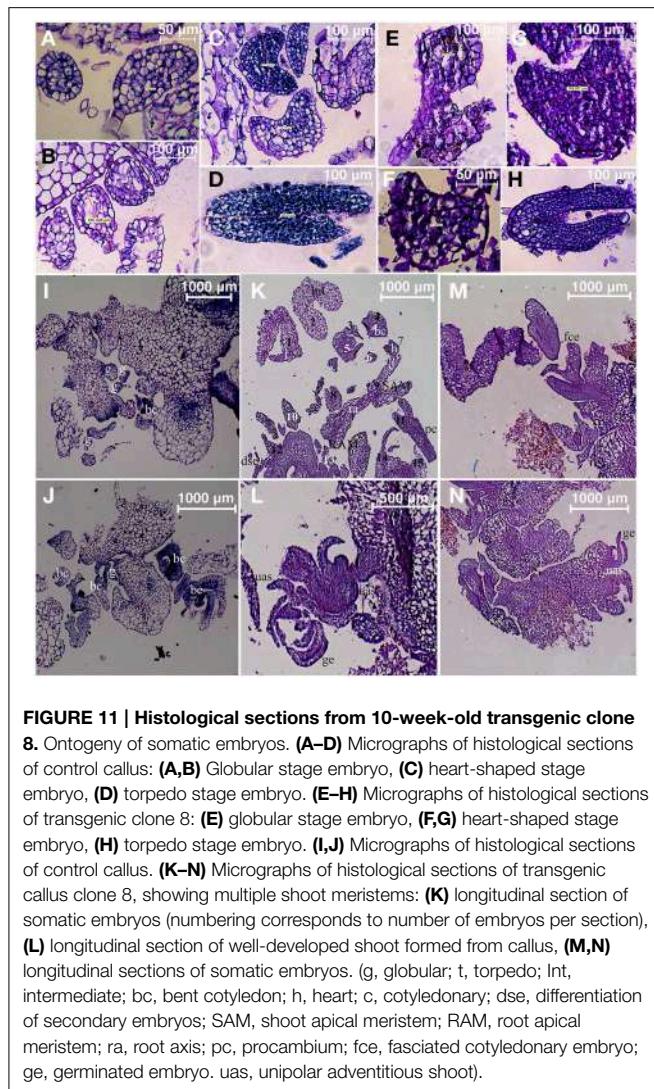
and, in turn, the highest expression of *PvASHH2h*, along with the greatest concentration of IAA (and to some extent 2iP). Clone 10 had the second highest expression of *PvASHH2h*, while clone 8 was lower. These results could be consistent with two sets of observations relating to these genes in *Arabidopsis*. First, the *ARABIDOPSIS TRITHORAX 1* (*ATX1*) gene, which is orthologous to *PvTRX1h*, participates in cell proliferation and cell patterning processes in the root apical meristem independently of auxin and the developmental abnormalities seen in *atx1-1* roots are unrelated to auxin response gradients (Napsucialy-Mendivil et al., 2014). Second, *ASHH2* (or *SDG8*) is involved in the activation of a subset of genes within the JA/ET signaling defense pathway (Berr et al., 2010) and in BR-regulated gene expression (Wang et al., 2014), and the *ashh2* loss-of-function mutant displays a reduced growth phenotype with compromised JA/ET and BR responses (Berr et al., 2010; Wang et al., 2014). These suggest that the two trithorax-Group (TrxG) histone methyltransferases carry out opposite non-redundant functions in callus (evidence provided in this study), roots, and plant development in general (Alvarez-Venegas et al., 2006; Xu et al., 2008; Berr et al., 2010; Napsucialy-Mendivil et al., 2014; Wang et al., 2014).

We also assessed the global patterns of histone methylation by Western blots, using antibodies against H3K4me3, as well as against the H3K36me3 mark. As noted earlier, greater *PvTRX1h* down-regulation resulted in higher *PvASHH2h* expression, and this resulted in re-establishment of the global histone H3K4me3 methylation levels (mainly for clone 12). This might be indicative of crosstalk between these two histone methyltransferases. We hypothesize that the changes in the global H3K4me3 mark in the transgenic clones, directly associated with the deposition of the same mark (as well as the H3K36me3 mark) by another specific histone methyltransferases (*PvASHH2h*), are involved in the synthesis of plant hormones and, in the case of IAA, are

related to the changes in transcript levels of the different genes involved in its synthesis.

The down-regulation of *PvTRX1h* permitted us to generate embryogenic calli. These calli overproduced several compounds, most notably IAA and cytokinins. Hormonal signaling pathways interact at the level of gene expression. For example, cytokinin and auxin are antagonistic during lateral root initiation. Cytokinins perturb the expression of *PIN* genes in lateral root founder cells, inhibiting the formation of an auxin gradient that is necessary for lateral root initiation (Laplaze et al., 2007). Also, target genes repressed by auxin are also repressed by brassinosteroids, and genes induced by auxin are induced by brassinosteroids, indicative of coordination between the signaling pathways (Santner and Estelle, 2009).

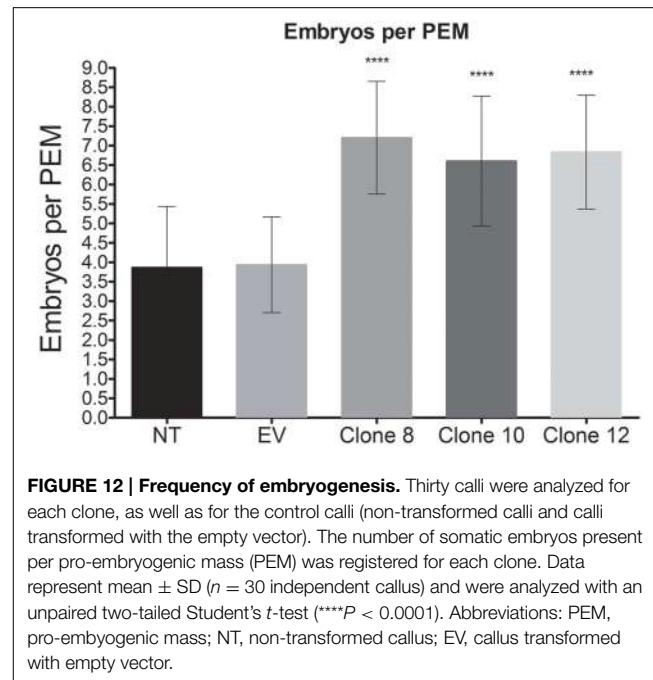
The increased levels of both free IAA and cytokinins in the *PvTRX1h*-RNAi transgenic calli may also be related to the unforeseen over-expression of the *PvASHH2h* gene. For example, down-regulation in rice of the *SDG725* gene, orthologous to *ASHH2*, which codes for a histone H3 lysine 36 methyltransferase, produces plants with phenotypes that are very similar to those of BR-deficient mutants (Sui et al., 2012). It is noteworthy that several BR biosynthesis-related genes are down-regulated in *SDG725* RNAi mutants (Sui et al., 2012). Accordingly, the up-regulation of the *PvASHH2h* gene in the *PvTRX1h* RNAi transgenic calli, and the up-regulation of several genes involved in the biosynthesis of BR (Figure 9) suggests that *PvASHH2h* has an active role in the regulation of BR-related genes and BR biosynthesis in common bean calli, as it does in rice and *Arabidopsis* (Normanly et al., 1997; Sui et al., 2012), although the epibrassinolide concentration was below the LOD in all transgenic calli. This also supports our contention that the *PvTRX1h* and *PvASHH2h* genes carry out opposite non-redundant functions in callus, roots, and in plant development in general. Altogether, the free IAA content enhancement and



the variation in cytokinins concentration for the clones analyzed support indirect regulation by PvTRX1h of biosynthesis of some of the plant hormones and somatic embryo development.

Conclusions

Down-regulation of the *PvTRX1h* gene in pro-embryogenic calli of *P. vulgaris* had multiple effects. Transgenic pro-embryogenic calli were able to differentiate and form somatic embryos with diverse phenotypes, an overproduction of somatic embryos was achieved in the transgenic clones, the concentration of different plant hormones was altered, and *PvTRX1h* appeared to regulate the expression of genes involved in auxin biosynthesis. These observations suggest that *PvTRX1h* regulates somatic embryogenesis and plant hormone synthesis. In addition, *PvTRX1h* and *PvASHH2h* carry out opposite non-redundant functions in embryogenesis, indicative of crosstalk among histone methyltransferases and plant hormone signaling. Furthermore, our results indicate that epigenetic changes such



as histone methylation have an active role in the regulation of plant hormone biosynthesis in common bean calli, as has been shown in rice and *Arabidopsis* for BR-related genes and BR biosynthesis. New approaches of this kind and the development of new technologies, particularly regeneration of common bean plants, will undoubtedly increase our knowledge of the crosstalk among histone methyltransferases, plant hormone signaling, and gene regulation of somatic embryogenesis.

Author Contributions

RA provided the idea of the work. RA, AB, JC, and RW designed the experiments. AB conducted the histological analysis, Western blots, RT-PCR, qRT-PCR, and sample preparation for UPLC-ESI-MS experiments. JC conducted the callus transformation and selection. RG, AB, and RW performed the UPLC-ESI-MS analysis. FL created the *PvTRX1hRiA* silencing vector. RA, AB, and RW participated in the interpretation of results and critically reviewed the manuscript. RA wrote the paper. 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://journal.frontiersin.org/article/10.3389/fpls.2015.00577>

Supplementary Figure SF1 | Map of the PvTRX1hRiA construction.

Construction of the PvTRX1hRiA silencing vector, driving the expression of an antisense sequence from the *PvTRX1h* gene, under control of the ectopic CaMV35S promoter.

Supplementary Figure SF2 | Establishment of the limit of detection (LOD) and the limit of quantification (LOQ) based in the signal intensity percentage, derived from the UPLC-MS intensity data, of transgenic callus clones compared with control callus of the different plant hormones analyzed.

In the cases where the signal was below the LOD and LOQ, "spiking" determinations were performed. **(A)** IAA content was determined for transgenic callus clones since IAA signal intensity was above the LOD (20.45 pmol/g FW) and LOQ (22.69 pmol/g FW), whereas in control callus IAA signal intensity was below the LOD and LOQ. **(B)** ABA content was determined only for transgenic callus clone 8 since ABA signal intensity was above LOQ

(10.27 pmol/g FW); however in transgenic callus clones 10 and 12, and control calli, ABA signal intensity were above the LOD (6.09 pmol/g FW) but below LOQ (10.27 pmol/g FW). **(C)** Zeatin content was determined for all transgenic callus clones and control callus since Zeatin signal intensity for all samples analyzed were above LOD (22.69 pmol/g FW) and LOQ (22.69 pmol/g FW). **(D)** 2iP content was determined for all transgenic callus clones and control calli since 2iP signal intensity for all samples analyzed were above the LOD (17.51 pmol/g FW) and LOQ (18.64 pmol/g FW).

Supplementary Table ST1 | List of the auxins (IAA), abscisic acid (ABA), cytokinins (2-iP and zeatin), and brassinosteroids (epibrassinolide) biosynthetic pathway genes analyzed by qPCR.**Supplementary Table ST2 | Primer list used for determination of expression levels by q-PCR and presence of the CaMV35s promoter.**

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The role of chromatin modifications in somatic embryogenesis in plants

Clelia De-la-Peña¹, Geovanny I. Nic-Can², Rosa M. Galaz-Ávalos³,
Randy Avilez-Montalvo³ and Víctor M. Loyola-Vargas^{3*}

¹ Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Mérida, Mexico, ² Facultad de Ingeniería Química, Campus de Ciencias Exactas e Ingeniería, Universidad Autónoma de Yucatán, Mérida, Mexico, ³ Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, Mexico

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*Correspondence:

Victor M. Loyola-Vargas,
Unidad de Bioquímica y Biología
Molecular de Plantas,
Centro de Investigación Científica
de Yucatán, Calle 43, No. 130 Colonia
Chuburná de Hidalgo, Mérida
CP 97200, Yucatán, Mexico
vmloyola@cicy.mx

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Somatic embryogenesis (SE) is a powerful tool for plant genetic improvement when used in combination with traditional agricultural techniques, and it is also an important technique to understand the different processes that occur during the development of plant embryogenesis. SE onset depends on a complex network of interactions among plant growth regulators, mainly auxins and cytokinins, during the proembryogenic early stages, and ethylene and gibberellins and abscisic acids later in the development of the somatic embryos. These growth regulators control spatial and temporal regulation of multiple genes in order to initiate change in the genetic program of somatic cells, as well as moderating the transition between embryo developmental stages. In recent years, epigenetic mechanisms have emerged as critical factors during SE. Some early reports indicate that auxins and *in vitro* conditions modify the levels of DNA methylation in embryogenic cells. The changes in DNA methylation patterns are associated with the regulation of several genes involved in SE, such as *WUS*, *BBM1*, *LEC*, and several others. In this review, we highlight the more recent discoveries in the understanding of the role of epigenetic regulation of SE. In addition, we include a survey of different approaches to the study of SE, and new opportunities to focus SE studies.

Keywords: DNA methylation, epigenetics, histone modification, somaclonal variation, somatic embryogenesis

Introduction

Somatic embryogenesis (SE) is a powerful tool for plant genetic improvement when it is used in combination with traditional agricultural techniques (Loyola-Vargas et al., 2008). SE onset depends on a complex network of interactions among plant growth regulators, mainly auxins and cytokinins, during the proembryogenic early stages, and ethylene and gibberellins and abscisic acids later in the development of somatic embryos. These growth regulators control the spatial and temporal expression of multiple genes in order to initiate change in the genetic program of the somatic cells, as well as the transition between embryo developmental stages (Fehér, 2015).

Plants as well as animals have sophisticated mechanisms to regulate cellular division, development and growth (Albert and Peters, 2009; Gonzalo, 2010; Wollmann and Berger, 2012; Nic-Can et al., 2013). Chromatin organization allows the expression or repression of genes depending on the degree of its compaction in a specific locus (Schones and Zhao, 2008; Tamaru, 2010). This chromatin compaction results from two main processes, histone modification and DNA methylation. Both are present in plants and animals; however, DNA methylation in plants is more complex than in animals. Other processes controlled by DNA methylation are the transcription of invading and mobile DNA elements, such as

viruses, transgenes, transposons, and retroelements (Law and Jacobsen, 2010; Feng and Jacobsen, 2011).

DNA methylation is carried out by the addition of a methyl group at the 5' position of the pyrimidine ring of cytosine in the DNA (5mC). In animals, this methylation occurs in a cytosine that is adjacent to a guanine (CpG) (Vanyushin, 1984). However, methylation in plants is not always in the CpG islands (Gruenbaum et al., 1981; Belanger and Hepburn, 1990); it can also be done in CpHpG and CpHpH (where H is any nucleotide except G; Finnegan et al., 1998; Feng et al., 2010).

During early embryo development, DNA methylation is continually changing in order to satisfy the cell requirements. In animals, *de novo* methylation is necessary for embryo implantation (Monk et al., 1987); if this methylation is not achieved, the survival of the embryo could be compromised (Okano et al., 1999). In plants, which form an embryo without egg fertilization, the dynamic of the methylation depends on embryo development (Nic-Can et al., 2013), as well as the species (Nic-Can and De-la-Peña, 2014). Plants are able to survive larger reductions in genomic 5mC than animals. This phenomenon is very relevant, since DNA demethylation produces an important increase in the rates of transposon insertion (Hirochika et al., 2000; Singer et al., 2001; Kato et al., 2003; Tsukahara et al., 2009). On the other hand, the exposure to *in vitro* culture conditions produces epigenetic variation at several levels (Kaeppeler and Phillips, 1993; Smykal et al., 2007; Valledor et al., 2007; Miguel and Marum, 2011; De-la-Peña et al., 2012; for a review see Neelakandan and Wang, 2012; Us-Camas et al., 2014).

Methyltransferases

Methylation of DNA is catalyzed by a set of enzymes named DNA (cytosine-5-)methyltransferase (DCMTases; EC 2.1.1.37).

With the exclusion of fungal enzymes, and based on the sequence homology within their C-terminal catalytic domains, most DCMTases can be grouped into four distinct families (Grace Goll and Bestor, 2005). Plants have all four classes of DCMTases, while other eukaryotic organisms have only two or three classes. In plants, these groups of DCMTases are named DNA methyltransferase1 (MET1), domains rearranged methyltransferase (DRM), DNA nucleotide methyltransferase2 (DNMT2) and chromomethylase3 (CMT3). This last group appears to be unique to plants. There is significant variability in the types and numbers of DCMTases in plants (Table 1; Figure 1); e.g., *Arabidopsis thaliana* has 11, *Glycine max* has nine, *Coffea canephora* has eight, and *Vitis vinifera* and *Theobroma cacao* have six.

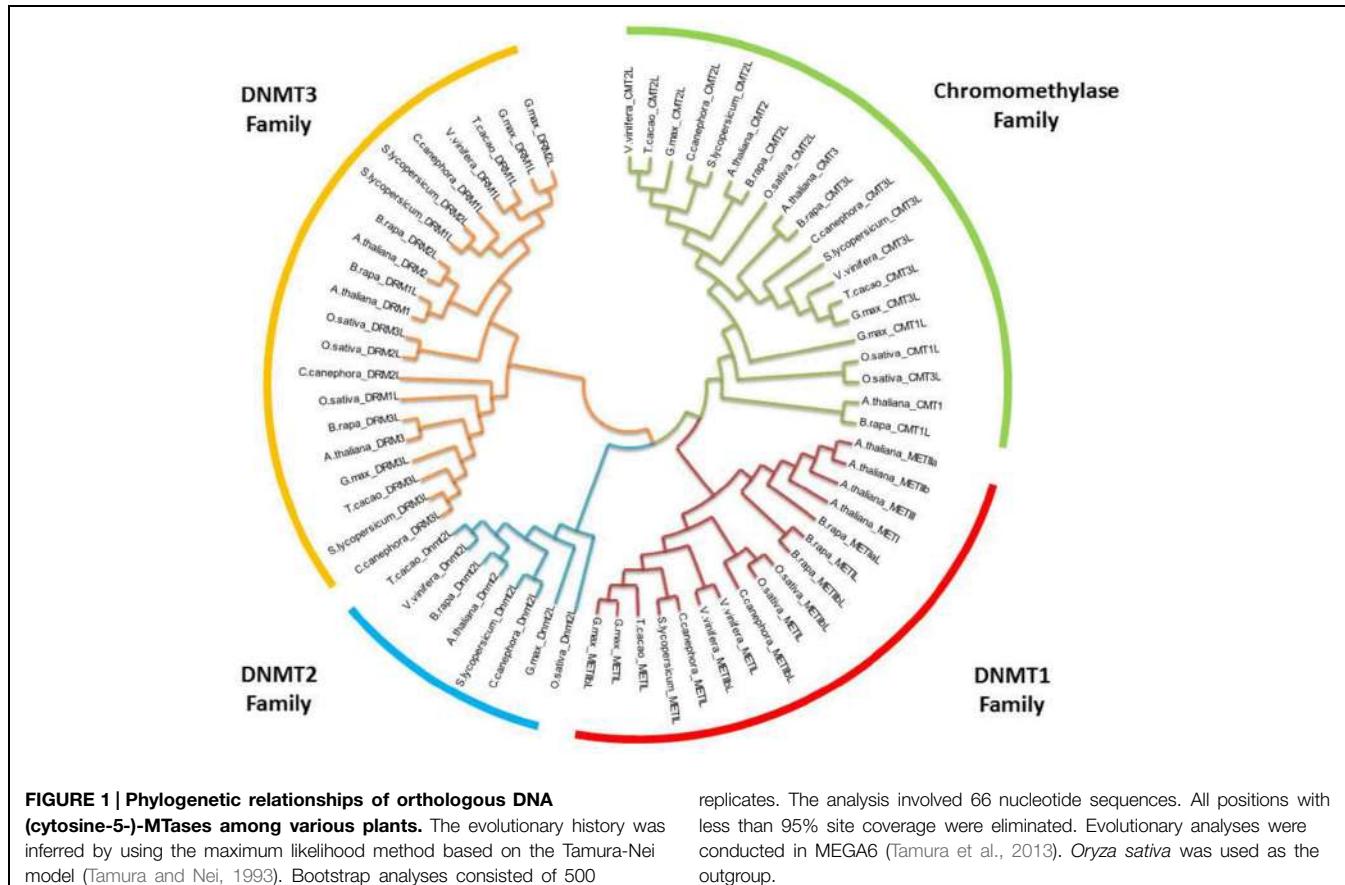
Using sequence similarity to Dnmt1 [the plant homolog of mammalian DNA (cytosine-5) methyltransferase 1], a DCMTase named MET1 was identified and cloned in *A. thaliana* (Jean Finnegan and Dennis, 1993). MET1 catalyzes the maintenance methylation of the CG islands in the heterochromatin (Cao and Jacobsen, 2002; Cokus et al., 2008; Lister et al., 2008), but may also play a role in *de novo* methylation (Finnegan and Kovac, 2000); DRM and CMT3 are in charge of the maintenance methylation of CHG and CHH isles (Lindroth et al., 2001; Law and Jacobsen, 2010; Du et al., 2012; Köhler et al., 2012), and DRM also methylates *de novo* CG, CHG, and CHH. It is dependent on RNAi-like machinery (Law and Jacobsen, 2010).

Methyltransferase enzymes have important motif characteristics to facilitate their main functions. The crystallization of the methyltransferase domain of *NtDMR* from tobacco shows a classic class I methyltransferase fold. The enzyme forms a homodimer with the dimer interface mimicking the mammalian Dnmt3a-Dnmt3L heterodimer interface (Zhong et al., 2014). This is very interesting because

TABLE 1 | Genes codifying for methyltransferases in some genome plants.

Species	Family	Methyltransferases			
		MET1	DNMT2	DRM	CMT3
		Substrate specificity			
		Maintenance CG&CHG	Broader specificity	De novo CG&CHG Maintenance CHH	Maintenance CHG&CHH
<i>A. thaliana</i>	Brassicaceae	4	1	3	3
<i>B. rapa</i>	Brassicaceae	3	1	3	3
<i>S. lycopersicum</i>	Solanaceae	1	1	3	2
<i>V. vinifera</i>	Vitaceae	2	1	1	2
<i>C. canephora</i>	Rubiaceae	2	1	3	2
<i>G. max</i>	Leguminosae	2	1	3	3
<i>T. cacao</i>	Malvaceae	1	1	2	2
<i>O. sativa</i>	Poaceae	2	1	3	3

The analysis for the presence of MTases was carried out using the complete sequence for *Coffea canephora* from Coffee Genome Hub (<http://coffee-genome.org/>), for *Arabidopsis thaliana* from TAIR10 (<http://www.arabidopsis.org/>) and for *Solanum lycopersicum*, *Vitis vinifera*, *Theobroma cacao*, *Brassica rapa*, *Glycine max*, and *Oryza sativa* from PHYTOZOME v10.2 (<http://phytozome.jgi.doe.gov/pz/portal.html>). We took into account all of the sequences that proved to contain more than one of the following functional annotations: PFAM/PF00145C-5/cytosine-specific DNA methylase, DNA (cytosine-5-)methyltransferase [EC:2.1.1.37] and (GO:0008168) [QuickGo from European Bioinformatics Institute].



this family of enzymes shows a strong conservation of the catalytic motifs in their C-terminal domains with mammalian Dnmt3A and Dnmt3B proteins (Figure 1) (Grace Goll and Bestor, 2005).

Chromomethylases are unique to flowering plants, and were identified by Henikoff and Comai (1998). These enzymes possess a chromodomain between motifs II and IV (Henikoff and Comai, 1998) and keep the eight conserved motifs characteristic of eukaryote cytosine methyltransferases (Finnegan and Kovac, 2000).

The sequence alignments and comparison of DCMTases present in the genome of eight plants, the genomes of which have been sequenced, was carried out (Figure 1). The cladogram reveals a clear division among the four groups of DCMTases present in plants. The family DNMT1 shares 46–60% similarity between *A. thaliana* and *C. canephora*. For the families DNMT2, DNMT3 and chromomethylase, these values are 65, 36–52%, and 53–60%, respectively, for both species.

A number of chemical and enzymatic studies show that the catalytic mechanism of DCMTases initiates with a nucleophilic attack of a conserved cysteine-81 (Cys) from the active site of the enzyme on carbon 6 (C6) of cytosine in DNA and generates a covalent enzyme-DNA intermediate (Figure 2). This nucleophilic attack activates an original inert carbon 5 (C5), and the flow of electrons to C5 produces a negative charge and leads to an attack on the methyl group of S-adenosyl-L-methionine (electrophile).

The nucleophilic attack increases the pK of the N3 of the cytosine and this nitrogen is protonated. This part of the reaction takes place with the Glu119 of the enzyme active site. Abstraction of the proton at the C5 position followed by β elimination allows reformation of the C5–C6 double bond and releases the active enzyme and DNA with a methylated cytosine (Figure 2) (Santi et al., 1983, 1984; Wu and Santi, 1987; Klimasauskas et al., 1994; Peräkylä, 1998; Liutkeviciute et al., 2011).

Inhibitors

Methyltransferases and their function in DNA methylation can be modified by a set of compounds that interfere in different steps of the methylation process (Goffin and Eisenhauer, 2002). The pyrimidine analogs 5-azacytidine (azaC) and the 5-aza-2'-deoxycytidine (decitabine) are cytosine analogs that, instead of the carbon atom at position 5, have a nitrogen atom (Figure 3) (Jones and Taylor, 1980). During the replication of DNA, (Lübbert, 2000), these compounds can be incorporated into the DNA, avoiding the methylation of DNA and resulting in a general genome hypomethylation (Santi et al., 1983). The 2-amino-5-ethoxy-carbonyl-pyrimidine-4(3H)-one is a pyrimidine analog that also inhibits the methylation of DNA (Figure 3) (Raugei et al., 1981). Another compound employed in epigenetic studies is ethionine (2-amino-4-ethylsulfanylbutyric acid) (Figure 3), used

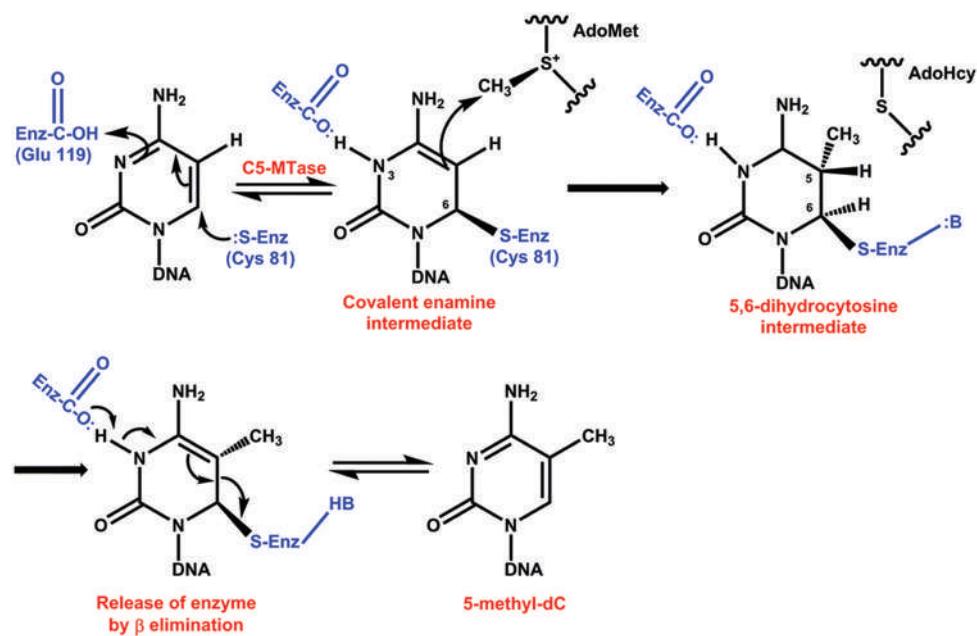


FIGURE 2 | Catalytic mechanism of DNA methyltransferases. The reaction initiates with a nucleophilic attack on carbon 6 of cytosine in DNA. This nucleophilic attack activates an original inert carbon 5. Abstraction of the proton at the C5 position followed by β elimination

allows reformation of the C5–C6 double bond and releases the active enzyme and DNA with a methylated cytosine (Santi et al., 1983, 1984; Wu and Santi, 1987; Klimasauskas et al., 1994; Peräkylä, 1998; Liutkevičiūtė et al., 2011).

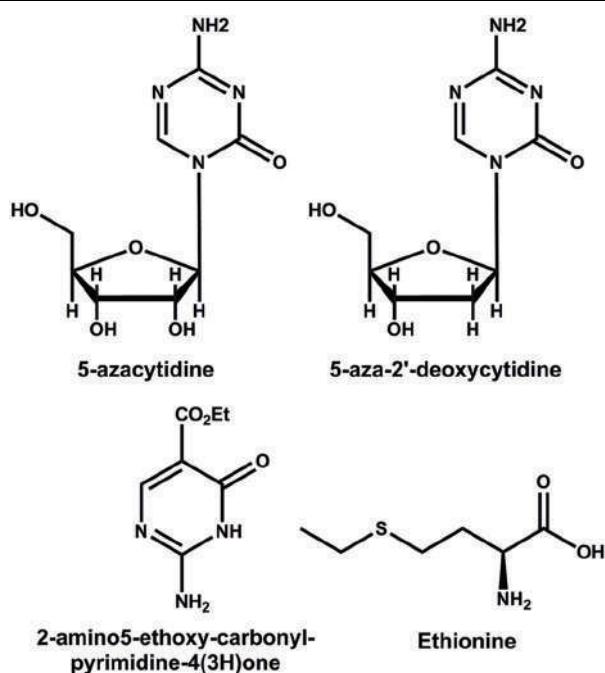


FIGURE 3 | Structures of some inhibitors of the methylation of DNA.

by cells to produce *S*-adenosyl-L-methionine, which functions as a competitive inhibitor of DNA methylation (Cox and Irving, 1977).

It has been suggested that the inhibitory mechanism of DNA methylation by pyrimidine analogs could be through the formation of a covalent bond between a catalytic nucleophile site of the DCMTases and the reactive 6 position of azaC that has replaced cytosine in DNA (Bouchard and Momparler, 1983; Santi et al., 1983; Jüttermann et al., 1994). The substitution of carbon by nitrogen at position 5 changes the reactivity of carbon at position 6, avoiding the reversibility of the bond between this carbon and a cysteine at the active site of the enzyme (Figure 4) (Santi et al., 1983, 1984). After repeated replication of cell cycles, the inhibitor depletes the cell of DCMTases, resulting in the hypomethylation of DNA (Santini et al., 2001). Another possible mechanism of action of these inhibitors would be through the damage to the structural stability of DNA (Lin et al., 1981; D'Incalci et al., 1985).

AzaC and decitabine can be also incorporated into DNA or RNA (Santini et al., 2001). AzaC is incorporated preferentially into RNA (Santini et al., 2001) and decitabine into DNA. The incorporation of azaC into RNA produces a ribosome malfunction and inhibits protein synthesis. All of these mechanisms have been studied in animal cells, but at present there has not been a study of plant cells in order to determine whether the inhibition mechanism is the same as that in animals.

Techniques to Determine DNA Methylation

DNA methylation is an important and widely used regulatory process among higher organisms. This led to the development

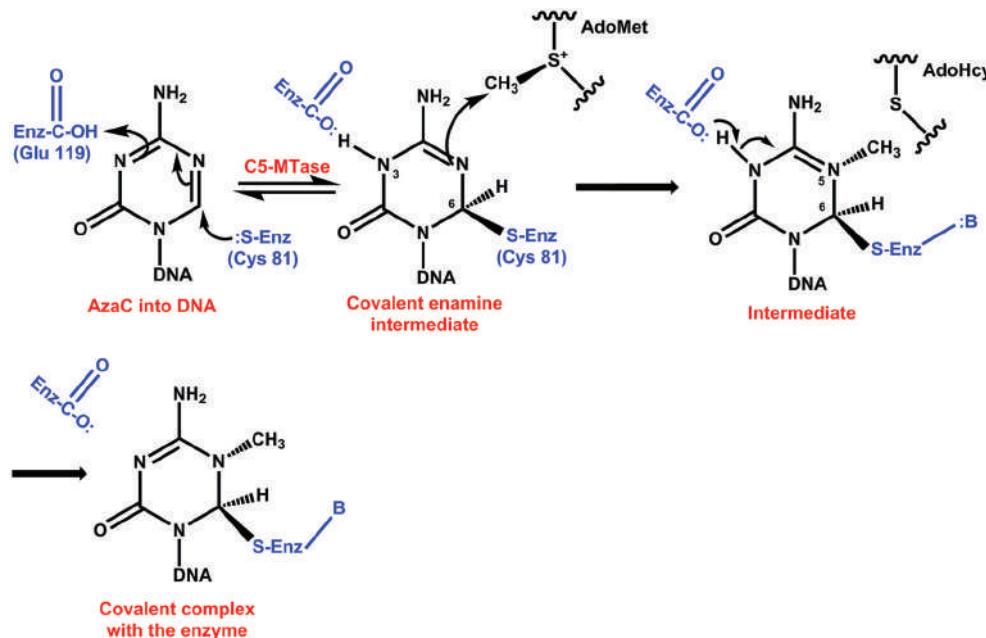


FIGURE 4 | Catalytic mechanism of the inhibition of DNA methylation by 5-azacytidine. After the formation of C6 and the enzyme, slow methyl transfer takes place; however, the absence of hydrogen at C5 avoids the β elimination and the enzyme remains attached to the complex (Santi et al., 1983, 1984; Santini et al., 2001).

of precise and efficient methods to determine the genomic DNA methylation content, as well as the specific sites of methylation in order to elucidate its role in biological processes such as SE.

The methods for the determination of methylation levels in DNA can be divided into at least into six general groups: global DNA methylation, regional DNA methylation, genome-wide analysis, DNA methylation analysis by sequencing, detection of specific methylation patterns, and individual CpG analysis (Figure 5). Some of these have been applied to study the process of both SE and zygotic embryogenesis (El-Tantawy et al., 2014; Nic-Can and De-la-Peña, 2014; Wolny et al., 2014; Pérez et al., 2015b); however, it is necessary to expand current strategies in order to have a more precise understanding of these important processes. For a complete analysis of all of the techniques used to study chromatin modifications, see Tost (2009), Kovalchuk and Zemp (2010), Tollesbo (2011), and Spillane and McKeown (2014).

Analysis of DNA Methylation by Bisulfite Sequencing

The bisulfite genomic sequencing method (Frommer et al., 1992) is both qualitative and quantitative. This method is based on the conversion of cytosines in single-stranded DNA into uracil by sodium bisulfite, which is recognized as thymine in subsequent PCR amplification and sequencing. The 5mCs do not react to this transformation and remain cytosines, allowing 5mCs to be distinguished from unmethylated cytosines. The first step in this method is to denature the double strand

of DNA; this is followed by the sulphonation of the cytosine residues at the C-6 position, hydrolytic deamination at C-4 to produce uracil-sulphonate, and desulphonation under alkaline conditions (Figure 6) (Wang et al., 1980; Grigg, 1996; Rein et al., 1998; Hajkova et al., 2002). The 5mC is unreactive due to the inability of bisulfite to access the C-6 position. This method has been used to determine the methylation status at a specific locus of genes involved in the SE of *Daucus carota* (Shibukawa et al., 2009) and *C. arabica* (Bobadilla Landey et al., 2013). The bisulfite method has become the basis for other methods, such as methylation-sensitive single nucleotide primer extension (Ms-SNuPE), combined bisulfite restriction analysis (COBRA), methylation-specific PCR (MSP), and others that would be interesting to apply during the transition of somatic cells into embryogenic ones.

Quantification of Global DNA Methylation

Global DNA methylation is frequently used to evaluate whether DNA methylation changes exist on a large scale during growth and development or if they are induced by different environmental signals in plants and animals. Global 5mC levels can be detected by several analytical techniques, such as reversed-phase high performance liquid chromatography (RP-HPLC), capillary electrophoresis, inductively mass coupled mass plasma spectrometry (ICP-MS), coupling liquid chromatography or gas chromatography, and electrospray ionization mass spectrometry (ESI-MS; Magaña Alcázar et al., 2008; Wrobel et al., 2009; Lopez Torres et al., 2011). Among these analytical techniques, RP-HPLC

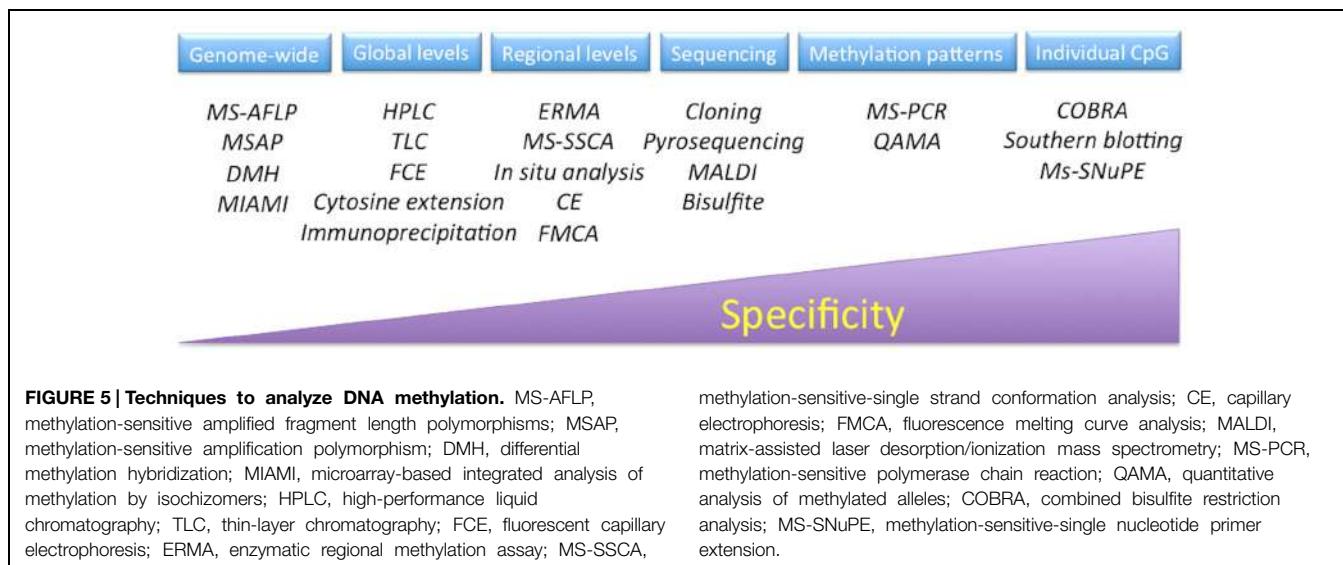


FIGURE 5 | Techniques to analyze DNA methylation. MS-AFLP, methylation-sensitive amplified fragment length polymorphisms; MSAP, methylation-sensitive amplification polymorphism; DMH, differential methylation hybridization; MIAMI, microarray-based integrated analysis of methylation by isochizomers; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; FCE, fluorescent capillary electrophoresis; ERMA, enzymatic regional methylation assay; MS-SSCA,

methylation-sensitive-single strand conformation analysis; CE, capillary electrophoresis; FMCA, fluorescence melting curve analysis; MALDI, matrix-assisted laser desorption/ionization mass spectrometry; MS-PCR, methylation-sensitive polymerase chain reaction; QAMA, quantitative analysis of methylated alleles; COBRA, combined bisulfite restriction analysis; MS-SNuPE, methylation-sensitive-single nucleotide primer extension.

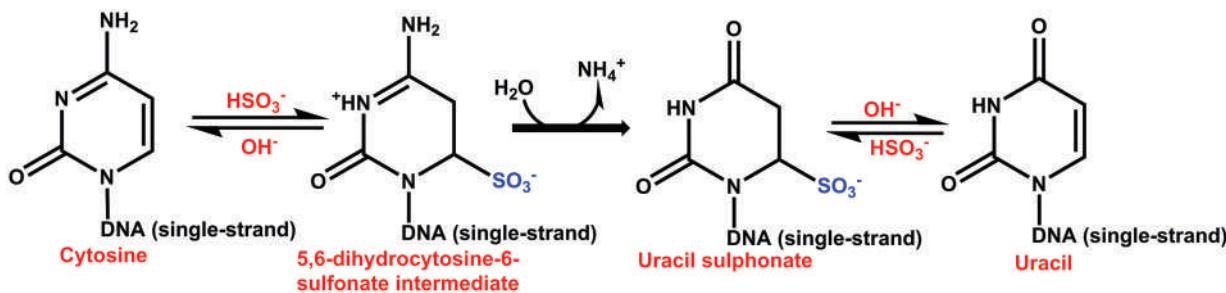


FIGURE 6 | Catalytic mechanism of the reaction of bisulfite with cytosine. The sulphonation is favored at low pH and low temperature. The hydrolytic deamination of cytosine-6-sulphonate to uracil-6-sulphonate is irreversible. The desulphonation of uracil-6-sulphonate to uracil is reversible and is favored at high pH (Wang et al., 1980; Frommer et al., 1992; Hajkova et al., 2002).

is the most common procedure. The level of 5mC is obtained through enzymatic digestion of DNA (DNase, nuclease P1 and phosphatase alkaline) to obtain free deoxynucleosides, followed by chromatographic separation, where the suitable separation of deoxynucleosides must be ensured. The use of the HPLC technique to analyze DNA methylation has been effective at determining the methylation changes throughout the whole SE process in *C. canephora* (Nic-Can et al., 2013) and during the SE of *Castanea sativa* (Viejo et al., 2010).

Methylation-Sensitive Amplified Polymorphism (MSAP)

Analysis of methylation-sensitive amplified polymorphism (MSAP) has been applied to several plants in order to identify genome-wide epigenetic variations. This technique is based on the use of a pair of methylation-sensitive restriction enzymes, HpaII and MspI, which are isochizomers, as well as the use of EcoRI. Both enzymes recognize the same sequence, 5'-CCGG-3'; however, their action is affected by the methylation pattern at the inner or outer cytosine (Reyna-López et al., 1997). MSAP

has proven to be an efficient method for detecting alterations in cytosine methylation in fixed genotypes (Li et al., 2008); this technique is relatively simple and has been often used to assess different systems of plant tissue cultures with the purpose of identifying genes under epigenetic control (Miguel and Marum, 2011). In addition, MSAP does not require a sequenced reference genome, but the scoring of MSAP data should be made carefully in order to determine the distribution of CpG methylation at the 5'-CCGG-3' sites through the genome (Abid et al., 2011). Xu et al. (2004) used this technique in *Rosa hybrida* cv. Carefree, and found that the demethylation of outer cytosines occurred at a high frequency during SE.

In Situ Analysis of DNA Methylation

As the realization of the importance of DNA methylation to different biological processes is growing (Martienssen and Colot, 2001; Bruce et al., 2007; Amasino, 2010), the number and sensitivity of techniques to measure 5mC is also receiving more attention. Many techniques, such as MSAP (Baurens et al., 2008), HPLC (De-la-Peña et al., 2012), high-performance capillary

electrophoresis (HPCE; Fraga et al., 2000; Meijón et al., 2009) and ELISA-based assays (Testillano et al., 2013) have been used effectively to measure global DNA methylation levels. However, some studies are applying a more sensitive spatial and temporal analysis to localize the precise distribution of 5mC (Meijón et al., 2009; Testillano et al., 2013).

In situ analysis using immunolocalization coupled to confocal microscopy in order to localize 5mC to an exact moment and cell have resolved many biological questions (Meijón et al., 2009; Kathiria and Kovalchuk, 2010; Testillano et al., 2013; Pérez et al., 2015b). This technique is designed to work with antibodies and a confocal microscope, and it is possible to detect the fluorescence signal with high sensitivity and good reproducibility (Kathiria and Kovalchuk, 2010). This technique has not been applied to the SE process, but it is clear that it would give interesting results about specific methylation sites during the transition from the globular to the heart stage.

Epigenetics of Somatic Embryogenesis

In recent years, epigenetic mechanisms have emerged as critical factors during the induction of both somatic (Nic-Can and De-la-Peña, 2014) and zygotic embryogenesis (Nodine and Bartel,

2010). The modifications present during the induction of SE and development of somatic embryos include methylation of DNA, as well as histone modifications (Table 2). The methylation of DNA is essential in order for SE to succeed. This epigenetic mechanism during the induction of SE has been documented in at least 18 species from 12 families (Table 2). However, in only four of these species have the modifications in histones been determined (Nic-Can et al., 2013; Rodríguez-Sanz et al., 2014b; Pérez et al., 2015a; Wickramasuriya and Dunwell, 2015).

In general, the methylation of DNA is lower in the embryogenic tissues than in the non-embryogenic tissues. For instance, in Siberian ginseng (*Eleutherococcus senticosus*), the non-embryogenic calli showed higher DNA methylation in the sites 5'-CCGG-3' (16.99%) than the embryogenic calli (11.20%) (Chakrabarty et al., 2003). A similar pattern has been determined in *Pinus nigra* Arn. ssp *Austriaca*, in which embryogenic lines showed low methylation levels (Noceda et al., 2009). Since the lowest level of DNA methylation is always found in the embryogenic cells (Palmgren et al., 1991), it is possible that DNA hypomethylation is involved in the signal that leads to the induction of SE. The transient expression of a carrot DNA methyltransferase gene, Met1-5, after the induction of SE by 2,4-dichlorophenoxyacetic acid (2,4-D) and before the formation of embryogenic cell clumps (Yamamoto et al., 2005), seems to

TABLE 2 | DNA methylation and histone modifications during the induction of somatic embryogenesis and development of somatic embryos.

Family	Species	Epigenetic modifications through SE development					Reference
		DNA methylation	H3K4me ³	H3K9me ²	H3K27me ³	H3K36me ³	
Apiaceae	<i>Daucus carota</i>						LoSchiavo et al. (1989), Palmgren et al. (1991), Shibukawa et al. (2009), Yamamoto et al. (2005)
Araliaceae	<i>Eleuterococcus senticosus</i>						Chakrabarty et al. (2003)
Arecaceae	<i>Elaeis guineensis</i>						Jaligot et al. (2000, 2002, 2004)
Brassicaceae	<i>Brassica napus</i>						Rodríguez-Sanz et al. (2014b), Solís et al. (2015)
	<i>Arabidopsis thaliana</i>						Wickramasuriya and Dunwell (2015)
Cucurbitaceae	<i>Cucurbita pepo</i>						Leljak-Levanic et al. (2004)
Fabaceae	<i>Medicago truncatula</i>						Santos and Fevereiro (2002)
	<i>Castanea sativa</i>						Viejo et al. (2010)
Fagaceae	<i>Quercus suber</i>						Rodríguez-Sanz et al. (2014a), Pérez et al. (2015a)
Myrtaceae	<i>Acca sellowiana</i>						Fraga et al. (2012), Cristofolini et al. (2014)
Pinaceae	<i>Picea abies</i>						Yakovlev et al. (2014)
	<i>Picea omorika</i>						Levanic et al. (2009)
	<i>Pinus pinaster</i>						Klimaszewska et al. (2009)
	<i>Pinus nigra</i>						Noceda et al. (2009)
	<i>Larix x eurolepis</i>						Teyssier et al. (2014)
Poaceae	<i>Hordeum vulgare</i>						El-Tantawy et al. (2014)
Rosaceae	<i>Rosa x hybrida</i>						Xu et al. (2004)
Rubiaceae	<i>Coffea canephora</i>						Nic-Can et al. (2013)

support this theory. However, these studies are in contradiction with the finding from *P. pinaster*, where there are no differences in the amount of DNA methylation of embryogenic and non-embryogenic lines. The DNA methylation values are between 17.8 and 19.1%, with no significant difference (Klimaszewska et al., 2009). Nevertheless, the determination of total 5mC can lead to underestimating the methylation/demethylation of specific sites of DNA. To avoid this problem, it is necessary to use more reliable techniques, such as MSAP. Using a modification of this technique, Xu et al. (2004) were able to show that in *R. hybrida* cv. Carefree, the demethylation of outer cytosines occurred at a high frequency during SE. This indicates that besides the total 5mC quantification, it is necessary to evaluate specific methylation sites in order to have more complete information about DNA methylation not only for SE but also for other systems.

The study of the epigenetic changes during the induction of SE and the development of the somatic embryos is not an easy task. Several factors can affect the changes in the methylation pattern of DNA. Among these factors are the age of the cell lines, the genetic background of the explant, the presence/absence of growth regulators, the culture medium components, the physiological conditions of the explant, the temperature of incubation, molecules secreted by the explants and others. For instance, it was found in *D. carota* that the presence of low levels of 2,4-D (2.26 µM) favored a low level of 5mC (i.e., 16%), whereas a 10-fold increase in the concentration of 2,4-D increased the methylation levels to 45%. A similar concentration of different auxins, such as 1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA), only increased the level of DNA methylation to 23% (LoSchiavo et al., 1989). This suggests that DNA methylation is affected not only by the presence of auxins, but also by the type of the auxin used. DNA methylation is also affected by the nitrogen source (Leljak-Levanic et al., 2004). In *Cucurbita pepo* embryogenic lines, it was found that a low amount of NH₄Cl present in the B₅ medium is enough to produce the highest rate of DNA methylation in an auxin-free medium (Leljak-Levanic et al., 2004).

Since the discovery of SE, it has been found that the stage of development of some tissues is crucial for the production of somatic embryos (Loyola-Vargas et al., 2008). Recently, Viejo et al. (2010) found that the induction of SE from zygotic embryos of *C. sativa* depends on a decrease in DNA methylation of the original explant. It seems that the induction of SE occurs when a decrease in DNA methylation happens during the zygotic embryo maturation. It is possible that the genotypic dependence of the induction of SE is related to the epigenetic status of the explant.

In general, it appears that the hypomethylation of the explant is a prerequisite for a successful induction of SE. However, the mechanism by which this hypomethylation affects the induction of SE is unclear. Using the SE of carrot as a model, two laboratories have reported contradictory results. Yamamoto et al. (2005) showed that azaC down-regulated the expression of the transcription factor *CARROT-LEAFY COTYLEDON1* (*DcLEC1*) during morphogenesis of embryos from epidermal carrot cells, while Shibukawa et al. (2009) showed that it is the

hypermethylation of a portion from -1,904 to -1,272 of the 5'-upstream region of the promoter of *DcLEC1* that actually down-regulates its expression. However, in both cases it is suggested that the expression of *DcLEC1* during carrot SE could be regulated by DNA methylation.

The use of pharmacological approaches in the study of metabolic pathways and their regulation has been very useful. The study of the epigenetic marks during the induction of SE and development of the somatic embryos is not an exception. The treatment of *Medicago truncatula* (Santos and Fevereiro, 2002) and *D. carota* (Yamamoto et al., 2005) embryogenic lines with azaC not only decreased the production of somatic embryos but also caused the loss of the SE. In the case of *M. truncatula*, it was found that the disruption of the SE response was due to an increase of demethylated rDNA (Santos and Fevereiro, 2002). However, in *D. carota* it was shown that the effects of azaC on SE depend on the embryogenic stage at which it is applied. Yamamoto et al. (2005) reported that this demethylating agent suppresses the SE if it is applied between 3 and 7 days after induction, but not if it is applied after day 7. These results suggest that certain levels of DNA methylation have to be maintained before the change from somatic cell to embryogenic cell. Similar to *D. carota*, the presence of azaC during the induction of SE in *C. canephora* decreased the DNA methylation and severely inhibited the embryogenic response when it was applied at both 7 and 14 days (Nic-Can et al., 2013). It was observed that the presence of azaC decreased the transcript levels of *LEC1* and *BABY BOOM1* (*BBM1*), impairing the embryogenic program. However, it was also observed that the SE response was improved and synchronized, even at a concentration of 20 µM of azaC, if it was added at 35 days after the embryogenic induction (Nic-Can et al., 2013). A similar reduction in DNA methylation has been observed in *P. omorika* after 1 week of exposure to azaC in the presence of 2,4-D and BA, with no effect on embryo development (Levanic et al., 2009). However, in *C. pepo* the use of azaC did not inhibit either the induction of SE or the amount of somatic embryos in the different developmental stages, compared with the controls without azaC (Leljak-Levanic et al., 2004). In *Acca sellowiana*, 200 µM of 2,4-D and 10 µM of azaC increased the formation of somatic embryos by more than 240% in accession 101 × 458, but not in accession 85. The same experiment, but using 50 µM of azaC, increased the formation of somatic embryos by more than 240% in accession 85 and decreased to 10% the formation of somatic embryos in accession 101 × 458 (Fraga et al., 2012). Meanwhile, the treatment of *P. pinaster* SE lines with 5 µM or greater of azaC reduced the growth of the cultures. However, DNA methylation remained around 18.7%. Furthermore, concentrations of 10 and 15 µM of azaC produced a slight increase in the number of mature somatic embryos (Klimaszewska et al., 2009). In *Brassica napus* and *Hordeum vulgare*, the incubation for 4 days with 2.5 µM of azaC increased embryo induction and modified the heterochromatin patterns (Solís et al., 2015). However, when the azaC treatment was longer, the number of embryos diminished.

It is known that stress can be an inducer of SE and this fact raises the question of whether/how both phenomena can

share common signaling pathways (Yang and Zhang, 2010). The change in the genetic program from microspore to SE could be due to heat (*B. napus*, *Nicotiana tabacum*; Touraev et al., 1996; Zhao et al., 1996; Solís et al., 2015) or cold (*H. vulgare*; El-Tantawy et al., 2014). However, other stresses such as osmotic shock (Kamada et al., 1993; Cabrera-Ponce et al., 2015), water deficit (Patnaik et al., 2005; Meneses et al., 2010), temperature (Decout et al., 1994; Kamada et al., 1994), heavy metals (Ikeda-Iwai et al., 2003; Patnaik et al., 2005), wounding (Nolan et al., 2006), nutrient starvation (Fuentes-Cerda et al., 2001; Mihaljevic et al., 2011), culture medium dehydration (Jin et al., 2014), ultraviolet radiation, and pH (Pasternak et al., 2002; Potters et al., 2007) might also play a major role in somatic embryo induction.

The use of massive transcriptome sequence during the SE induction in *Zea mays* (Salvo et al., 2014), *Gossypium hirsutum* (Jin et al., 2014) and *A. thaliana* (Wickramasuriya and Dunwell, 2015) have revealed the close relationship between the signaling pathways leading to stress and morphogenic responses. Both processes share the expression of many stress-morphogenic-related genes. These findings have led to the question of whether SE is a stress response of plants in order to survive extreme *in vitro* environmental conditions. An important addition to the understanding of the SE-stress relationship has been done recently. It is possible to hypothesize that the transition from a vegetative to an embryogenic stage requires a change in the genome organization and, therefore, an active role for chromatin modifications. During the microspore SE in *B. napus*, a decrease in DNA methylation occurs while during the embryo differentiation the DNA methylation increases (Solís et al., 2012). Besides DNA methylation, histone modifications play an important role in the microspore embryogenesis of *B. napus*. Rodríguez-Sanz et al. (2014b) suggest that the marks H3K9me2 and HKMT might participate in the embryo cell differentiation and heterochromatinization and the marks H3Ac, H4Ac, and HAT in events that take place during cell reprogramming and embryo development.

The expression pattern of *BnMET1a*-like genes, which codified for DNA methyltransferases, is highly correlated with the variations in DNA methylation. A DNA hypomethylation in *Quercus suber* has been also documented during the SE from microspores and immature zygotic embryos (Rodríguez-Sanz et al., 2014a). A similar decrease in DNA methylation has been found during the *H. vulgare* microspore SE induction (El-Tantawy et al., 2014). It is worth noting that the SE process in *H. vulgare* is induced with a cold stress instead of a heat stress as happens in *B. napus*, although the response is the same. The transcriptome of *Picea abies* under two epitype-inducing temperatures (18 vs. 30 °C) has also revealed that 35 expressed transcripts, orthologous to epigenetic-related genes, are involved in epigenetic regulation (Yakovlev et al., 2014). These data suggest that temperature-dependent gene expression during the induction of SE could originate from modifications in the chromatin structure.

On the other hand, it was found that explants under embryogenic conditions release organic molecules that inhibit the embryogenic response of somatic cells and also affect DNA methylation levels. There are some reports indicating that

phenolic compounds interfere with the SE process (Kouakou et al., 2007; Umehara et al., 2007; Nic-Can et al., 2015) and also inhibit the activity of DNA methyltransferases (Causevic et al., 2005). Recently, it has been shown that *C. arabica* leaves' explants release several phenolic compounds into the media, which seem to be directly related to the plant's poor response to direct SE (Nic-Can et al., 2015). Among these compounds, caffeine and chlorogenic acid, which represent 98% of the phenolic compounds, accumulate in the conditioned medium of *C. arabica*. Moreover, the addition of the phenolic compounds, either as conditioned medium or in a pure form, drastically interferes with the SE process in two highly embryogenic species (*C. canephora* and *D. carota*). Global DNA methylation analysis showed that conditioned medium of *C. arabica* stimulates the loss of DNA methylation even more than azaC does. Therefore, the instability of DNA methylation levels because of the accumulation of phenolic compounds could be one of the major causes of the disturbance of cellular metabolism needed to create embryonic complex structures from somatic cells.

Epigenetic Changes During the Development of Somatic Embryos

In general, variation in DNA methylation is related to developmental changes in response to growth regulator treatments. This methylation is essential during the early development of somatic embryos of *C. pepo* (Leljak-Levanic et al., 2004). The DNA methylation level in *Larix x eurolepis* differs at each step of the development of somatic embryos; it goes from 45.8% in the original embryogenic line to 61.5% after 1 week of maturation. DNA methylation decreases to 53.4% after 8 weeks of maturation (Teyssier et al., 2014). In *C. canephora*, it was also observed that DNA methylation levels increase as the embryo develops; for instance, when the somatic cells of the leaves' explants begin the cellular dedifferentiation, the content of methylated cytosines is about 23.7%, whereas during the later developmental embryo stages high levels of DNA methylation are established (Nic-Can et al., 2013). On the other hand, the modification of the DNA methylation pattern with azaC or hydroxyurea (hyper-methylating agent) significantly reduced both the relative growth rate and the embryogenic potential (Teyssier et al., 2014).

The treatment of embryogenic lines with a variety of auxin/cytokinin ratios before placement onto a maturation medium containing 40 μM ABA changes the methylation of DNA in the original embryogenic line. The decrease of 2,4-D concentration or its exclusion causes a reduction in the methylation and improves the maturation of somatic embryos in the presence of ABA (Levanic et al., 2009).

Since, in many cases, the elimination of the auxin from the culture medium is necessary for SE to occur, it is possible that the changes in the epigenetic marks form the initial step in the development of somatic embryos. In general the lack of quantitative differences in global cytosine methylation does not necessarily mean that a locus-specific methylation has an important effect on SE induction or development. Thus, more

detailed studies are necessary for a deep comprehension of the role of DNA methylation during the induction of SE and the development and transition among the different stages of development of the somatic embryos. One further step in the study of the changes in the epigenetic mechanism is the study of histone marks during the induction of SE.

Recently, a few groups have shown the dynamic activity in the modification of histones that leads to the modulation of the expression of genes that previously have been proposed to be involved in the SE process. For instance, in *C. canephora*, it was found that during the early events of SE the levels of the histone repressive marks H3K9me2 and H3K27me3 decrease, and these events were correlated with the beginning of the expression of *LEC1*, *BBM1* and *WOX4* (Nic-Can et al., 2013). Using chromatin immunoprecipitation (ChIP) assays, it has been found that during the cellular dedifferentiation, the H3K27me3 is removed from *LEC1* loci, allowing the expression of this transcription factor, whereas the expression of *BBM1* was related to the increase of both histone marks H3K4me3 and H3K36me2. In the case of *WOX4*, it was found that its transcriptional repression, especially during the maturation phase of somatic embryos, was correlated with the increase of H3K9me2. This indicates that the chromatin is dynamically regulated to change the transcriptional program of the somatic cells before and during the development of somatic embryos (Nic-Can et al., 2013).

The H3K9me2 mark has also been involved in embryo cell differentiation and heterochromatization events during the microspore embryogenesis in *B. napus* (Rodríguez-Sanz et al., 2014b). Using immunolocalization, it was observed that the levels of H3K9me2 were low in microspores before the induction of SE; however, an increase of more than two times occurs during the late stages of embryogenesis, particularly in the differentiated peripheral cells, indicating a high chromatin condensation. In contrast to H3K9me2, it was observed that the levels of acetylation of H3 and H4 (H3Ac and H4Ac) marks, which are related to transcriptional activity, were more abundant in the microspores before SE induction, especially the H4Ac, suggesting that these modifications might be related to the totipotency acquisition, cellular reprogramming and embryo development. In addition, these patterns were related to changes in the expression profiles of the histone methyltransferase and histone acetyltransferase genes as well as embryogenic development (Rodríguez-Sanz et al., 2014b).

The expression pattern of several genes related to chromatin modification and remodeling [two histone deacetylases (HDACs), *HDA6* and *HDA19*, two histone monoubiquitinases (*HUB1* and *HUB2*), a histone H3 kinase (*AUR3*), *PICKLE* and *VP1/ABSCISIC ACID INSNSITIVE 3-LIKE 1 (VAL1)*], have been studied during the SE process of *Q. suber* (Pérez et al., 2015a). It was found that *QsHDA19* decreases its expression as soon as the callus begins its differentiation, followed by a steady increase from immature cotyledonary embryo to an embryo with the cotyledons fully differentiated. On the other hand, a transient decrease in *QsHDA6*, *QsPICKLE*, and *QsVAL1* gene expression was observed in the transition from callus to the end of the mature embryo. *QsHUB1* and *QsHUB2* showed a transient increase expression from white callogenic structures

and globular embryos to immature cotyledonary embryos. The highest expression was observed in white opaque cotyledonary embryos, while *QsAUR3* was preferentially expressed in immature cotyledonary embryos. According to previous reports, histone deacetylases are related to transcriptional repression and chromatin condensation, whereas the monoubiquitination has been associated with transcriptional activation, and the histone kinase is an important mitosis regulator (Turner, 1991; Houben et al., 2005; Cao et al., 2008). In addition, *VAL1* and *PICKLE* are suggested to regulate the repression of the seed transcriptional program (Zhang and Ogas, 2009). All of these results suggest that these epigenetic components play a key role during the development and maturation of *Q. suber* somatic embryos.

Recently, the high resolution of the transcriptome sequencing in *Arabidopsis* has showed that there are important changes in the expression of chromatin-associated genes, which could help to understand the molecular mechanisms that lead to the acquisition of cellular totipotency (Wickramasuriya and Dunwell, 2015). For instance, Chupeau et al. (2013) reported that during the early events of differentiation from protoplast to plantlets a transient up-regulation of histone H3.3 variant occurs due to the incorporation of distinct sets of histone variants in the nucleosomes, particularly because of the enrichment of transcriptional active regions. Moreover, different gene-encoded proteins involved in histone modifications, DNA methylation and demethylation, as well as chromatin remodeling, are also up-regulated, indicating that they play an important role in the overall reprogramming of plant cells.

Lately, it has been found that HDACs are expressed throughout the SE, whereas histone acetyltransferases accumulate more in somatic embryos than in leaf tissues (Wickramasuriya and Dunwell, 2015). The authors suggest that some members of HDAC family are important for SE in *Arabidopsis*, probably through the regulation of the histone modifications in order to maintain a high methylation status during SE.

Together all of these results suggest that plant chromatin is dynamically regulated during SE, but how the somatic cells break the epigenetic barrier to reach the totipotent status is still a matter of study. It would be interesting to explore how the key genes of the induction of SE are switched on throughout chromatin remodeling in different species or induction conditions. Since stress, like heat in *B. napus* and cold in *H. vulgare*, is one of the inducers of SE, it would be very important to determine whether/how the same epigenetic marks are responsible for both cases.

Epigenetic Changes in Regenerated Plants

Since the rise of commercial micropropagation, somaclonal variation (SV) has been present as a serious problem, producing many variants among the regenerate plants and, on the other hand, creating a source of variation to achieve new agronomically important cultivars. When the plants come from somatic embryos, the variation can be high. It has been suggested that

this variation is due, at least in part, to changes in the DNA methylation pattern. These changes in the DNA methylation of regenerant plants from somatic embryos have been documented in several species. For instance, in maize, a high frequency of DNA methylation variation among regenerants was found (Kaeplinger and Phillips, 1993) and, in *Elaeis guineensis*, DNA methylation could be involved in the occurrence of 5% of SV. The DNA methylation present in the leaves of somaclonal regenerants is lower than in the non-variant plants (Jaligot et al., 2000, 2002). Also, the “mantled” SV in somatic embryo-derived oil palm plants (development of abnormal flowers) is associated with a decrease in global DNA methylation (Matthes et al., 2001; Jaligot et al., 2004). This mantle abnormality can be heritable and with time can show reversion to the normal phenotype. The uses of the restriction enzyme HpaII suggest that the loss of methylation occurs most frequently at the internal C (5'-CCGG-3'; Matthes et al., 2001). Regenerated plants from embryogenic callus of *Hordeum brevisubulatum* present a variation frequency of 9.3%. The degree of variation varies among the plants and the variation is present in both protein coding genes. Transposon/retrotransposons were found to underlie the genetic and epigenetic variations (Li et al., 2007).

Furthermore, some factors, such as cryopreservation of the embryogenic tissues, can modify the level of methylation of DNA. It has been determined that plant recovery from cryopreserved somatic embryo clusters of peach palm (*Bactris gasipaes*) showed an increased DNA methylation level when compared with non-cryopreserved somatic embryo clusters. However, 24 weeks after

regrowth, the global methylation profile decreases to the initial level (Heringer et al., 2013).

Conclusion

DNA methylation is an important epigenetic mechanism that has been studied with different approaches. Some of the most used techniques to study DNA methylation have been qualitative and/or quantitative, and the information from each has contributed to the understanding of many important biological questions, such as the mechanisms of SE induction and embryo development. However, it is necessary to explore the role of the different methyltransferases during the SE process, because so far it is poorly understood which of/how these enzymes participate in SE. It is clear that pharmacological assays with azaC have provided some of the answers about whether and how DNA methylation is involved during SE and how it can affect gene expression. The importance of revealing how epigenetics function in SE could help to increase plant productivity and improve agronomical breeding practices.

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Meristem micropropagation of cassava (*Manihot esculenta*) evokes genome-wide changes in DNA methylation

Shedrack R. Kitimu^{1†}, Julian Taylor², Timothy J. March³, Fred Tairo⁴, Mike J. Wilkinson¹ and Carlos M. Rodríguez López^{1*}

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Serena Varotto,

University of Padova, Italy

Fumihiro Sato,

Kyoto University, Japan

*Correspondence:

Carlos M. Rodríguez López,
Plant Research Centre, School of Agriculture Food and Wine, Faculty of Sciences, University of Adelaide, Waite Campus, Adelaide, SA 5064, Australia

carlos.rodriguezlopez@adelaide.edu.au

†Present address:

Shedrack R. Kitimu,

Sokoine University of Agriculture, P.O BOX 3000, Morogoro, Tanzania

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¹ Plant Research Centre, School of Agriculture Food and Wine, Faculty of Sciences, University of Adelaide, Adelaide, SA, Australia, ² Biometry Hub, School of Agriculture Food and Wine, Faculty of Sciences, University of Adelaide, Adelaide, SA, Australia, ³ School of Agriculture Food and Wine, Faculty of Sciences, University of Adelaide, Adelaide, SA, Australia, ⁴ Mikocheni Agricultural Research Institute, Dar es Salaam, Tanzania

There is great interest in the phenotypic, genetic and epigenetic changes associated with plant *in vitro* culture known as somaclonal variation. *In vitro* propagation systems that are based on the use of microcuttings or meristem cultures are considered analogous to clonal cuttings and so widely viewed to be largely free from such somaclonal effects. In this study, we surveyed for epigenetic changes during propagation by meristem culture and by field cuttings in five cassava (*Manihot esculenta*) cultivars. Principal Co-ordinate Analysis of profiles generated by methylation-sensitive amplified polymorphism revealed clear divergence between samples taken from field-grown cuttings and those recovered from meristem culture. There was also good separation between the tissues of field samples but this effect was less distinct among the meristem culture materials. Application of methylation-sensitive Genotype by sequencing identified 105 candidate epimarks that distinguish between field cutting and meristem culture samples. Cross referencing the sequences of these epimarks to the draft cassava genome revealed 102 sites associated with genes whose homologs have been implicated in a range of fundamental biological processes including cell differentiation, development, sugar metabolism, DNA methylation, stress response, photosynthesis, and transposon activation. We explore the relevance of these findings for the selection of micropropagation systems for use on this and other crops.

Keywords: methylation sensitive GBS, genotyping by sequencing, micropropagation, cassava, somaclonal variation, DNA methylation, epigenetics, methylation-sensitive amplified polymorphisms

Introduction

Epigenetic control of gene expression plays an important role in development (Meyer et al., 2013). Indeed, normal development in complex higher organisms is dependent upon both spatial and temporal control of gene expression (Zhang et al., 2010), much of which is facilitated by dynamic operation of various epigenetic regulatory systems (Morgan et al., 2005). DNA methylation, and more specifically cytosine methylation (i.e., the incorporation of a methyl group to carbon 5 of the cytosine pyrimidine ring to form 5-methylcytosine) is present across many eukaryotic phyla, including plants, mammals, birds, fish, and invertebrates and provides an

important source of epigenetic control for gene expression (Su et al., 2011). In plants, cytosine methylation can occur in three motif contexts (CG, CHG, or CHH, where H = a nucleotide other than G; Rodríguez López and Wilkinson, 2015). DNA methylation occurring within promoters or coding regions typically act to repress gene transcription and can be evoked by small interfering RNA-directed DNA Methylation (RdDM; see Matzke et al., 2007; Verdel et al., 2009). *De novo* DNA methylation directed by RdDM has been implicated in various types of plant stress responses (e.g., Agorio and Vera, 2007; Tricker et al., 2012, 2013) and developmental progression (e.g., Ruiz-García et al., 2005; Kinoshita et al., 2007).

The vegetative multiplication of elite genotypes to generate the large numbers of plants necessary for commercial production is an essential element for the commercial cultivation of all clonal crops and also of many perennial seed crops. The deployment of *in vitro* propagation methods rather than more traditional propagation approaches (such as field cuttings) can greatly increase the clonal multiplication throughput (Robert et al., 1992; Quiroz-Figueroa et al., 2006) and so accelerate the time to production. However, some *in vitro* multiplication techniques are associated with high levels of (usually) unwanted variability; known collectively as 'somaclonal variation' (e.g., Peraza-Echeverria et al., 2001). These aberrant regenerated plants can arise from both genetic and/or epigenetic-mediated alterations to gene expression and have sometimes led to significant economic losses. For example, around 5% of commercial oil palm (*Elaeis oleifera*) plants regenerated via somatic embryogenesis bore somaclonal abnormalities that included the mantled inflorescence syndrome (Jaligot et al., 2000). The appearance of these off-types was later associated with changes to their global DNA methylation status (Matthes et al., 2001) and linked to the use of specific plant hormones, growth regulators and nutrients in the culture media (Varga et al., 1988; Morcillo et al., 2006). The nature of the *in vitro* propagation system used to produce regenerated plants can have a profound effect on the likelihood of producing significant quantities of somaclonal variant plants. *In vitro* propagation systems that pass through an intermediate callus phase (such as in somatic embryogenesis) and so rely on a two stage process to generate new plants, are especially prone to evoking genetic and epigenetic change among the regenerated plants (Miguel and Marum, 2011). First, cells from the explant material must de-differentiate to form unspecialized callus cells. Second, some of these callus cells must re-differentiate in a manner that allows for the creation of the specialized cells needed to form tissues and organs. It appears that in some cases at least, one or both of these processes is incomplete. Certainly, Rodríguez López et al. (2010a) showed that the C-methylation profiles of leaves from plants recovered from somatic embryogenesis in cocoa (*Theobroma cacao*) retained many of the features of the explant tissue (staminoids) as well as only some of those found in the leaves of the mother plant. This finding suggests that at least the epigenetic DNA methylation landscape (and therefore the global gene regulation patterns) had not been entirely wiped (de-differentiated) in the

callus cells prior to the formation of new adventitious plant tissues.

Induced changes to DNA methylation and associated perturbations to gene expression has been reported for genes associated with organogenesis (De-La-Peña et al., 2012) and other developmental processes in plants (Nic-Can et al., 2013). In comparison, *in vitro* regeneration protocols that preserve meristem anatomy and function, and which generate new plants from the activation of previously dormant meristems are known as micropropagation systems and are widely viewed as being genetically analogous to field cuttings. These systems are thought to generate daughter ramet plants that are morphologically and genetically faithful replicates of the original explant material (Kahn, 2012). To date, however, little is known about the degree to which the epigenetic profiles (and so associated cell regulatory processes) of regenerated plants from micropropagation represent faithful replicates of the original mother plant. In this study we combine methylation-sensitive amplified polymorphism (MSAP) and methylation-sensitive genotype by sequencing (msGBS; Xia et al., 2014) to assess the epigenetic fidelity of meristem micropropagation and to seek specific methylation signatures associated to *in vitro* propagation in cassava (*M. esculenta*).

Materials and Methods

Plant Material

Five varieties of cassava (*M. esculenta* Crantz) namely Kiroba, Kizimbani, Kibandameno, Mfaransa, and Mzungu were used in this study. *In vitro* micropropagated samples were obtained from the tissue culture facility of the Mikocheni Agriculture Research Institute (MARI) Dar es Salaam, Tanzania and were propagated as described by (Konan et al., 1997). Field samples of the same genotypes were grown at the Sugarcane Research Institute-Kibaha (SRI-KIBAHA), Tanzania. Samples were collected from young leaves (last leaf emerged from bud), newly mature leaves (first fully expanded leaf) and primary root tips from three individual plants representing each cassava variety both from field cutting and meristem culture samples. All samples were kept in dry ice in the field and stored at -80°C until required for DNA extraction.

DNA Isolation

DNA was extracted from all samples at MARI using a DNeasy plant mini kit (Qiagen) according to the manufacturers' instructions. DNA concentration and quality was estimated using a Nano-Drop 1000 Spectrophotometer (Thermo Scientific). DNA was lyophilised prior to transport to the Plant Research Centre in Adelaide, Australia for use in subsequent MSAP or msGBS analyses. Upon arrival, all DNA samples were re-suspended in nuclease free water (Sigma), and re-quantified using the Thermo Scientific NanoDropTM 1000 Spectrophotometer. DNA concentrations were standardized to produce working solutions of 10 or 20 ng/ μl .

Methylation-Sensitive Amplification Polymorphism Procedure

A modification of the MSAP technique (Reyna-Lopez et al., 1997) was used as described by Rodríguez López et al. (2012). In brief, genomic DNA was digested with a combination of the methylation insensitive restriction enzyme *EcoRI* and one of two isoschizomer enzymes that exhibit differential sensitivity to DNA methylation (*HpaII* and *MspI*; **Table 1**). Adapters were ligated to the digested gDNA and then used as template for the first of two consecutive selective PCR amplifications in which the primers were complementary to the adaptors but possessed unique 3' overhangs (**Table 1**). *HpaII/MspI* selective primers were end labeled using a 6-FAM reporter molecule for fragment detection using capillary electrophoresis. A total of six primer combinations (**Table 1**) were tested in a pilot study using eight randomly selected DNA samples.

Sample Fractionation by Capillary Electrophoresis

Single base resolution separation of the MSAP products was achieved by capillary electrophoresis on an ABI PRISM 3130 (Applied Biosystems, Foster City, CA, USA) housed at the Australian Genome Research Facility Ltd, Adelaide South Australia. Sample fractionation was performed as follows: 2 μ l of the labeled MSAP products were combined with 15 μ l of HiDi formamide (Applied Biosystems, Foster City, CA, USA) and mixed with 0.5 μ l of GeneScanTM 500 ROXTM Size Standard (Applied Biosystems, Foster City, CA). Samples were heat-denatured at 95°C for 5 min and snap-cooled on ice for 5 min. Samples were fractionated at 15 kV for 6 s and at 15 kV for 33 min at 66°C.

Methylation Sensitive Genotyping by Sequencing

We performed the methylation-sensitive modification of the genotype by sequencing (GBS) technique (Poland et al., 2012) as described by Xia et al. (2014). In brief, a two-enzyme approach was used to generate restriction products. In this experiment, only one enzyme combination was used (*MspI* with *EcoRI*). The

selected enzyme combination was based on the results obtained using the MSAP approach. Two hundred nanogram of genomic DNA from each of the 86 selected samples [comprising three replicate per tissue/variety and growing condition (i.e., *in vitro* or field) see Supplementary Table S1] were used in a reaction volume of 20 μ l containing 2 μ l of NEB Smart cut buffer, 8 U of HF-*EcoRI* (High-Fidelity) and 8 U of *MspI* (New England BioLabs Inc., Ipswich, MA, USA). Reactions were prepared in a 96 well plate containing 87 reactions (86 DNA samples plus one Negative control water sample) and conducted on a BioRad 100 thermocycler at 37°C for 2 h and then 65°C for 20 min for enzyme inactivation. A set of 96 barcoded adapters with an *MspI* overhang and a common Y adapter with an *EcoRI* overhang were designed for the ligation reaction using barcode script made by Thomas P. van Gurp¹. Adapters were annealed prior to ligation as described by Poland et al. (2012). A full list of adapters for *MspI* (with corresponding barcodes and cassava samples) and *EcoRI* is listed in Supplementary Table S1. The ligation reaction (40 μ l) was carried out on the same PCR plate adding to the restriction products T4 Ligase (200 U) and T4 Ligase buffer (NEB T4 DNA Ligase #M0202), 0.1 pmol of the respective barcoded *MspI* adapter and 15 pmol of the common Y-adapter. Ligation was completed at 22°C for 2 h followed by an enzyme inactivation step of 20 min at 65°C. Five micro liter from each ligation reaction were pooled into a single tube and then divided into two equal volumes for column clean-up using PureLink[®] PCR Purification Kit (Life Technologies) according to manufacturer's instructions. Samples were re-suspended in 60 μ l of nanopure water. Both clean-ups were then combined and divided again into eight samples for PCR amplification. Each 25 μ l PCR consisted of 10 μ l of DNA digested/ligated library), 5 μ l of 5x NEB MasterMix, 2 μ l of 10 uM Forward and Reverse primers at 10 uM (Supplementary Table S1). Reactions were performed in a BioRad T100 thermocycler for eight cycles consisting of 95°C (30 s), 62°C (30 s), 68 °C (30 s). All eight PCR products were pooled and then purified first using a PureLink[®] PCR Purification Kit (Life Technologies) according to manufacturer's instructions (resuspended in 30 μ l). Excess adaptor was finally removed using Ampure XP magnetic beads (Beckman) by mixing 30 μ l of the pooled PCRs with 22.5 μ l of beads. Captured fragments were eluted in 30 μ l of water. Next, 125 bp paired-end sequencing was performed in one Illumina HiSeq 2000 v4lane (Illumina Inc., San Diego, CA, USA) by QBI Centre for Brain Genomics.

Statistical Analysis

Analysis of Genetic/Epigenetic Variability using MSAP

The MSAP technique uses *MspI* or *HpaII* as isoschizomers; both can cleave the motif CCGG in the absence of methylation. *MspI* can also cleave hemi-methylated dsDNA (mC in one DNA strand only) or fully methylated DNA sequences where the internal cytosine is methylated C^mCGG. However, it cannot digest hemi-methylated and fully methylated at the external cytosine site, viz: mCCGG and mCmCGG motifs (Walder

TABLE 1 | Primer sequences used for MSAP.

Oligo name	Function	Sequence
Ad <i>HpaII/MspI</i>	Reverse Adaptor	GACGTAGAGTCTAGAA
Ad. <i>HpaII/MspI</i>	Forward Adaptor	CGTTCT AGACTCATC
Ad. <i>EcoRI</i>	Reverse Adaptor	AATTGGTACGCAGTCCTAC
Ad <i>EcoRI</i>	Forward Adaptor	CTCGTAGACTGCGTACC
Pre. <i>EcoRI</i>	Preselective primer	GACTGCGTACCAATTCA
Pre. <i>HpaII/MspI</i>	Preselective primer	GATGAGTCCTGAGCGGC
<i>EcoRI</i> 5*	Selective primer	GACTGCGTACCAATTCAA
<i>EcoRI</i> 10	Selective primer	GACTGCGTACCAATTCA
<i>HpaII</i> 2.2*	Selective primer	GATGAGTCCTGAGCGGCC
<i>HpaII</i> 2.3	Selective primer	GATGAGTCCTGAGCGGCC
<i>HpaII</i> 2.4	Selective primer	GATGAGTCCTGAGCGGCC

*Indicates the selective primers used to analyze all samples.

¹www.deenabio.com/gbs-adapters

et al., 1983; Reyna-Lopez et al., 1997). In contrast, HpaII is more sensitive to methylation but can cleave hemimethylated DNA at the external cytosine position (mCCGG; Mann and Smith, 1977; Reyna-Lopez et al., 1997). Direct comparison of MspI profiles with those generated by the more methylation-sensitive HpaII therefore does not provide a definitive contrast between genetic variation and that attributable to changes in methylation (Fulneček and Kovařík, 2014). For these reasons, simple comparisons were made between profiles generated from various tissues of plants grown in the two settings (micropropagation and field cuttings) under the reasonable assumption that consistent differences will arise from differential methylation (driven by RdDM) rather than by repeated chance mutations.

MSAP profiles were visualized using GeneMapper Software v4 (Applied Biosystems, Foster City, CA, USA). Two matrices containing allelic information were generated. First, a qualitative analysis was carried out in which epiloci were scored as “present” (1) or “absent” (0) to form a presence/absence binary matrix. In this case, the selection of MSAP fragments was limited to allelic sizes between 100 and 580 bp to reduce the potential impact of size homoplasy (Caballero et al., 2008). Profile polymorphisms between DNA samples from the same cassava variety but extracted from different tissues (young leaves, newly mature leaves, and primary root tips) were retained as inter-tissue methylation differences. Polymorphisms between DNA samples from *in vitro* culture plants and from field grown plants were considered as *in vitro* culture induced methylation differences. Second, a matrix containing peak heights of fragments with allelic size between 50 and 550 bp was created for quantitative analysis (Rodríguez López et al., 2012). In both cases, different levels of hierarchy were generated to group the samples. Samples were first grouped according to cassava variety. Then, samples were divided into field grown and *in vitro* grown. Finally, samples were separated into the three different tissues of origin (young leave, mature leave, and roots).

For the analysis of the MSAP qualitative data, GenAlex v6.4 software (Peakall and Smouse, 2006) was used to infer pairwise epigenetic PhiPT distances (estimation of genetic/epigenetic distances) between different cassava samples. Analysis of molecular variance (AMOVA) was then performed using the same software to test the significance of the estimated PhiPT between tissues (Michalakis and Excoffier, 1996). An allele frequency table was generated using GenAlex 6.4 to find *in vitro*/field specific qualitative markers for each cultivar and for all cultivars. Finally, the visualization of the patterns of tissue epigenetic variations in this study was done by constructing a Principal Coordinates Analysis (PCoA).

For each variety, the peak height intensities of the epiloci generated using MSAP were compared between field grown and *in vitro* tissue samples as well as compared between samples from different tissue origins within field and *in vitro* groups. Preceding comparative analysis the data was filtered by removing epiloci containing excessively low peak height intensities across the complete set of samples. From this reduced set of epiloci the peak height libraries were

normalized using the model based weighted trimmed mean method derived in Robinson and Oshlack (2010). For each pair of tissue groups being investigated, the normalized peak heights were extracted and compared using the approach described in Robinson and Smyth (2007, 2008). This approach initially assumes the normalized peak heights are distributed as a negative binomial with a common dispersion calculated across the complete set of epiloci for the two groups. From this, individual epiloci dispersions were calculated using the empirical Bayes methods of Robinson and Smyth (2007). An exact statistical test was then conducted for each epiloci to determine differences in peak heights between the two groups (Robinson and Smyth, 2008). The *p*-values obtained from these tests were then appropriately adjusted for multiple comparisons using the false discovery rate (FDR) method of Benjamini and Hochberg (1995). All analyses were performed using the differential expression analysis R package edgeR (Robinson et al., 2010) available in the R statistical computing environment (R Development Core Team Foundation, 2015).

Analysis of Genetic/Epigenetic Variability using GBS Data

For the processing of Illumina HiSeq 2000 v4 data, the sequences from the unfiltered fastq Illumina output were separated into samples using the barcode sequence and trimmed to 64 bp using the software TASSEL (sourceforge.net/projects/tassel/). Only sequences with one of the exact used barcodes followed by the expected sequence of three nucleotides remaining from an MspI cut-site (5'-CGG-3') were retained for analysis. Sequences present in the negative water control were also removed from the analysis. Finally only sequences present in three or more different samples were kept for analysis. A matrix of sequence abundance of was then generated for further analysis.

Using the differential expression analysis procedure outlined in Section “Analysis of Genetic/Epigenetic Variability Using MSAP,” the sequence abundances were compared between field grown and *in vitro* tissue samples and also compared between samples from different tissue origins within field and *in vitro* groups. Sequences presenting significantly different number of reads between all *in vitro* and all field grown samples for each variety were isolated. Finally, only those sequences that presented the same variation (increase or reduction of number of reads in all varieties when comparing *in vitro* against field grown samples) in at least four of the five studied varieties were considered micropropagation induced markers. Due to the extremely low probability of a mutational event leading to the generation of these markers happening in all plants from all varieties during culture we can consider that such markers are differentially methylated regions (DMRs) induced by the micropropagation procedure. Detected DMRs were then selected for blast analysis against the cassava (BLASTN, nucleotide query to cassava nucleotide database genome blast tool in Phytozome. Top hits indicating differential methylation of a genic region were sought by comparing exons, introns, and flanking sequences (5 kb upstream of the Transcription Start Site and 5 kb downstream of the Transcription Termination Site).

Results

Analysis of Genetic/Epigenetic Variability using MSAP

Estimation of Genetic/Epigenetic Differences Based on Qualitative Analysis

Methylation-sensitive amplified polymorphism profiles generated a total of 164 loci (13 unique to *Hpa*II, 22 unique to *Msp*I, and 129 common to both enzymes) for the 86 samples of five cassava cultivars used in this study. PCoA analysis created from a simple presence/absence similarity matrix of the combined MSAP profiles revealed clear separation between *in vitro* propagated cultivars and their field counterparts for all cultivars (Figure 1). Calculated genetic/epigenetic distances between field and *in vitro* samples were significant for all cultivars (Table 2). In general, calculated distances between *in vitro* and field samples were higher for samples restricted using *Msp*I (Table 2). All pairwise PhiPT between *in vitro*-grown samples and those grown in the field tissues were significant for all varieties using both *Hpa*II and *Msp*I (Table 2). In general, genetic/epigenetic distances were higher between tissues recovered from plants grown in the field than between those taken from *in vitro*-grown plants (Table 3). The reduced divergence between tissues taken from *in vitro*-grown material was also evident from the PCoA analysis, with samples from different tissues of *in vitro* propagated plants occupying less eigen space than those of the same tissues obtained from field grown plants (Figure 1). However, the level of variability observed within tissue types did not differ significantly between *in vitro*-grown and field-grown material.

We further analyzed the differences existing between each tissue derived from the field grown plants and all samples from plants grown *in vitro*. The aim here was to investigate which of the *in vitro*-grown tissues generated MSAP profiles were most similar to the field samples. Distance estimates were significant for all pairs, but it was consistently smaller between young leaves from field grown plants and bulked *in vitro* tissues (Table 4).

Estimation of Genetic/Epigenetic Differences Based on Quantitative Analysis

We selected 106 markers for quantitative analysis of MSAP profiles based on peak height data. In general, both enzymes, yielded more markers separating between tissues from field-grown plants than those taken from *in vitro* material (62 vs. 44 for *Msp*I and 44 vs. 42 for *Hpa*II; Table 5; For a list of all fragments and their levels of significance see Supplementary Table S2 for *Msp*I and Supplementary Table S3 for *Hpa*II). However the number and scale of these differences varied between cultivars.

A total of 14 and 15 markers for *Msp*I and *Hpa*I respectively were found to be significantly different ($p < 0.005$) between all *in vitro*- and field-derived material. Two of these markers generated using *Hpa*II, epiloci 55 and 101 bp, were able to diagnose *in vitro* from field samples of three varieties, Kiroba, Kibandameno, and Kizimbani (Figure 2). The epilocus 55 bp generated using *Msp*I

was also significantly different between propagation systems for the same varieties but not epilocus 101 bp (Figure 2).

Analysis of Epigenetic Variability using GBS Data

In total, we generated 236,624,193 raw reads from the HiSeq 2000 v4 lane, of which 71,723,843 (32.3%) passed quality filter and contained the expected exact matches to sequences of the barcode adapter, *Msp*I restriction product site and the *Eco*RI adapter, and which appeared in at least three biological samples but were absent from the negative (water) control. On average, 754,980 high quality reads were produced per DNA sample. Collectively, this included 357,271 unique sequence tags across all samples. The number of these markers that differed significantly in abundance between *in vitro*-grown and field-grown samples varied considerably between varieties: 3,298 for Kiroba; 25,683 for Kizimbani; 2,029 for Mfaransa; 34,098 for Mzungu and 17,702 for Kibandameno. Most of these sequences were more abundant in the field-grown samples (Table 6) and the overwhelming majority was variety-specific responses. We next sought to identify candidate generic epimarks that differentiate between propagation systems across all varieties. When the most stringent filter for differential abundance was applied (i.e., reads with an FDR lower than 0.05; the phase of differential abundance being conserved across all genotypes and tissues, and the absence of variety-specific SNPs) just 105 (0.03%) of unique differential sequences featured in the profiles of all varieties and showed a common pattern of phasing (Supplementary Table S4). There was also a marked difference in the phase of these marks, with just four tags being more abundant among *in vitro*-grown samples compared with 101 that were more numerous in the field-grown plants.

When compared against the cassava nucleotide database genome using the blast tool in Phytozome², 102 differentially methylated sequences generated one or multiple hits against the cassava genome. Eighty nine top hits were associated to a gene (i.e., mapped within a window of 5 kb from the gene). BLAST results indicate that the homologs of these genes are involved in many processes, including cell differentiation, plant development, sugar metabolism, nucleic acid methylation, stress response, photosynthesis, signaling and transposon activation (Supplementary Table S5). Of the 89 differentially methylated genes 45 have been previously mentioned in the literature as having homologous genes that are: regulated by DNA methylation (14) or other epigenetic mechanisms (10), methylated or differentially methylated under different growing conditions (4), implicated in the regulation of DNA methylation (9) or other epigenetic mechanisms (1) and DNA binding proteins affected by methylation of their target sequences (3).

Discussion

Micropropagation via nodal cuttings relies on the regeneration of pre-existing meristems and so is widely considered to be

²<http://www.phytozome.net>

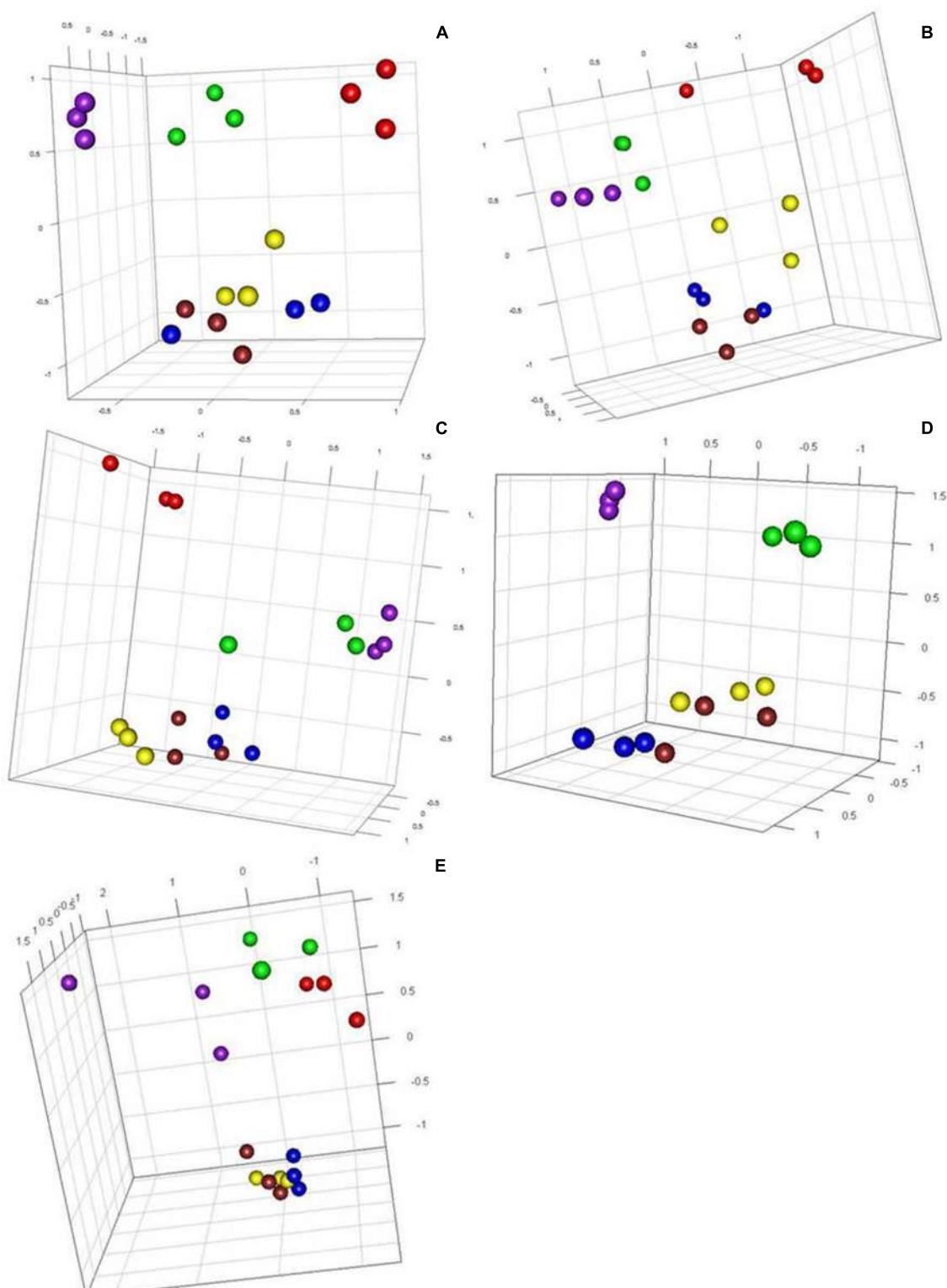


FIGURE 1 | Principal component analysis showing tissue separation in five cassava varieties using generated epigenetic profiles. Effects of tissue culture and field environment on epigenetic variation. Principal coordinate diagrams based on the Euclidean analysis of methylation-sensitive amplified polymorphism (MSAP) distances obtained from three

different tissues of Kiroba (**A**) and Mzungu (**B**), Kizimbanji (**C**), Mfaransa (**D**) and Kibandameno (**E**) varieties (*in vitro* and field cuttings) using primer combination *Hpa*I2.2/*Eco*R15. Green, field young leaves; Red, field mature leaves; Purple, field roots; Yellow, *in vitro* young leaves; Blue, *in vitro* mature leaves; and Brown, *in vitro* roots.

TABLE 2 | Summary of calculated genetic/epigenetic distance (PhiPT) between *in vitro*- and field-grown samples.

	<i>HpaII</i>		<i>MspI</i>	
	PhiPT	p-value	PhiPT	p-value
Mzungu	0.159	0.006	0.188	0.001
Kiroba	0.130	0.002	0.310	0.001
Kibandameno	0.273	0.001	0.374	0.001
Mfaransa	0.577	0.001	0.541	0.002
Kizimbani	0.160	0.003	0.243	0.001

Pairwise genetic/epigenetic distance between samples grown *in vitro* and those grown in the field from each variety were calculated using MSAP profiles obtained by restricting gDNA from five cultivars of cassava with *EcoRI* and *HpaII* or *MspI* using GenAlex 6.5.1 AMOVA. p-values were calculated based on 1000 permutations.

TABLE 3 | Effect of *in vitro* culture on epigenetic differentiation between tissues.

Tissues	<i>HpaII</i>		<i>MspI</i>		
	Cultivar	Field	In vitro	Field	In vitro
Ylv-Mlv	Kibandameno	0.208	0.070	0.237	0.000
	Mzungu	0.000	0.042	0.239	0.192
	Mfaransa	–	0.112	–	0.209
	Kizimbani	0.171	0.000	0.114	0.071
	Kiroba	0.259	0.178	0.260	0.118
	Average	0.1595	0.0804	0.2125	0.118
Ylv-Rt	Kibandameno	0.447	0.296	0.308	0.197
	Mzungu	0.000	0.162	0.461	0.178
	Mfaransa	0.401	0.260	0.538	0.148
	Kizimbani	0.142	0.272	0.654	0.286
	Kiroba	0.264	0.098	0.250	0.090
	Average	0.2508	0.2176	0.4422	0.1798
Mlv-Rt	Kibandameno	0.196	0.301	0.049	0.362
	Mzungu	0.108	0.344	0.459	0.386
	Mfaransa	–	0.408	–	0.375
	Kizimbani	0.263	0.139	0.550	0.250
	Kiroba	0.357	0.201	0.344	0.212
	Average	0.231	0.2786	0.3505	0.317

Calculated pairwise PhiPT values (epigenetic distances) between tissues from field cuttings and *in vitro* propagated lines. Distances were calculated using GenAlex 6.5.1 AMOVA on MSAP profiles obtained by restricting genomic DNA with *MspI* and *HpaII*. Ylv, young leaves, Mlv, mature leaves, Rt, roots.

analogous to field cuttings because they do not pass through a state of disorganized (dedifferentiated) tissue state (Dale and McPartlan, 1992). Nevertheless, ramets recovered from nodal micropropagation can still exhibit signs of increased morphological variability (somaclonal variation) when compared to those recovered from field cuttings (Debnath, 2005). Us-Camas et al. (2014) suggested that such observations might be explained by the stressful environment experienced by *in vitro*-grown plants (i.e., high relative humidity, low ventilation rate, high concentrations of sugars and plant growth regulators, and low light availability). Under these conditions, cultured plants cells are also forced to change their molecular make ups in order to generate different cell types. Cell division to generate tissues and organs require a precise coordination of genetic

and epigenetic processes (Miguel and Marum, 2011; Smulders and de Klerk, 2011). For micropropagation systems that rely on dedifferentiation and *de novo* organogenesis, *in vitro* culture can often yield occasional regenerants that are phenotypically off-type (e.g., Lakshmanan and Taji, 2000; Rout et al., 2000; Da Silva et al., 2015). In contrast, those recovered from meristem micropropagation are widely reported to remain more faithful to the phenotype of the parental plant in range of species (e.g., Villordon and LaBonte, 1996) including cassava (e.g., Santana et al., 2009). There is nevertheless a large body of evidence indicating that changed growing conditions often induces modulations in global methylation patterns in culture (for review see Pastor et al., 2013) and this leads to the plausible expectation of epigenetic divergence between plants cloned by meristem micropropagation and field cuttings. Evidence supporting this assertion came from a study by Baranek et al. (2010), who used MSAP profiles to compare daughter plants recovered from field cuttings and micropropagated nodal segments of two grape vine varieties. The authors found consistent differences between the two systems in their clustering on resultant dendograms. However, the work failed to further characterize the variation in terms of tissue type or to provide sequence identity for the differential epimarks. Characterizing such epigenetic differences may prove useful not only for the mere detection of putative somaclonal variants (Causevic et al., 2006) but for use in epiallele discovery, and as a tool for directed crop epigenetic improvement. In this study we combine MSAP and msGBS (Xia et al., 2014) to survey for C-methylation perturbations associated with the micropropagation of elite clones of cassava (*M. esculenta*).

Analysis of Genetic/Epigenetic Variability using MSAP

Both quantitative (Figure 1) and qualitative analysis (Table 5) of MSAP generated profiles showed clear separation of all tissues in all five varieties studied. Higher levels of diversity and divergence were observed when using *MspI* than *HpaII* (Tables 2 and 4). Care should be exercised before tentatively assigning this variability as likely to have arisen through genetic or epigenetic causes. Moreover, polymorphic markers between propagation systems that appear in the profiles generated of both isoschizomer restriction enzymes (*HpaII* and *MspI*; Figure 2) could be caused by either a genetic or an epigenetic change. Conversely, a polymorphic marker detected by only one of the enzymes can only be epigenetic in nature (Pérez-Figueroa, 2013). Application of this reasoning implies that variation at the propagation system diagnostic 55 epilocus could be explained by both genetic and epigenetic changes whereas that of epilocus 101 was due to differential methylation arising from the tissue culture conditions (Figure 2). However, since the chance of a genetic mutation occurring at exactly the same location on more than one occasion is extremely low (Rodríguez López et al., 2010b) combined with the fact that these two markers polymorphic were found in three different cultivars implies that both markers probably have an epigenetic origin rather than one caused by genetic mutation.

The variability in MSAP profiles seen between DNA extracted from the same tissue type was both modest and consistent,

TABLE 4 | Epigenetic distance between field cutting tissues and all tissues from *in vitro* conditions.

	Kizimbani	Mzungu	Kiroba	Mfaransa	Kibandameno
Young lv	0.236*(0.007)	0.276*(0.004)	0.308*(0.006)	0.559*(0.026)	0.502*(0.007)
Mature lv	0.350 (0.001)	0.286 (0.003)	0.423 (0.007)	Δλ	0.521 (0.004)
Root	0.528 (0.005)	0.398 (0.006)	0.389 (0.007)	0.655 (0.006)	0.576 (0.007)

Calculated pairwise tissue PhiPT values (epigenetic distances) obtained from MSAP profiles obtained by restricting genomic DNA from ten cultivars of cassava with Mspl using GenAlex 6.5.1 AMOVA. The values show the distance between individual tissues of field cutting lines from bulks of all tissues of *in vitro* propagated lines. Young lv: distance between field derived young leaves and bulked *in vitro* tissues, Mature lv: distance between field derived mature leaves and bulked *in vitro* tissues, and Root: distance between field derived root and bulked *in vitro* tissues. Probability values based on 9999 permutations are shown in parenthesis. Asterisks indicate lower PhiPT values.

TABLE 5 | Number of significantly different quantitative epimarkers across all cultivars.

Cultivar	Mspl			HpaII		
	Field vs. <i>in vitro</i>	Tissues (<i>in vitro</i>)	Tissues (Field)	Field vs. <i>in vitro</i>	Tissues (<i>in vitro</i>)	Tissues (Field)
Mfaransa	6	10	7	6	9	2
Mzungu	0	9	19	1	9	7
Kizimbani	3	1	27	2	7	7
Kiroba	8	13	8	3	12	11
Kibandameno	6	11	1	7	5	17

Quantitative markers were generated from MSAP profiles peak heights obtained by restricting genomic DNA from five cultivars of cassava (*in vitro* and *in the field*) with Mspl and HpaII. Column Field vs. *in vitro* shows the number of significantly different ($p > 0.005$) epimarkers between samples of the same cultivar grown *in vitro* or *in the field*. Columns Tissues (*in vitro*) and Tissues (Field) show the number of significantly different ($p > 0.005$) epimarkers between tissues grown either *in the field* or *in vitro*.

regardless of the propagation system used to produce the plants (**Figure 1**). This finding suggests that these DNA methylation changes induced by micropropagation are not random, as would be expected for genetic somaclonal variation (Bairu et al., 2011) and so more likely to be associated with methylation events associated with cell and tissue differentiation. Circumstantial evidence in support of this inference can be taken from the PhiPT distance estimate, which showed that samples from *in vitro* nodal micropropagation ramets were always (epigenetically) closer to young leaves of their field counterparts (**Table 4**).

Genetic variation induced during *in vitro* nodal micropropagation cannot be ruled out in this study. However, our results suggest that the majority, if not all, the variability detected using MSAPs is epigenetic in nature. This is supported by the lack of higher levels of variation between micropropagated samples than in field grown samples and the fact that the observed variability seems to be conserved between different plants and between different varieties (**Figure 1**) and also by previous studies that show that micropropagated plants using this approach present high levels of genetic stability (Lata et al., 2010) but measurable levels of epigenetic variability (Baranek et al., 2010).

Our MSAP results suggest that (1) *in vitro* nodal micropropagation introduces *de novo* variability in the global methylation patterns; (2) micropropagation induced epigenetic variability does not seem to be random.

Analysis of Epigenetic Variability using GBS Data

Most studies of the epigenetic basis of somaclonal variation have used MSAPs to characterize culture-induced epigenetic

variation. This technique is reliable and does not require previous knowledge of the studied organism. Conversely, it presents the disadvantage that the generated markers are anonymous. It is possible to isolate and sequence the differential markers (Massicotte et al., 2011), although the process can be cumbersome, expensive and time-consuming (Schrey et al., 2013), especially when many markers and samples are involved. The use of Next-generation sequencing can significantly reduce the cost of epiallele sequence characterization. The recent development of GBS (Elshire et al., 2011; Poland et al., 2012) and its methylation-sensitive version (ms-GBS; Xia et al., 2014) has allowed for a simple, time and cost effective system for the sequencing of multiple DMRs in non-model organisms.

Our study uncovered 105 unique sequences (Supplementary Table S4; 0.03% of those generated) with different levels of methylation between propagation systems. Although total sequence reads were similar between systems, the vast majority of differential tags (101/105) were more abundant among ramets recovered from field cuttings, suggesting again lower global levels of methylation in field grown plants. This is in contradiction with previous evidence suggesting that *in vitro* culture is related to low DNA methylation (Valledor et al., 2007). However, deciphering global hyper/hypomethylation from restriction products is not a reliable approach (Fulneček and Kovařík, 2014). What is more, other studies have shown that methylation levels during *in vitro* propagation are related to the donor tissue (Fang et al., 2009; Wang et al., 2012), to the length of the culture (Díaz-Sala et al., 1995; Rodríguez López et al., 2010a,b), and the media components (LoSchiavo et al., 1989; Arnholdt-Schmitt, 1993).

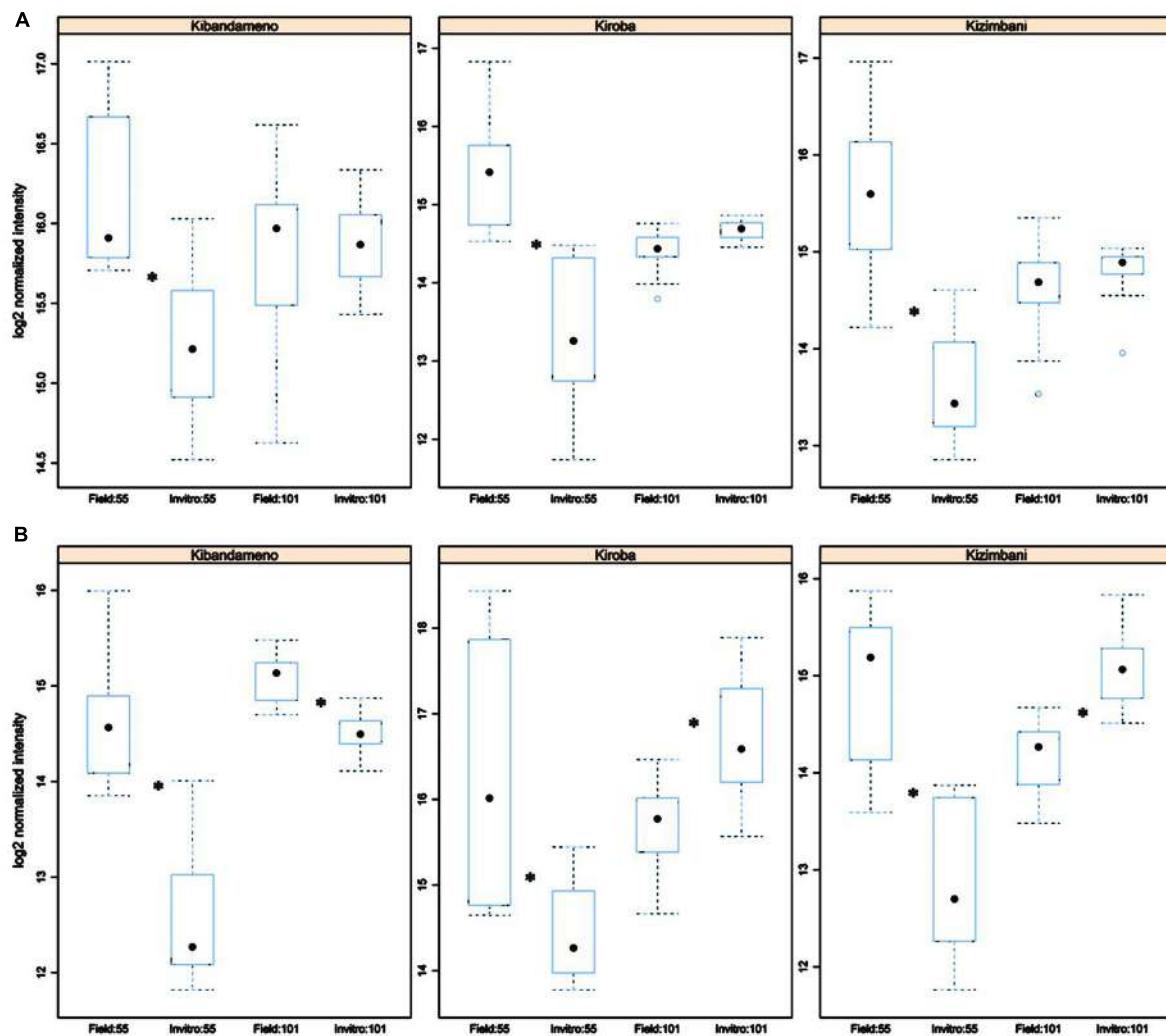


FIGURE 2 | Differential peak intensity of informative epiloci (55 and 101 bp) between *in vitro* tissues and field tissues for three cassava cultivars (Kibandameno, Kiroba, Kizimban). Peak intensities were obtained from MSAP profiles generated restricting genomic DNA from three different tissues (i.e., young leaf, mature

leaf, and roots) in three plants grown either *in vitro* or in the field with *MspI* (A) and *HpaII* (B) and amplifying using primer combination *HpaII*2.2/*EcoRI*15. Box plots show the average normalized intensity scores for a 55 and a 101 bp MSAP fragment selected using. Asterisk indicates $p < 0.005$.

TABLE 6 | Number of significantly different msGBS sequences between *in vitro* and field grown samples.

Cultivar	<i>In vitro</i>	Field	Total
Mfaransa	185	1844	2029
Mzungu	16021	17888	34098
Kizimban	2245	23438	25683
Kiroba	1918	1380	3298
Kibandameno	5068	12634	17702

"*In vitro*" and "Field" columns indicate the number of sequences with a higher number of reads from samples grown under each condition.

BLAST analysis against the cassava genome of the micropropagation induced DMRs generated in this study yielded significant hits for 102 sequences of which the 89

top hits were each associated to a gene (i.e., mapped within a window of 5 kb from the gene; Supplementary Table S5). BLAST results indicate that the homologs of these fragments are involved in many processes, including cell differentiation, plant development, sugar metabolism, nucleic acid methylation, stress response, photosynthesis, cell wall modifications, signaling and transposon activation (Supplementary Table S5). However, it is important to remember that the mere presence of differential methylation in or around a gene is not sufficient evidence to infer that expression of the gene is actually regulated by methylation. There are nevertheless enticing hints to suggest that these candidate methylation markers for propagation system may indeed also play a role in metabolic divergence between field cuttings and meristem micropropagated plants.

A series of studies have implicated DNA methylation in the regulation of genes controlling pathways in plant developmental progression or tissue differentiation (Messeguer et al., 1991), during embryogenesis, seed formation (Xiao et al., 2006), apical dominance regulation, flowering, and floral and leaf formation (Finnegan et al., 1996). Several differentially abundant loci identified in the present study showed high sequence homology to loci in cassava that have been previously implicated in cell differentiation and development: CWF19, XPMC2, EXO70, TAP42-like, Sterile alpha motif (SAM) domain-containing protein, AP3M, Enhancer of polycomb-like transcription factor protein, cassava protein containing a transcription factor UCC1, GFS9, GT-2, EMB71, and ARF2 (Supplementary Table S5). Even if it were shown that the changes in methylation among loci identified are causally linked to changes in gene expression, further work would still be required to establish whether such changes are sufficient to cause a biological meaningful change in cell metabolism and phenotype. Once again, however, there are some grounds to reason that at least some loci might.

A number of studies have shown that DNA methylation plays a central role in gene expression and plant development under stress (for extensive reviews see Chinnusamy and Zhu, 2009; Kinoshita and Seki, 2014). Not surprisingly perhaps, abiotic stresses like those encountered under *in vitro* culture conditions have been found to impose an effect on DNA methylation and have been correlated with subsequent organogenesis (Us-Camas et al., 2014). However, the comparative paucity of marks that appear at higher abundance in meristem culture (just 4 of the 105 generic marks; Supplementary Table S4) suggests that changes of this type lay in the minority. Explanation is therefore required for the far more commonly encountered appearance of marks among the field cutting samples only. Perhaps the most plausible hypothesis for this divergence lay in the more variable living environment experienced by field cutting plants when compared with the more homogeneous environment in culture. Field-grown plants are continuously exposed to pathogens throughout their lifetime and their DNA epigenetic patterns become altered by infection (Alvarez et al., 2010; De-La-Peña et al., 2012). It is therefore, not difficult to presume that *in vitro* grown plants will not experience the same pathogen or abiotic stress-induced DNA methylation marks as will those grown exposed to pathogens in the field. In our study, 13 of the 105 differentially methylated loci mapped to locations of the cassava genome associated to known stress response genes [i.e., Calcium-dependent lipid-binding (CaLB domain) family protein, Plastocyanin-like domain, Leucine-Rich Repeat Receptor-Like Protein Kinase (LRR-RK), Disease resistance protein (TIR-NBS-LRR class) family, ATBCB, ATHCHIB, ATTTM2, MLP-Like Protein 28, ATMGL, ATBZIP1, XTH1, Peroxidase superfamily protein, ATATG18F and HT1; Supplementary Table S5]. Plants have evolved two different strategies involving LRR proteins to perceive microbial pathogens. LRR-RKs are transmembrane host-encoded pattern-recognition receptors that directly recognize pathogens while NBS-LRR indirectly recognize pathogen effectors by sensing their effects on plant target proteins (Yu et al., 2013). The

latest has been shown to be regulated by DNA methylation (Yu et al., 2013). Previous studies have shown that overexpression of NBS-LRRs induces a severe drop in fitness (Tao et al., 2000). Our results show several of the 105 candidate loci associated to both types of LRRs, so it would be tempting to speculate that the agronomic performance of micropropagated plants could be potentially affected if the observed changes on DNA methylation led to the overexpression of such genes. Calcium lipid-binding domain (CaLB domain) proteins are repressors of abiotic stress response in plants (de Silva et al., 2011) and have been shown to be regulated by environmental conditions through DNA methylation (Dubin et al., 2015). Curiously, our results showed one differentially abundant sequence matched to the xyloglucan endotransglucosylase/hydrolase1 (XTH1) gene. Previous studies have shown that XTHs have a function on cell wall modifications and that changes on their DNA methylation levels are associated to colonization of potato plants by beneficial bacterial endophytes (Da et al., 2012).

In all, 45 of the 89 differentially abundant sequences matched to genes that have been previously reported to be: regulated by DNA methylation (14) or other epigenetic mechanisms (10), methylated or differentially methylated under different growing conditions (4), associated to the regulation of DNA methylation (9) or other epigenetic mechanisms (1) and DNA binding proteins affected by methylation of their target sequences (3) (For references see Supplementary Table S5). Which (if any) of these genes is playing a role in a possible divergence in cell metabolism and phenotype between plants replicated by meristem-propagation and field cutting warrants further attention. Looking further ahead, identifying the developmentally important genes whose expression is sensitive to culture growth conditions may ultimately allow for the development of new culture regimes that yield regenerants with the lowest possible incidence of off-types. In the shorter term, however, the provision of methylation marks that consistently diverge in abundance between plants propagated by meristem culture and those recovered by field cuttings could have utility in the optimization of *in vitro* meristem propagation protocols and also in the diagnosis of the origin of clonal stocks.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00590>

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Maize miRNA and target regulation in response to hormone depletion and light exposure during somatic embryogenesis

Elva C. Chávez-Hernández, Naholi D. Alejandri-Ramírez, Vasti T. Juárez-González and Tzvetanka D. Dinkova*

Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México, Mexico City, Mexico

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Clelia De-la-Peña,
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Reviewed by:

Abu Hena Mostafa Kamal,
National Agriculture and Food
Research Organization, Japan
Soumitra Paul,
Krishnagar Government College, India

*Correspondence:

Tzvetanka D. Dinkova,
Departamento de Bioquímica,
Facultad de Química, Conjunto E,
Universidad Nacional Autónoma de
México, 04510 Mexico City, Mexico
cesy@unam.mx

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Maize somatic embryogenesis (SE) is induced from the immature zygotic embryo in darkness and under the appropriate hormones' levels. Small RNA expression is reprogrammed and certain miRNAs become particularly enriched during induction while others, characteristic to the zygotic embryo, decrease. To explore the impact of different environmental cues on miRNA regulation in maize SE, we tested specific miRNA abundance and their target gene expression in response to photoperiod and hormone depletion for two different maize cultivars (VS-535 and H-565). The expression levels of miR156, miR159, miR164, miR168, miR397, miR398, miR408, miR528, and some predicted targets (*SBP23*, *GA-MYB*, *CUC2*, *AGO1c*, *LAC2*, *SOD9*, *GR1*, *SOD1A*, *PLC*) were examined upon staged hormone depletion in the presence of light photoperiod or darkness. Almost all examined miRNA, except miR159, increased upon hormone depletion, regardless photoperiod absence/presence. miR528, miR408, and miR398 changed the most. On the other hand, expression of miRNA target genes was strongly regulated by the photoperiod exposure. Stress-related miRNA targets showed greater differences between cultivars than development-related targets. miRNA/target inverse relationship was more frequently observed in darkness than light. Interestingly, miR528, but not miR159, miR168 or miR398, was located on polyribosome fractions suggesting a role for this miRNA at the level of translation. Overall our results demonstrate that hormone depletion exerts a great influence on specific miRNA expression during plant regeneration independently of light. However, their targets are additionally influenced by the presence of photoperiod. The reproducibility or differences observed for particular miRNA-target regulation between two different highly embryogenic genotypes provide clues for conserved miRNA roles within the SE process.

Keywords: hormone depletion, maize, miRNA, photoperiod response, polyribosomes, somatic embryogenesis

Introduction

Maize (*Zea mays* L.) is one of the most important widely cultivated cereal crops, used as valuable source for human food, livestock feed, and raw material for the industry (Huang et al., 2002). Besides its agricultural and economic relevance, it has been a major model system in plant genetics and improvement. Common methods for maize transformation involve a process known as somatic

embryogenesis (SE). SE begins with callus induction, a process characterized by re-organization and re-structuring of somatic cells in the presence of the synthetic auxin 2,4-D (Zimmerman, 1993). The second step of SE is plant regeneration. Plants can be regenerated when somatic embryos are depleted of external hormones and are exposed to photoperiod (Garrocho-Villegas et al., 2012). SE has been advantageously used in many plant species for clonal propagation, plant transformation and genetic improvement (Stasolla and Yeung, 2003).

Plant regeneration through maize SE was first reported by Green and Phillips (1975) using immature embryos as the initial explant. Various conditions for callus induction and plant regeneration have been tested since then, encountering the embryogenic potential highly dependent on explant and maize genotype used (Obert et al., 2009; Shen et al., 2012). The immature embryo's developmental stage and size, usually from 12 to 18 days upon pollination, appear as particularly relevant to generate embryogenic callus type II associated with high plant regeneration frequency over long subculture periods (Armstrong and Green, 1985). A few inbred lines have been reported with good embryogenic potential (Armstrong and Green, 1991; Jakubeková et al., 2012; Shen et al., 2012). Creole varieties VS-535 and H-565 (Costeño mejorado), derived from the germplasm of Mexican landrace Tuxpeño, are highly embryogenic and their plant regeneration frequency could be maintained for over 2 years of subculture (Garrocho-Villegas et al., 2012). However, while VS-535 has been successfully used for more than 15 years in tissue culture, H-565, released in 2008, has shown variable behavior in callus proliferation and plant regeneration responses through SE.

The mechanisms underlying gene activation/repression in the SE process have been poorly characterized (Salvo et al., 2014). Recently, a growing number of reports evidenced essential regulatory roles for microRNAs (miRNAs) in plant developmental and differentiation processes, including zygotic embryogenesis (Nodine and Bartel, 2010; Willmann et al., 2011), hormone signaling (Guo et al., 2005; Reyes and Chua, 2007) and stress response (Sunkar and Zhu, 2004). MicroRNAs are small 21–22 nt RNAs derived from longer precursors by Dicer-like (DCL) endonuclease activity and recruited to protein complexes by Argonaute (AGO) to target specific mRNA repression, either through degradation or translation inhibition (Llave et al., 2002; Li et al., 2013b).

In the past few years, several publications reported the miRNA presence/regulation during plant SE (Luo et al., 2006; Zhang et al., 2010, 2012; Chen et al., 2011; Wu et al., 2011, 2015; Li et al., 2012; Shen et al., 2012, 2013; Lin and Lai, 2013; Qiao and Xiang, 2013; Yang et al., 2013). Most of these reports used a high-throughput sequencing technology to compare the presence of conserved and species-specific miRNAs in the embryogenic callus (EC) before and during different stages of SE. Overall, an up-regulation of specific miRNAs was observed at particular stages of the somatic embryo differentiation. For most of the analyzed plant species, the proliferative embryogenic callus is characterized by low expression of many miRNAs related to flowering and leaf development, while stress-related miRNAs are increased in comparison to the original explant. Although

miRNA expression patterns are altered upon callus induction, between embryogenic and non-embryogenic callus, as well as during SE and differentiation, the enriched or decreased miRNA species are particular for each of the plant species analyzed. Therefore, it is important to approach the miRNA regulation in the context of each plant SE system, taking into account the specific conditions and genotypes used in dedifferentiation as well as in plant regeneration induction.

Shen et al. (2013) analyzed by deep sequencing miRNA expression patterns upon callus induction from the maize inbred line 18-599R. They identified miR528, miR156, miR166, miR168, miR390, miR164, miR167, miR398, miR397, miR408, and miR319 as the most abundant during dedifferentiation. These miRNAs increased as the embryos were dedifferentiated into calli, with the exception of miR166 and miR167 that decreased. A degradome analysis indicated that most of their targets are involved in hormone signaling transduction pathways. In a previous study performed on VS-535-derived EC, we found that development-related miRNAs such as miR156, miR159, miR164 and miR168 decreased as the length of subculture increased, while stress-related miRNAs such as miR397, miR398, miR408, and miR528 remained highly expressed (Dinkova and Alejandri-Ramirez, 2014). However, the regulation on miRNA expression and their target mRNAs has not been explored in maize plant regeneration through SE. In this study, we addressed the impact of light exposure and hormone depletion as plant regeneration cues on maize miRNA levels and target gene expression. Taking into account the relevance of maize genotype on plant regeneration success through SE, we investigated how general the observed changes were by comparing two closely related cultivars, VS-535, and H-565.

Materials and Methods

Callus Induction and Subculture

To induce embryogenic callus type II, immature embryos were collected at 15–18 days after pollination from two closely related maize cultivars, VS-535 and H-565. These cultivars have been derived from the Tuxpeño landrace germplasm (Márquez-Sánchez, 2008). Tuxpeño (VS-535) was previously shown to display high embryogenic potential during *in vitro* culture (Garrocho-Villegas et al., 2012), while Costeño mejorado (H-565) was introduced more recently (Márquez-Sánchez, 2008) and its behavior in tissue culture has been variable (unpublished data). The ears from the middle part of the husk (similar developmental conditions) were gently washed, first with 70% ethanol (1 min); then with 50% bleach solution (15 min); and three times with sterile deionized water. Next, the immature embryos were dissected and placed on a Petri dish with sterile deionized water and 1 g L⁻¹ Cefotaxime. Thirty embryos (embryo axis side down) were placed per Petri dish on N6I medium (Supplementary Material, Data sheet 1) and maintained for 3 weeks under darkness at 25 ± 2°C. Upon this time, pro-embryogenic masses were selected for subculture on proliferation medium N6P (Supplementary Material, Data sheet 1). Every 3 weeks the embryogenic callus was subcultured on fresh N6P medium.

Plant Regeneration

To test the effects of hormone depletion and light on miRNA-mediated regulation during plant regeneration, the embryogenic callus was subjected to stage hormone depletion under darkness or light photoperiod (16 h light/8 h dark). During the first stage, the 2,4-D and kinetin concentrations were half-reduced and during the second stage (2 weeks after the first regeneration subculture) hormones were omitted from N6P. Samples were collected 1 week upon each subculture and stored at -70°C until used. Three biological samples were collected from each stage for RNA extraction. Regenerating callus was maintained on N6P devoid of hormones with every 2 weeks-subcultures until plantlets appeared under photoperiod. Plantlets were subcultured on MS medium (Murashige and Skoog, 1962; Supplementary Material, Data sheet 1).

Total and Polysomal RNA Isolation

Total RNA was isolated from triplicates with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. For polyribosome fractionation 5 g of embryogenic callus were pulverized in liquid nitrogen with mortar and pestle. The powder was suspended in 25 mL of lysis buffer (200 mM Tris-HCl pH 8.5, 50 mM KCl, 25 mM MgCl₂, 2 mM ethylene glycol tetra-acetic acid and 0.05 mg mL⁻¹ cycloheximide) and clarified by centrifugation at 15,000 rpm for 15 min. The supernatant was layered onto 4 mL sucrose cushion buffer (50 mM Tris-HCl pH 8.5, 25 mM KCl, 10 mM MgCl₂, 60% sucrose and 0.05 mg mL⁻¹ cycloheximide) and centrifuged at 45,000 rpm in a 75Ti rotor (Beckman Coulter, Mexico City, Mexico) for 3 h to concentrate ribosomes. The ribosomal pellet was suspended in 0.5 mL of DEPC water, layered onto 15–60% continuous sucrose gradient and centrifuged in SW-40 rotor (Beckman) at 36,000 rpm for 2.0 h. Fractionation and absorptivity at 260 nm of the gradient was performed in an Auto Densi-flow system (Labconco, Kansas City, MO, USA) connected to Econo UV Monitor EM-1 (BioRad). RNA was isolated from each fraction as described previously (Martinez-Silva et al., 2012). The RNA quality was tested by agarose gel electrophoresis and the concentration was measured with Nanodrop.

Northern Blot

Ten micrograms of total RNA were separated by electrophoresis on 15% polyacrylamide gels including 7 M urea, transferred to Hybond-N+ membranes (GE Healthcare Life Sciences, USA) and hybridized in ULTRAhyb®-Oligo hybridization buffer (Ambion, USA) with oligonucleotide probes for each miRNA (Supplementary Material, Table S1) end-labeled with [γ -32P]ATP (PerkinElmer, USA) by T4 polynucleotide kinase (NEB, USA).

miRNA Target Prediction

Targets of zma-miRNAs were predicted by psRNATarget program (<http://plantgrn.noble.org/psRNATarget/>) with default parameters (Dai and Zhao, 2011), except for the Maximum expectation parameter which was set at five to get a higher prediction coverage. The selected genomic library for the target search was: "Zea mays (maize), transcript, NSF-funded Maize

Genome Sequencing Project, Release 5a, filtered set." Target gene descriptions were retrieved from biomart (Kasprzyk, 2011). Some of the predicted miRNA targets (Supplementary Material, Table S2) were chosen to evaluate their expression levels during plant regeneration (Table 1) according to the following criteria: (1) appropriate miRNA:target pairing in the seed region; (2) annotation of the target as protein coding transcript; and (3) experimental evidence as miRNA target in maize and/or in other plant species.

qRT-PCR

Total RNA was extracted from two independent biological samples, treated with RQ1 RNase-Free DNase (Promega, USA) and reverse-transcribed using the ImProm-II™ reverse transcription system (Promega, USA). Each RNA sample was reverse-transcribed in two replicate reactions. Quantitative PCR (qPCR) was performed on the two biological samples for each genotype with three technical replicates per cDNA, using the Express GreenER qPCR reagents (GE Healthcare Life Sciences, USA) in a 7500 Real-time PCR System (Applied Biosystems, USA). Specific primers for qPCR were designed using Primer3Plus (Untergasser et al., 2007) and are available in Supplementary Material, Table S3. Relative abundance was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001). Target levels were normalized by rRNA 18S as internal housekeeping control and then compared to the levels found for the initial tissue (100% hormones, darkness). qRT-PCR fold-change data were summarized as Mean + Standard Error. The results obtained for each condition were compared performing a two-way (light \times hormone)-ANalysis Of VAriance (ANOVA). The significance of mean difference within and between the groups was retrieved using Tukey Honestly Significance Difference (HSD) at $P < 0.05$.

Results

Plant Regeneration through Somatic Embryogenesis

Staged hormones depletion combined with light photoperiod promotes plant regeneration through SE in maize (Jakubeková et al., 2012; Garrocho-Villegas et al., 2012). Both signals exert important effects at molecular, biochemical, and physiological levels leading to a developmental switch from highly proliferating dedifferentiated tissues to fully differentiated plantlets. In agreement with this, clear morphological changes were observed upon hormone depletion in both, light photoperiod and darkness (Figure 1). Particularly, globular compact structures appeared at 50% hormone (2,4-D and kinetin) reduction in light and 0% hormones in darkness (Figure 1E and Figure 1C, respectively). The light presence rapidly (< 24 h) induced pigment deposition in the callus. For both conditions, the globular structures became elongated, but while under light they eventually resulted in plantlets, in darkness only organogenesis was appreciated (Figure 1G vs. Figure 1D). Fully developed plantlets were derived from 2 years-subcultured EC at 6–7 weeks upon hormones depletion for both, VS-535 and H-565. However, qualitative differences during plant regeneration were evident

TABLE 1 | Analysis of miRNA targets.

miRNA	Target	Description	Hybrid	E	UPE	
miR156a-5p	GRMZM2G126018_T01	Squamosa promoter binding like transcription factor family; protein isoform 1 (SBP23)	mirRNA	CACGAGUGAGAGAAGACAGU	1	18.45
			Target	: : : : : : : : : : : :		
miR159a-3p	GRMZM2G139688_T01	Zea mays GAMYB transcription factor	mirRNA	GUCUCGAGGGAAAGUUAGGUU	2.5	17.184
			Target	. . : : : : : : : : : :		
miR164a-5p	GRMZM2G393433_T01	CUC2; Putative NAC domain transcription factor superfamily	mirRNA	CGUGCACGGGACGAAGAGGU	1	17.089
			Target	: : : : : : : : : : : :		
miR168a-5p	GRMZM2G039455_T01	Argonaute-like protein	mirRNA	AGGGCUAGACGUGGUUCGCU	3.5	19.106
			Target	: : : : : : : : : : : :		
miR397a-5p	GRMZM2G146152_T01	LAC2; Multicopper oxidase, Laccase, Cupredoxin	mirRNA	GUAGUUGCGACGCGAGAUACU	3	23.155
			Target	: : : : : : : : : : : :		
miR398a-3p	GRMZM2G058522_T01	SOD-4A; Superoxide dismutase; [Cu-Zn] 4AP (SOD9)	mirRNA	GCCCCCGCUGGACUCUUGUGU	3.5	17.156
			Target	. . : : : : : : : : : :		
miR408a	GRMZM2G384327_T03	Zea mays gamma response I protein	mirRNA	CGGGUCCUUCUCCGUCACGUC	3.5	21.422
			Target	: : : : : : : : : : : :		
miR528a-5p	GRMZM2G106928_T01	SOD-1A; Superoxide dismutase [Cu-Zn]	mirRNA	GCGAGAGAAGAGGCCGUGCAG	2.5	11.294
			Target	: : : : : : : : : : : :		
miR528a-5p	GRMZM2G107562_T01	Plastocyanin-like Blue (type 1) copper ion binding protein	mirRNA	UUCCUCCGCACGCCUUUCCA	2.5	18.145
			Target	: : : : : : : : : : : :		

miRNA targets were predicted with psRNATarget (<http://plantgrn.noble.org/psRNATarget>). The Expectation (E) value refers to the score of complementarity between each miRNA and target transcript. The UnPair Energy (UPE) value refers to the energy required for secondary structure melting around the target site. A lower UPE implies higher possibility for establishing a contact between miRNA and target mRNA, as well as for AGO-mediated cleavage.

between these genotypes. For instance, the greenish color was more intense for VS-535 callus and a greater proportion of plantlets were obtained per gram of tissue for this variety (data not shown).

Specific miRNA Expression Is Mostly Affected by Hormones Depletion

Previous studies in maize long-term subcultured EC indicated that miR156, miR159, miR164, miR168, and miR319 importantly reduce their levels in subcultures maintained for more than 18 months (Dinkova and Alejandri-Ramírez, 2014). On the other hand, the stress-related miR397, miR398, miR408 and miR528, become enriched upon callus induction and remain at high levels once the proliferation is established. This is in accordance with the proposal that specific miRNA expression in the undifferentiated EC associates with their proliferation maintenance, suggesting a switch from this expression pattern during plant differentiation (Luo et al., 2006). To evaluate which stimulus promotes miRNA expression changes, we tested the effect of hormone depletion in darkness or light for two independent maize 2 year-subcultured EC, owing that only in the presence of light plant regeneration could be achieved.

In VS-535-derived EC, most of the analyzed miRNAs increased in response to hormone half reduction (50%), regardless the presence of light (Figure 2A; lanes b and d).

Although similar behavior was observed in H-565-derived callus, fold changes with respect to 100% hormones were distinct if compared to VS-535 (Figure 2B). For instance, at least two-fold increase in miR156, miR164, miR168 and miR408, was observed in VS-535, 50% hormones, while a modest 1.2–1.4-fold increase under the same conditions was evident for H-565 (Figure 2 and Supplementary Material, Figure S1). The next stage, 0% hormones, implied a sharp reduction for most miRNAs in VS-535, while in H-565 the reduction was lower or not observed depending on the miRNA. The levels of miR156 and miR164 decreased by three-fold (with respect to 50% hormones) for VS-535, but showed no change for H-565 (Figure 2, lanes c vs. b and e vs. d). Curiously, miRNA levels in the initial tissue (100% hormones) at 2 year-subcultures were also different between maize genotypes (data not shown). The effect of hormone depletion on miRNA expression was greater in the presence of light, at least for VS-535. miR398 decreased almost by 40-fold between 50 and 0% hormones in VS-535 against a modest two-fold decrease in H-565.

An exemption to most miRNA expression patterns was miR159, showing little or no change in response to hormones depletion in both cultures. In addition, while miR168 importantly increased (around two-fold) in 50% hormones for VS-535, it remained at similar levels for H-565. Regarding the differences observed between genotypes, it is important to stress out that

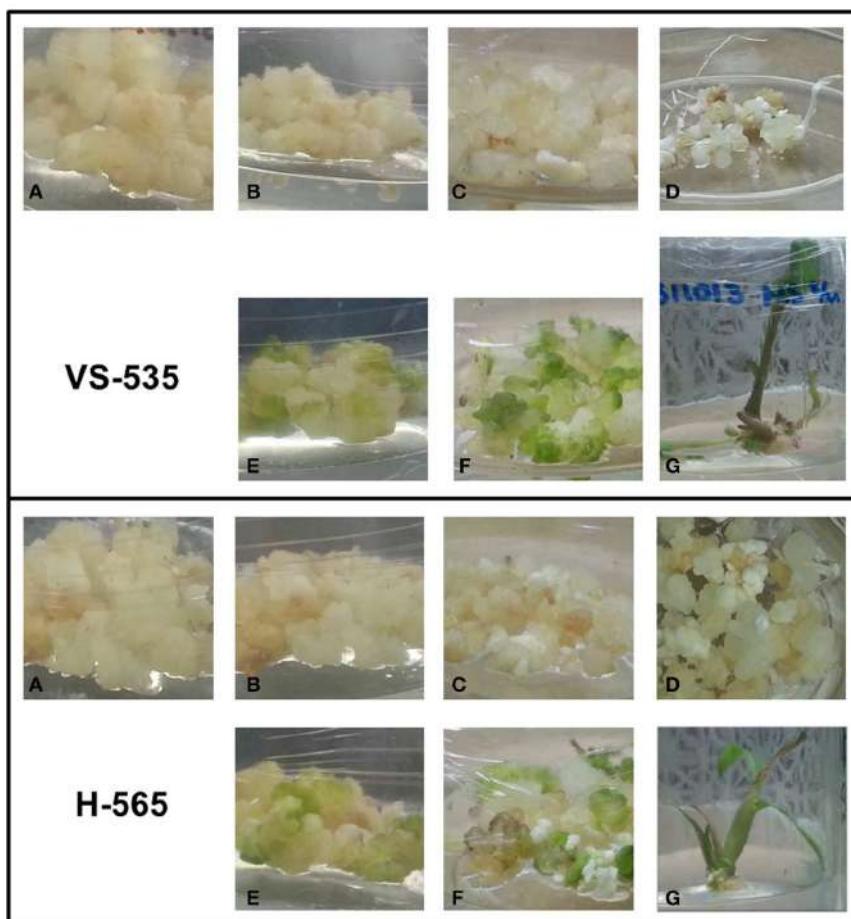


FIGURE 1 | Maize plant regeneration through somatic embryogenesis (SE). Embryogenic callus (**A**), derived from VS-535 or H-565 maize cultivars, was subcultured on N6P medium with half the hormone (2,4-D and kinetin) concentration (**B,E**) and 2 weeks later on N6P medium without hormones (**C,F**). Further subcultures were performed every 2 weeks until plant

regeneration was achieved. Cultures were kept in darkness (**A–D**) or under a photoperiod of 16 h light/8 h dark (**E–G**). Organogenesis was observed in darkness (**D**) while in photoperiod plantlets were formed (**G**). Plantlets were subcultured on MS medium. Tissue was collected 1 week after the subculture.

tissues cultured *in vitro* are highly heterogeneous. Although for RNA analysis, EC was always sampled a week upon subculture, and visibly embryogenic differentiating tissues were selected, the heterogeneity inherent to each cultivar could not be avoided. The miRNA expression levels achieved during hormone depletion were maintained or further decreased in plantlets regenerated from VS-535 (Figure 2, lane f). Particularly, the stress-related miR397, miR398, miR408, and miR528 showed about two-fold reduction in VS-535 fully developed plantlets with respect to dedifferentiated tissues (100% hormones). However, in H-565-derived plantlets miR397 and miR398 remained expressed at higher levels.

Light Is a Major Stimulus Affecting Development-related miRNA Target Levels during Maize SE

Several of the miRNA targets analyzed in this study (*SBP23*, *GAMYB*, *CUC2*) encode for transcription factors known to participate in plant developmental switches including zygotic

embryogenesis (Table 1). Others represent enzymes involved in plant stress response (targets of miR397, miR398, miR408, miR528) or the miRNA biogenesis pathway itself (miR168). According to this, we separated the results from qRT-PCR in development-related and stress-related miRNA targets (Figures 3, 4). The levels of *GRMZM2G039455_T01*, an AGO-like transcript also termed *AGO117* or *AGO1c* (Qian et al., 2011) were considered within the development-related targets, but according to its proposed function in miRNA biogenesis it actually corresponds to any of the subdivisions.

Development-related target mRNA levels displayed mostly contrasting behavior between darkness and photoperiod. The Squamosa Promoter Binding protein (SBP)-like transcript *GRMZM2G126018_T01* (*SBP23*) targeted by miR156 showed significantly higher levels upon hormone half reduction under darkness than in the presence of light (Figure 3, b–c vs. d–e). Under darkness, SBP23 inversely mirrored miR156 changes due to hormone depletion (Figure 3A, lanes b–c; and Figure 3B, lanes a–b). However, its levels were higher in this condition

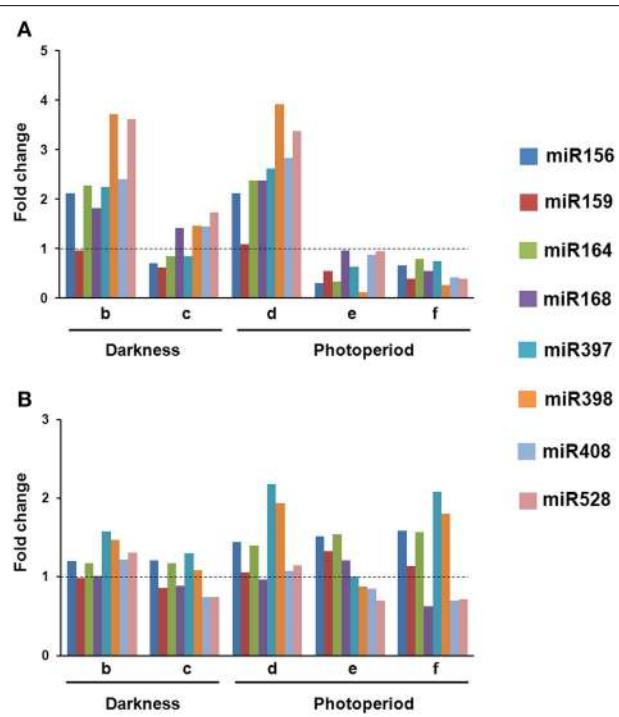


FIGURE 2 | Changes in miRNA levels occur upon hormone depletion under both, darkness and photoperiod conditions. Northern Blot assays were performed to evaluate miRNA abundance during plant regeneration from somatic embryos in two maize cultivars: VS-535 (A) and H-565 (B). Northern blot signals were acquired by densitometry and normalized according to 5S rRNA (Supplementary Material, Figure S1). The value corresponding to EC in 100% hormones (2,4-D and kinetin) was set as 1. Fold changes in 50% hormones (b, d); 0% hormones (c, e) and plantlet (f) with respect to 100% hormones were plotted on charts.

than in the presence of light. Such behavior correlates with the differentiation status of the tissue and with major influence of light on *SBP23* expression. Since miRNA changes were essentially similar between darkness and light, but levels of the target displayed significant differences between these conditions, additional levels of regulation are likely operating to down-regulate *SBP23* during differentiation. Although we only tested one SBP-like target of miR156, 11 out of 26 SBP-like maize genes were predicted as targets for this miRNA, all of them exhibiting the same target sequence as *SBP23* (Supplementary Material, Table S2). Targeting of SBP-like transcripts by miR156 is highly conserved in plants (Rhoades et al., 2002; Xie et al., 2006; Gandikota et al., 2007) and has been reported as relevant for vegetative to reproductive phase changes and plastochron length (Wu and Poethig, 2006; Wang et al., 2008). For maize, it has been shown that miR156 gene family overexpression renders plants with increased number of leaves and delayed flowering (Chuck et al., 2007).

Similar to *SBP23*, a miR164 previously validated target, *GRMZM2G393433_T01* (NAC-domain transcription factor *CUC2* or *NAC107*, Zhai et al., 2013; Liu et al., 2014) was more abundant in darkness than light for either genotype (Figure 3, lanes a–c). However, while for H-565 the miRNA/target levels

followed an inverse tendency with a consistent decrease of *CUC2* upon hormone removal and light exposure, for VS-535 a correlation was observed only under light (Figure 3A, lanes d–f). Global transcriptome analyses indicated that *CUC2* expression was restricted to immature inflorescence and ear in maize (Sekhon et al., 2011; Fan et al., 2014). Therefore, it is interesting that during SE this transcript was particularly abundant at 100% (H-565) or 50% (VS-535) hormones in darkness, both stages characterized by undifferentiated tissues (Figure 1).

Although miR159 and miR168 displayed slight changes in response to plant regeneration stimuli, their target levels (*GRMZM2G139688_T01*, *Giberellic Acid-responsive MYB*, *GA-MYB* for miR159 and *GRMZM2G039455_T01*, *AGO1c* for miR168) showed significant fluctuations at least for 0% hormones in darkness and photoperiod (Figure 3, lanes c and e). For both genotypes *GA-MYB* levels increased at 0% hormones in darkness, while strongly decreased for the same condition in light photoperiod. This correlates with the observation of drastic morphological changes in somatic embryos (Figure 1). miR159 is one of the most abundant miRNAs in maize EC (Shen et al., 2013) and its levels change the least (Figure 2). Nevertheless, an inverse correlation between *GA-MYB* and slight miR159 fluctuations were usually observed for H-565 and to a lesser extent for VS-535, suggesting that miR159 expression during SE might be important to keep *GA-MYB* levels low when not needed. Regarding normal maize plant development, *GA-MYB* expression has been detected in 24 h germinating seed and during seed development in the endosperm and pericarp, in meiotic tassel and in anthers (Sekhon et al., 2011). In addition, miR159-mediated regulation on *MYB* transcripts might depend on the tissue and developmental stage, at least in *Arabidopsis* (Woodger et al., 2003; Alonso-Peral et al., 2012).

miR168 is known as master regulator of the general miRNA pathway since it is required for fine-tuning the *AGO1* levels in *Arabidopsis* (Vaucheret et al., 2006). In maize, at least five *AGO1* putative genes were previously identified (Qian et al., 2011) in contrast to only one *AGO1* encoding gene in *Arabidopsis*. *AGO1c* shows the greatest identity with the *Arabidopsis* *AGO1* homolog and has conserved the miR168 target sequence (Table 1). Similar to miR156 and *SBP23*, there was poor correlation between miR168 and *AGO1c* transcript levels. While the greatest increase in miR168 was observed for VS-535 at 50% hormones under photoperiod, *AGO1c* levels reached a two-fold increase in the same sample (Figure 3A, lane d). It was then decreased by four-fold at 0% hormones under the same condition when also miR168 decreased (Figure 3A, lane e). A greater reduction in this *AGO1c* transcript was observed for H-565, 0% hormones and photoperiod (25-fold) without accompanying changes in miR168 (Figure 3B, lane e). This suggests a complex regulation on miRNA biogenesis in maize, probably involving additional *AGO1* isoforms during plant regeneration.

Targets of Stress-related miRNAs Display Essentially Contrasting Patterns between Maize Genotypes during SE

Stress-related miRNAs are highly expressed in the process of EC induction for one Chinese maize genotype (Shen

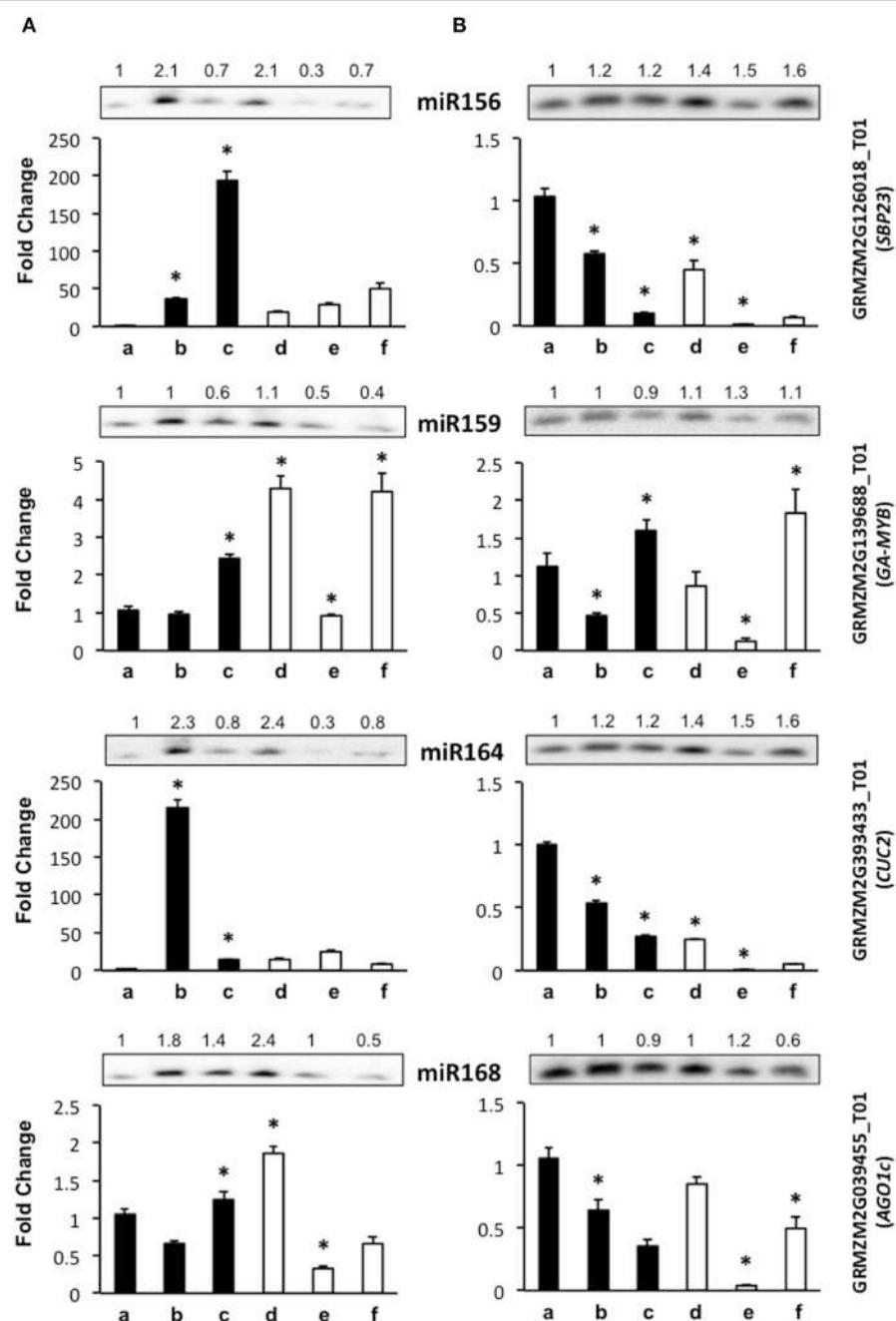


FIGURE 3 | The abundance of development-related miRNA targets is greatly affected by light presence during maize plant regeneration through SE. Transcript levels of development-related miRNAs were analyzed by qRT-PCR in the same samples as miRNAs for VS-535 (A) and H-565 (B). The 18S rRNA was used as reference control and plotted values represent the expression of each mRNA relative to 100% hormones (a). Fold changes are shown accordingly for 50% hormones (b, d); 0% hormones (c, e) and plantlet (f).

Filled bars represent darkness and empty bars, photoperiod. The corresponding miRNA Northern blots are shown at the top of each chart. miR156 target: *GRMZM2G126018_T01* (*SBP23*); miR159 target: *GRMZM2G139688_T01* (*GA-MYB*); miR164 target: *GRMZM2G393433_T01* (*CUC2*); miR168 target: *GRMZM2G039455_T01* (*AGO1c*). Error bars represent + Standard Error; $n = 6$. Statistically significant differences were identified using two-way ANOVA and Tukey as described in Methods at $*P < 0.05$.

et al., 2013). Stress has been also regarded as one of the main stimuli promoting dedifferentiation as well as SE. Major targets for stress-related miRNAs are copper proteins, multi-copper oxidases, superoxide dismutases and laccases

(Jones-Rhoades and Bartel, 2004; Yamasaki et al., 2007; Abdel-Ghany and Pilon, 2008). Here we tested the mRNA levels for maize *GRMZM2G146152_T01* (laccase-like, *LAC2*) as target for miR397, *GRMZM2G058522_T01* (*SOD9*) as validated target for

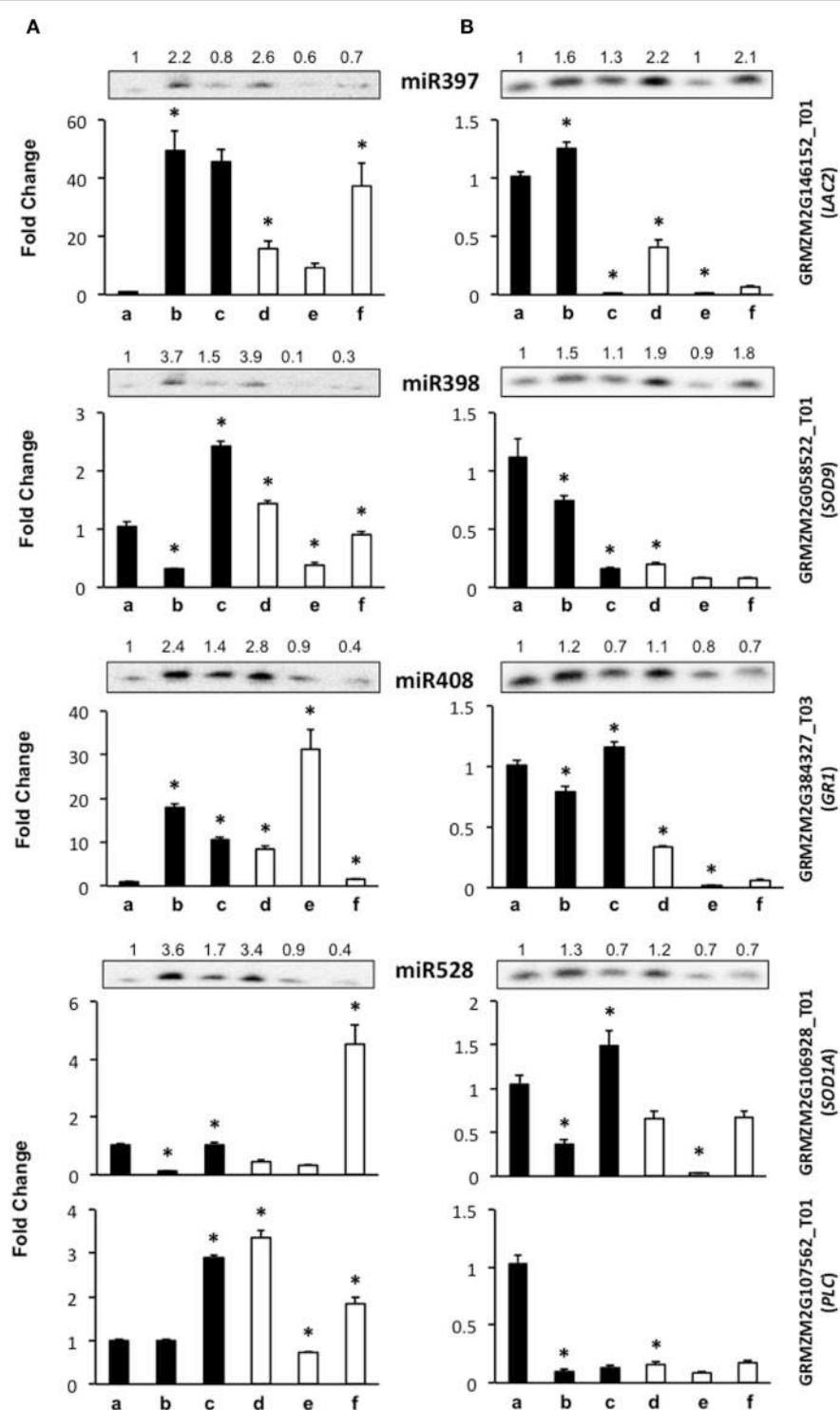


FIGURE 4 | The abundance of stress-related miRNA targets is dependent on the genotype during maize plant regeneration through SE. Transcript levels of miRNA stress-related targets were analyzed by qRT-PCR in the same samples as miRNAs for VS-535 (**A**) and H-565 (**B**). The 18S rRNA was used as reference control and plotted values represent the expression of each mRNA relative to 100% hormones (a, set to 1). Fold changes are shown accordingly for 50% hormones (b, d); 0% hormones (c, e) and plantlet (f). Filled bars

represent darkness and empty bars, photoperiod. The corresponding miRNA Northern blots are shown at the top of each chart. miR397 target: *GRMZM2G146152_T01* (*LAC2*); miR398 target: *GRMZM2G058522_T01* (*SOD9*); miR408 target: *GRMZM2G384327_T03* (*GR1*); miR528 targets: *GRMZM2G106928_T01* (*SOD1A*) and *GRMZM2G107562_T01* (*PLC*). Error bars represent + Standard Error; $n = 6$. Statistically significant differences were identified using Two-Way ANOVA and Tukey as described in Methods at $*P < 0.05$.

miR398 (Shen et al., 2013), *GRMZM2G106928_T01* (*SOD-1A*) and *GRMZM2G107562_T01* (plastocyanin-like protein, *PLC*) as targets for miR528, and *GRMZM2G384327_T03* (Gamma Response 1 protein, *GR1*), as previously predicted miR408 target (Li et al., 2013a). It is worth mentioning that although stress-related miRNAs have been identified as very highly expressed during germination, immature embryo dedifferentiation and stress-response, their target identification has remained elusive in maize. Our selection included both, validated and predicted targets according to the properties indicated in **Table 1**.

The level of miR397 target, *LAC2*, showed contrasting changes between VS-535 and H-565 genotypes throughout hormone depletion. For VS-535, it increased upon 50% hormone reduction under darkness or light (to a lesser extent), and remained unaltered after complete hormone removal (**Figure 4A**). On the contrary, for H-565 it was strongly reduced in 0% hormones, darkness or light (**Figure 4B**). For other stress-related miRNA targets, such as *SOD9* (miR398), *SOD1A* (miR528) and *GR1* (miR408), lower levels were also detected in H-565 than in VS-535, particularly in differentiating tissues under light (**Figure 4A** vs. **Figure 4B**, lanes e and f). There was poor correlation between miR397 and *LAC2* levels for either of the embryogenic cultivars, suggesting this transcript is regulated by additional mechanisms during plant regeneration. We observed an inverse correlation between miR398 (increase) and *SOD9* (decrease) upon 50% reduction in hormones for both cultivars in darkness, but only for H-565 in light (**Figure 4A** vs. **Figure 4B**). This indicates even for a validated target it is difficult to find strong miRNA/target correlation under light, particularly upon full hormone depletion. Similarly, *SOD1A* showed a clear inverse correlation with miR528 levels for both, VS-535 and H-565, in darkness but not in light (**Figure 4**, upper panels corresponding to miR528). It is interesting to notice that although *SOD9* and *SOD-1A* catalyze the same reaction, their high and differential transcript expression supports a non-overlapping function in maize plant regeneration through SE. Similar to other stress-related miRNA targets, *GR1* levels were different between the tested maize genotypes (**Figure 4**). It increased upon hormone depletion in darkness or light for VS-535, while strongly decreased in the presence of light for H-565 (**Figure 4A** vs. **Figure 4B**). A correlation between *GR1* and miR408 was observed for H-565, but not for VS-535, suggesting this target regulation during SE could be genotype-specific.

miR528 is one of the most abundant, stress-related miRNAs in SE for maize (Shen et al., 2013), rice (Luo et al., 2006; Chen et al., 2011), and citrus (Wu et al., 2015). In our miR528 target prediction analysis, several copper-binding protein encoding transcripts, many uncharacterized genes and even transcription factors were found (Supplementary Material, Table S2). Taking into account the expected relevance for miR528-mediated regulation in SE, we tested, in addition to *SOD1A*, the expression levels of *GRMZM2G107562_T01* that codes for a plastocyanin-like protein (PLC). Unlike *SOD1A*, *PLC* showed a very weak correspondence with miR528 changes. However, consistent with other stress-related miRNA targets, its levels differed between VS-535 and H-565 throughout the hormone depletion stages (**Figure 4A** vs. **Figure 4B**, lower panels corresponding to

miR528). Curiously, *PLC* levels were high in darkness, in spite of its predicted photosynthesis-related function (Abdel-Ghany and Pilon, 2008). In this regard, a light-independent induction of photosynthesis genes has been documented throughout embryogenesis stages (from globular to torpedo) in *Arabidopsis*. Genes involved in energy production comprised the largest up-regulated functional group, strongly biased toward components of the photosynthetic apparatus (Spencer et al., 2007). Taking this into account, high expression of *PLC* is probably required at early stages of SE.

Polyribosomal Distribution of miR528 in Maize EC

According to the lack of consistent correlation between levels of conserved development- or stress-related miRNAs and their targets (predicted or confirmed) during maize SE and plant regeneration, the regulation by additional mechanisms was evident. Primary regulation at transcription levels was already described for several of the analyzed targets (*SBP23*, *GA-MYB*, *CUC2*), particularly in response to developmental cues and hormones' presence. However, another level of regulation might be exerted by miRNAs at translation without affecting the transcript levels (Brodersen et al., 2008; Lanet et al., 2009; Iwakawa and Tomari, 2013). To assess the possible role of some conserved miRNAs at translation level, polyribosomal fractions were obtained from 2 years-subcultured VS-535 EC and the

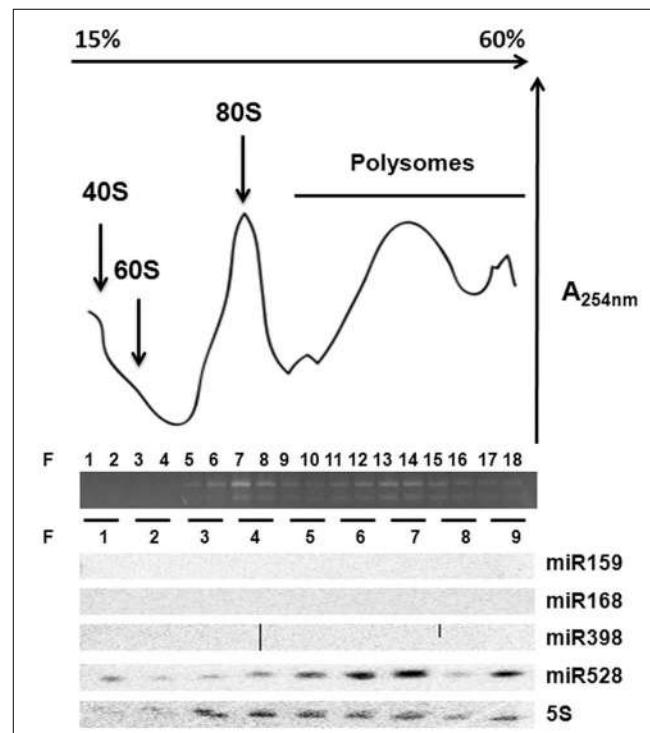


FIGURE 5 | miR528 is localized on polyribosomes. Ribosomal profiling of two year-subcultured EC after sucrose density gradient fractionation. Total RNA abundance and integrity across fractions (F) was measured by absorbance at 254 nm and agarose gel electrophoresis. Northern blotting of selected miRNAs was performed upon RNA isolation from every two-fraction pools resulting in nine fractions. The 5S ribosomal RNA was used as control for the Northern blotting.

presence of miR159, miR168, miR398, and miR528 was analyzed (**Figure 5**). Only miR528 was detected in polyribosomal fractions (F 10–18) suggesting this miRNA could regulate some of its targets at translation level. In *Arabidopsis*, the presence of miR168 and miR398 in polyribosomes was previously demonstrated in 10 days-old seedlings (Lanet et al., 2009). However, we could not detect these miRNAs in maize polyribosomes from EC, although they had detectable levels in total RNA obtained from this tissue (**Figure 2**, Supplementary Material, Figure S1).

Discussion

In the past few years, several publications reported conserved and species-specific miRNA levels during plant SE (Luo et al., 2006; Zhang et al., 2010, 2012; Chen et al., 2011; Wu et al., 2011, 2015; Li et al., 2012; Shen et al., 2012, 2013; Lin and Lai, 2013; Qiao and Xiang, 2013; Yang et al., 2013). These studies found that miRNA patterns change upon callus induction, between embryogenic and non-emбриogenic callus, as well as during SE and differentiation in a plant species-dependent fashion. For example, while miR171, miR390, and miR398 are preferentially expressed in EC before induction of plant differentiation in rice (Luo et al., 2006); these miRNAs are increased during the differentiation process in citrus (Wu et al., 2011). Even miRNAs from the same family, i.e., miR156a and miR156b, may display differential expression patterns during the SE differentiation process, as demonstrated in a recent study on *Larix leptolepis* (Zhang et al., 2012). Hence, the current knowledge based on global miRNA approaches highlights the relevance of exploring particular miRNA landscapes and their target regulation in the context of species-specific SE conditions.

miRNA Expression in Maize SE

Separated analysis on hormone depletion and light effects during maize SE indicated that miRNA expression patterns are affected mostly by hormones, rather than the light presence. By analyzing the process in two independent embryogenic genotypes we expected to confirm whether the observed miRNA changes are inherent to the process rather than the cultivar. However, we noticed a great influence of the genotype, first on the initial miRNA level appreciated in the 2 years-subcultured EC, and second on the degree of changes registered during hormone depletion. In this sense, miRNA expression regulation resulted more dramatic in VS-535 than in H-565. Strikingly lower levels were detected for all tested miRNAs in VS-535 at 0% hormones and regenerated plantlets under light, whereas H-565 maintained the presence of some of the stress-related miRNAs (miR397 and miR398) higher. A recent study explored miRNA differential expression during immature embryo dedifferentiation in the presence of 2,4-D using a highly embryogenic maize inbred line 18-599R (Shen et al., 2013). The authors suggested miR164, miR169, miR528, and miR529 might be primarily participating in the process of EC induction through the regulation of targets involved in auxin and gibberellin signaling. However, other miRNAs, significantly up-regulated in the dedifferentiation process, were miR156k, miR168, miR397, miR398, and miR408. We found the same miRNAs transiently increased by the

reduction of hormones concentration in half during plant regeneration. However, in the absence of hormones their levels were reduced. These observations support the occurrence of specific miRNA expression readjustments in embryogenic tissues in response to hormone changes in the environment.

One important question is whether the high concentration of specific miRNAs associates with the embryogenic potential of the callus. Shen et al. (2013) found that the initial increase in miRNAs was either enhanced or maintained during the dedifferentiation process. However, in maize EC subcultured for long periods (up-to 2 years) we have found a gradual reduction in miR156, miR164 and miR168 levels without impairment on the callus embryogenic potential (Dinkova and Alejandri-Ramirez, 2014). In agreement, the data presented here support an initial burst on certain miRNA levels preceding their further decrease during maize SE. Hence, miRNA expression response to hormone changes could be a major factor impacting on the embryogenic potential of maize cultivars during both, dedifferentiation and plant regeneration.

Correlation between miRNA and Target Levels in Maize SE

miRNAs are known to regulate their target mRNAs by degradation, translation inhibition or both. In plants, an inverse correlation between miRNA and target levels is commonly observed, suggesting that mRNA degradation is the preferred regulatory mechanism. However, global miRNA and degradome sequencing data have shown that not always the degradation products of predicted or even validated targets could be detected in the libraries (Shen et al., 2013; Wu et al., 2015). Conversely, these studies have identified novel targets for conserved miRNAs such as miR156 and miR164, or known targets with novel miRNA sites. An increasing number of reports have also revealed the lack of inverse relationship between miRNAs and their targets, depending on the tissue or the process analyzed (Brodersen et al., 2008; Wu et al., 2011; Alonso-Peral et al., 2012).

Here we found the expected inverse correlation between a miRNA and its predicted and/or validated target is highly dependent on environmental (light presence) and internal (genotype) signals during maize plant regeneration through SE (**Figure 6**). Major differences were observed for development-related miRNA target levels between genotypes under darkness, but not under light where plant regeneration took place. On the other hand, inverse relationship between miRNA/target levels was more easily found in darkness than in light. Therefore, for most of the analyzed targets we propose there is a major influence of additional regulation under a photoperiod, making it difficult to appreciate the effect of miRNAs. On the other hand, increments of both, miRNA and target, upon hormone 50% reduction (e.g., miR156, miR164, miR168) suggest miRNA up-regulation might be required to control the levels of transcripts induced during SE.

Accessing miRNA-mediated regulation through quantitative transcript evaluation has been widely used in plants, particularly during SE (Li et al., 2012; Yang et al., 2013; Wu et al., 2011, 2015). However, an inverse miRNA/target relationship has not always been observed, even in the presence of miRNA-mediated

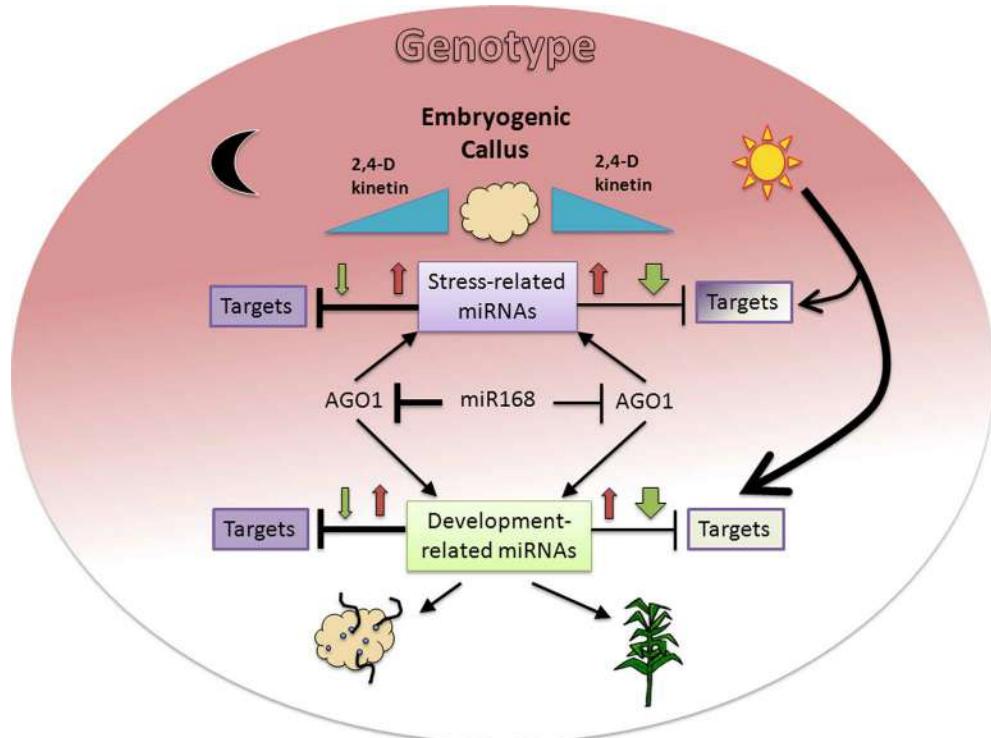


FIGURE 6 | Proposed model for miRNA and target regulation responses to environmental and genotype dependent cues in maize plant regeneration through SE. Hormone (2,4-D and kinetin) depletion (blue triangles) affects miRNA expression patterns with initial increase (red arrows) at 50% and further decrease (green arrows) at 0% hormones in darkness or light presence. The presence of light induces a greater decrease of miRNAs in the absence of hormones, resulting in lesser effect on their targets. However the same condition represents a major regulation on target expression, particularly for development-related targets (bigger arrow). In

addition to environmental cues, the genotype (colored background gradient) has an important role on both, miRNA and target regulation with greater effect on stress-related miRNAs and undifferentiated tissues (dark color). A regulatory miR168-AGO1 loop might also participate in the differential regulation observed between darkness/light and genotypes. The fading background color toward development-related miRNA targets and the physiological outcomes for either darkness (organogenesis) or light (plant regeneration) is consistent with the embryogenic potential of both maize genotypes assayed in this study.

degradation evidence (degradome or 5'RACE fragments). Our finding on miR528 distribution in maize EC polysomal fractions supports the notion that target regulation is probably exerted at multiple levels depending on the developmental process, miRNA, and analyzed target. In agreement, we observed such expected inverse correlation between miR528 and *SOD1A* target, but not for *PLC* target. Therefore, for future studies, it would be relevant to include miRNA-target evaluations at protein in addition to transcript levels.

Physiological Relevance of Development-related miRNA Regulation in Maize SE

miR156 and miR164 have been found as SE-abundant miRNAs in several species, including maize (Li et al., 2012; Shen et al., 2013; Dinkova and Alejandri-Ramirez, 2014; Wu et al., 2015). The miR156-mediated suppression of *SBP* transcripts is probably required for early SE, as demonstrated for *Arabidopsis* zygotic embryogenesis (Nodine and Bartel, 2010). Similarly, miR164 initial increase during SE is consistent with maintaining low levels of its *CUC2* target during plant regeneration under light. In plants, conserved miR164 targets are NAC transcription factors

(Mallory et al., 2004). The NAC family of proteins includes NAM, ATAF1-2, and CUC2. Proteins belonging to the NAC family are involved in many plant developmental processes, such as flowering, embryogenesis, senescence, auxin signaling, secondary wall thickening and others (Fan et al., 2014). NAM was reported as related to the shoot apical meristem and primordium formation in *Petunia hybrida* (Souer et al., 1996) and CUC2 has been involved in *Arabidopsis* shoot apical meristem development (Aida et al., 1997). This context is consistent with the observed *CUC2* reduction in the absence of hormones and in fully differentiated tissues. Therefore, although miR164 and miR156 targets might display contrasting behavior in undifferentiated tissues (darkness, hormones' presence) between maize genotypes, their expression regulation is apparently required for plant regeneration through SE, regardless the genotype (Figure 6).

One possibility underlying the contrasting behavior of miRNA targets between darkness and light could be their transcription responsiveness to photoperiod. Another is a differential function of miRNA-mediated silencing pathways depending on light presence/absence during SE. It was previously reported that

AGO1 expression is highly induced early in carrot SE and further decreased after the globular-staged embryo (Takahata, 2008). In maize, the presence of several putative *AGO1* genes, not all of them exhibiting the miR168 target sequence, further adds a complexity level to the interpretation of miRNA-mediated regulation. According to a global transcriptome analysis for the maize B73 line, *AGO1c* transcript (target of miR168) is highly expressed in differentiating tissues, such as the shoot apical meristem, immature cob, and tassel (Sekhon et al., 2011). It remains high for few days upon pollination, while in the mature and germinating maize seed *AGO1c* and miR168 are both decreased. Another *AGO1* isoform also exhibiting the miR168 target sequence (*GRMZM2G441583_T01*, *AGO113* or *AGO1a*) remains highly expressed during seed maturation and germination (Sekhon et al., 2011). The behavior of *AGO1c* in maize SE is consistent with the transient increase observed in carrot and in early zygotic embryogenesis. We did not analyze other *AGO1* transcripts during maize plant regeneration, but it remains possible that they were differentially expressed depending on the genotype and regulated during SE.

Physiological Relevance of Stress-related miRNA Regulation in Maize SE

Stress-related miRNAs and their targets have been associated with sweet orange callus embryogenic potential (Wu et al., 2011, 2015) and SE in other species (Li et al., 2012). Highly proliferating tissues are thought to produce an excess of reactive oxygen species (ROS) making transcript accumulation of stress-related genes such as superoxide dismutase, cupredoxin, and multi-copper oxidases necessary to minimize cell damage and promote SE. Members of the copper superoxide dismutase (CSD, SOD) family are miRNA targets in several plant species (Jones-Rhoades and Bartel, 2004; Jovanović et al., 2014; Naya et al., 2014). These enzymes are in charge of destroying oxygen reactive species accumulating during fast plant growth (Ravet and Pilon, 2013). On the other hand, an *Arabidopsis* ortholog of the predicted maize miR408 target, *GR1*, is expressed in mitotically active tissues, such as the shoot apical meristem and floral primordium from unstressed plants in a similar to cell cycle-related gene expression profiles (Deveaux et al., 2000). At $GR1$ is responsive to genotoxic stress, such as gamma radiation, and has been proposed to block mitotic cell divisions in irradiated cells to prevent premature entry into mitosis before completion of DNA repair. Therefore, the control of this transcript by miRNA makes sense in highly proliferating undifferentiated callus (Luo et al., 2006; Shen et al., 2013). Here we found that during maize hormone depletion, targets of stress-related miRNAs

often displayed higher expression under darkness. In addition, their expression patterns under light showed striking differences between genotypes contrary to what observed for development-related targets. Accordingly, we propose that while stress-related miRNAs are required to control their targets in undifferentiated tissues, their participation during the plant regeneration process could be dependent on the genotype and the physiology of the EC used to initiate SE (Figure 6).

Conclusion

The present study provides important novel information about the separate effects of hormone depletion and light presence on miRNA patterns and their target regulation during plant regeneration from maize embryogenic callus. While development or stress-related miRNAs are responsive to hormone concentration, their targets are additionally influenced by the presence of photoperiod. The reproducibility or differences observed for particular miRNA-target regulation between two different highly embryogenic genotypes provide clues for conserved miRNA roles within the SE process. Future work should aim to approach the mechanism underlying regulation of SE-related miRNAs on their targets and its relevance for the plant regeneration success.

Author Contributions

Conceived and designed the experiments: EC, NA, TD. Performed the experiments: EC, NA, VJ. Analyzed the data: EC, NA, VJ. Contributed reagents/ materials/ analysis tools: EC, NA, VJ. Wrote the paper: EC, TD. Revised the manuscript: EC, TD. Steered the whole study: TD.

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Supplementary Material

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The rice enhancer of zeste [E(z)] genes *SDG711* and *SDG718* are respectively involved in long day and short day signaling to mediate the accurate photoperiod control of flowering time

Xiaoyun Liu¹, Chao Zhou¹, Yu Zhao¹, Shaoli Zhou¹, Wentao Wang¹ and Dao-Xiu Zhou^{1,2 *}

¹ National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China

² Institut de Biologie des Plantes, UMR8618, Université Paris-Sud 11, Orsay, France

Edited by:

Raúl Alvarez-Venegas, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico

Reviewed by:

Hairong Wei, Michigan Technological University, USA

Donna Glassop, Commonwealth Scientific and Industrial Research Organisation, Australia

***Correspondence:**

Dao-Xiu Zhou, Institut de Biologie des Plantes, UMR8618, Université Paris-Sud 11, 90405 Orsay, France
e-mail: dao-xiu.zhou@u-psud.fr

Recent advances in rice flowering studies have shown that the accurate control of flowering by photoperiod is regulated by key mechanisms that involve the regulation of flowering genes including *Heading date1* (*Hd1*), *Early hd1* (*Ehd1*), *Hd3a*, and *RFT1*. The chromatin mechanism involved in the regulation of rice flowering genes is presently not well known. Here we show that the rice enhancer of zeste [E(z)] genes *SDG711* and *SDG718*, which encode the polycomb repressive complex2 (PRC2) key subunit that is required for trimethylation of histone H3 lysine 27 (H3K27me3), are respectively, involved in long day (LD) and short day (SD) regulation of key flowering genes. The expression of *SDG711* and *SDG718* is induced by LD and SD, respectively. Over-expression and down-regulation of *SDG711* respectively, repressed and promoted flowering in LD, but had no effect in SD. By contrast, down-regulation of *SDG718* had no effect in LD but delayed flowering in SD. *SDG711* and *SDG718* repressed *OsLF* (a repressor of *Hd1*) respectively in LD and SD, leading to a higher expression of *Hd1* thus late flowering in LD and early flowering in SD. *SDG711* was also found to be involved in the repression of *Ehd1* in LD. *SDG711* was shown to directly target to *OsLF* and *Ehd1* loci to mediate H3K27me3 and gene repression. The function of the rice E(z) genes in LD repression and SD promotion of flowering suggests that PRC2-mediated epigenetic repression of gene expression is involved in the accurate photoperiod control of rice flowering.

Keywords: *Oryza sativa*, heading date, epigenetics, epigenomics, histone methylation, PRC2, *OsCLF*, *OsiEZ1*

INTRODUCTION

The control of flowering time is a critical step for successful grain production in rice. Day length is a key factor controlling rice flowering. Most rice cultivars recognize 13.5 h light/10.5 h dark as a critical photoperiod to separate long day (LD) from short day (SD) periods, day length shorter than 13.5 h will greatly induce rice flowering (Itoh et al., 2010; Tsuji et al., 2013). Despite some genes are shared between *Arabidopsis* and rice flowering regulatory pathways, there are considerable differences between the regulation of flowering of both species such as absence of the vernalization pathway in rice (Shrestha et al., 2014). *Heading date1* (*Hd1*), the rice ortholog of *Arabidopsis CONSTANS* (*CO*), has a dual role in flowering time control. It promotes flowering at SD and represses flowering in LD (Yano et al., 2000). (*Hd3a* is a rice ortholog of the *Arabidopsis* florigen gene *FLOWERING LOCUS T* (*FT*; Yano et al., 2000; Kojima et al., 2002; Tamaki et al., 2007). *Hd1* activates *Hd3a* in SD, but in LD *Hd1* is converted by phytochrome B to a repressor of *Hd3a* (Yano et al., 2000; Hayama and Coupland, 2004). *Hd1* expression is controlled by *Oryza sativa GIGANTEA* (*OsGI*), a key factor of circadian rhythms in rice flowering control (Yano et al., 2000; Hayama et al., 2003). There is a second, *Hd1*-independent, photoperiod inductive pathway in rice. *Early*

hd1 (*Ehd1*), a B-type response regulator that is activated by a SD flowering promoter (i.e., *OsMADS51*), activates the expression of *Hd3a*, *RFT1* (another Rice *FT* gene), and *OsMADS14*, and mainly confers SD-dependent flowering promotion (Doi et al., 2004; Kim et al., 2007). In LD, *Ehd1* is activated by *Ehd2/OsId1/RID1* (*Rice Indeterminate1*) and *OsMADS50*, but repressed by *Ghd7* (*Grain number, plant height and heading date7*; Lee et al., 2004; Matsubara et al., 2008; Park et al., 2008; Wu et al., 2008; Xue et al., 2008; Itoh et al., 2010; Tsuji et al., 2013). *Ehd1* activates the expression of *Hd3a* and *RFT1* which activates *MADS14* and *MADS15* (Komiyama et al., 2009). The tight control of expression of the flowering promoter *Ehd1* and the flowering repressor *Ghd7* allows to measure the slight differences in day lengths to control *Hd3a* and *RFT1* transcription with a critical day length threshold (Itoh et al., 2010; Tsuji et al., 2013). Unlike *Hd1* that is conserved with *Arabidopsis CO*, *Ehd1*, and *Ghd7* are evolutionarily acquired rice-specific genes. Therefore, the control of florigen expression is regulated by key mechanisms that involve the regulation of *Hd1* expression, the conversion of *Hd1* function on *Hd3a* expression, and modulation of *Ehd1/Ghd7* expression.

Recent studies have established a close relationship of epigenetic regulation with flowering. For instance, during the

process of vernalization in *Arabidopsis*, polycomb repressive complex 2 (PRC2)-mediated trimethylation of histone H3 lysine 27 (H3K27me3) represses the expression of *Flowering Locus C* (*FLC*) to stimulate flowering (Sung and Amasino, 2004; De Lucia et al., 2008). In rice, SDG724 mediates H3K36me2/3 deposition at *OsMADS50* and *RFT1*, promoting flowering and establishing a difference of functionality between paralogs *RFT1* and *Hd3a* under LD or SD conditions (Sun et al., 2012). SDG725 also mediates H3K36me2/3 deposition at *Ehd2*, *Ehd3*, *OsMADS50*, *Hd3a*, and *RFT1*, promoting flowering under LD or SD conditions (Sui et al., 2013). The PRC2 complex was first discovered in *Drosophila*, which has four core proteins: ENHANCER OF ZESTE [E(z)], SUPPRESSOR OF ZESTE 12 [Su(z)12], EXTRA SEX COMBS (ESC) and P55 (Schuettengruber and Cavalli, 2009). The E(z) protein has the H3K27 methyltransferase activity (Cao et al., 2002; Czermin et al., 2002). *Arabidopsis* contains three E(z) genes [*CURLY LEAF* (*CLF*), *SWINGER* (*SWN*), and *MEDEA* (*MEA*)], three Su(z)12 genes [*FERTILIZATION INDEPENDENT SEED 2* (*FIS2*), *VERNALIZATION 2* (*VRN2*) and *EMBRYONIC FLOWER 2* (*EMF2*)], and one ESC gene [*FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*)] (Luo et al., 1999; Ohad et al., 1999; Gendall et al., 2001; Yoshida et al., 2001; Hennig et al., 2003), which form three PRC2-like complexes. The FIS complex, which contains MEA/SWN, FIS2, FIE, and MSI1 (Multicopy Suppressor of IRA1), functions during gametogenesis and seed development. The others are involved in flowering control: the EMF complex that is comprised of CLF/SWN, EMF2, FIE, and MSI1 and is involved in the suppression of early flowering and the VRN complex that plays critical roles in the vernalization pathway by maintaining the high level of H3K27me3 on the *FLC* locus after vernalization (Hennig and Derkacheva, 2009). The VRN complex is associated with VERNALIZATION INSENSITIVE 3 (VIN3, a PHD-domain containing protein) and VIN3-like proteins to form PHD-VRN PRC2 complexes (Wood et al., 2006; De Lucia et al., 2008). The VIN3 protein enhances H3K27me3 throughout the target loci to a level sufficient for stable silencing.

The rice genome contains two genes for E(z) (*OsiEZ1* and *OsCLF*), *Su(Z)12* (*OsEMF2a* and *OsEMF2b*) and ESC (*OsFIE1* and *OsFIE2*; Luo et al., 2009). No morphological changes are observed in *osclf* and *osfie1* mutants, while *osfie2* and *osemf2b* mutants display earlier flowering at LD and abnormal flower organs (Luo et al., 2009). Recent results have shown that rice VIN3-like proteins OsVIL2, OsVIL3, or RICE LEAF INCLINATION 2 (LC2, hereafter referred to as LC2) promote rice flowering through the photoperiod pathway (Wang et al., 2013; Yang et al., 2013). These results suggest that PRC2 and PRC2-associated genes are involved in photoperiod regulation of flowering in rice. However, how PRC2-mediated gene repression is involved in accurate photoperiod control of rice flowering is not clear.

In this work we show that the two rice E(z) genes, *OsCLF* (or *SDG711*, *Os06g16390*, here after referred to as *SDG711*) and *OsiEZ1* (or *SDG718*, *Os03g19480*, here after referred to as *SDG718*), displayed distinct function in photoperiod regulation of flowering in rice. *SDG718* is induced in SD and represses *OsLF*, a repressor of *Hd1* (Zhao et al., 2011), leading to a higher expression of *Hd1* (that activates *Hd3a* in SD) and thus early flowering.

SDG711 is induced in LD and represses *OsLF*, *Ehd1*, and other flowering-promoting genes leading to late flowering in LD. The data suggested that the two E(z) genes are involved respectively, in LD and SD signals to differentially control key flowering genes expression and contribute to the accurate photoperiod control of flowering time in rice.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWING CONDITIONS

Rice cultivar (*Oryza sativa* spp. *japonica*) “Zhonghua 11” (ZH11) and “DongJin” (DJ) were used for genetic transformation in this study. T-DNA insertion line of *SDG711* (3A-60654.R) was obtained from the Postech rice mutant database (<http://signal.salk.edu/cgi-bin/RiceGE>). Insertion was confirmed by PCR using the specific primers F and R and a T-DNA left side primer RB2. The primers used for genotyping and real-time PCR analysis are listed in Table S1. The rice plants were grown either in a paddy field in summer in Wuhan (day length >13.5 h) or in controlled growth chambers for 6 week-old under SD (10 h light at 30°C/14 h dark at 25°C) or 8 week-old LDs (14 h light at 30°C/10 h dark at 25°C) conditions as described previously (Yang et al., 2013).

EXPRESSION ANALYSIS BY NORTHERN BLOTS AND RT-PCR

Total RNA was isolated from rice callus, stems, leaves, flag leaves, shoots, panicles, endosperm, and roots using TRIzol reagent (Invitrogen). Three µg of total RNA were reverse-transcribed in a reaction of 20 µl by using DNase I and SuperScript III (Invitrogen) according to the manufacturer’s instruction to obtain cDNA. For northern blotting analysis, fifteen micrograms of total RNA extracted from field-grown rice leaves was separated in 1.2% (w/v) formamide-denaturing agarose gels, then transferred to nylon membranes. Gene-specific probes were labeled with ³²P-dCTP using the Random Primer kit (Invitrogen) and hybridized to the RNA blots. The probe of *SDG711* was amplified from *SDG711* cDNA using primers Insitu-*SDG711*-F and Insitu-*SDG711*-R (Table S1), resulting in a fragment of 505 bp of the cDNA.

Real-time PCR was performed in an optical 96-well plate that included SYBR Premix EX Taq and 0.5 µl of Rox Reference Dye II (Takara), 1 µl of the reverse transcription reaction, and 0.25 µM of each gene-specific primer in a final volume of 25 µl on a PRISM 7500 PCR instrument (Applied Biosystems). The reactions were performed at 95°C for 10 s, 45 cycles of 95°C for 5 s, and 60°C for 40 s. Disassociation curve analysis was performed as follows: 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. Data were collected using the ABI PRISM 7500 sequence detection system following the instruction manual. The relative expression levels were analyzed using the 2- $\Delta\Delta CT$ method (Livak and Schmittgen, 2001). The rice *ACTIN1* gene was used as the internal control. Accession numbers of genes analyzed in this study: *SDG711*: LOC_Os06g16390; *SDG718*: LOC_Os03g19480; *OsLF*: LOC_Os05g46370; *Ehd1*: LOC_Os10g32600; *RFT1*: LOC_Os06g06300; *Hd1*: LOC_Os06g16370; *Hd3a*: LOC_Os06g06320; *RID1*: LOC_Os10g28330; *OsGI*: LOC_Os01g08700; *Ghd7*: LOC_Os07g15770; *OsMADS14*: LOC_Os03g54160; *OsMADS15*: LOC_Os07g01820; *OsMADS50*: LOC_Os03g03070;

OsMADS51: LOC_Os01g69850. The primers for real-time PCR are listed in Table S1.

VECTOR CONSTRUCTION AND PLANT TRANSFORMATION

For over-expression (OX) vector, the *SDG711* full-length cDNA was amplified from DJ leaf mRNA using primer set OXSDG711-F and OXSDG711-R, then inserted into the OX vector pU1301 under the control of the maize ubiquitin gene promoter within *Kpn*I sites (Sun and Zhou, 2008). For RNAi vectors, gene-specific sequences of *SDG711* and *SDG718*, spanning from 2352 bp to 2860 bp relative to the translation start site and from 918 bp to 1599 bp relative to the translation start site, respectively, were amplified from cDNA using primer sets RiSDG711-F/RiSDG711-R and RiSDG718-F/RiSDG718-R, then inserted into the RNAi vector pDS1301 (Huang et al., 2007). Sequence amplified using the primers sets are listed in Table S1. The constructs were transformed into DJ (*SDG711* OX and RNAi) and ZH11 (*SDG718* RNAi) plants by *Agrobacterium tumefaciens* (strain *EHA105*) – mediated transformation as previously described (Huang et al., 2007).

WESTERN BLOTTING ANALYSIS

For Western blot analysis, histone-enriched fractions were extracted from wild type (WT), mutant, and transgenic leaves as described previously (Huang et al., 2007). Antibodies used in Western blot are: anti-H3K27me3 (07-449, Millipore), anti-H3K27me2 (ab24684, Abcam), anti-H3K27me1 (ab113671, Abcam), anti-H3 (ab1791, Abcam), anti-H3K4me3 (07-473, Millipore), anti-H3K4me2 (07-430, Millipore), anti-H3K4me1 (07-436, Millipore), anti-H3K9me3 (ab8898, Abcam), anti-H3K9me2 (07-441, Millipore), anti-H3K9me1 (ab9045, Abcam) H3K36me1 (ab9048, Abcam), anti-H3K36me2 (ab9049, Abcam), and anti-H3K36me3 (ab9050, Abcam). Anti-*SDG711* was prepared by immunizing rabbits with *SDG711* protein produced in *Escherichia coli* (in pET-28a vector) and purified with His-tag protein purification beads (V8550, GE Healthcare). The anti-serum was affinity-purified with protein-A agarose beads purchased from Millipore (16-157).

CHROMATIN IMMUNOPRECIPITATION (ChIP)

The chromatin immunoprecipitation (ChIP) experiment was performed as described (Huang et al., 2007). Rice leaves (0.8–1.0 g fresh weight) were harvested at the end of the dark period of 8 week-old LD grown and 6 week-old SD grown plants and crosslinked in 1% formaldehyde under vacuum. Chromatin was extracted and fragmented to 200–750 bp by sonication, and ChIP was performed using the following antibodies: H3K27me3 (07-449, Millipore), H3K4me3 (07-473, Millipore), and Anti-*SDG711*. The precipitated and input DNA samples were then analyzed by real-time PCR with gene-specific primers listed in Table S1. All assays were performed at least three times from two biological replicates.

RESULTS

THE RICE E(z) HOMOLOGOUS GENES DISPLAY DISTINCT PHOTOPERIODIC EXPRESSION PATTERNS

Phylogenetic analysis has previously shown that rice E(z) homologous genes, *SDG711* and *SDG718*, are closely related to *Arabidopsis*

CLF and *SWN*, respectively (Luo et al., 2009; Figure S1). The two rice genes share 51% amino acid sequence identity, with much higher conservations in the catalytic SET domain and the protein interaction domains (SAND, Cys-rich). Analysis of expression of the genes by real-time PCR indicated that *SDG711* was widely expressed in different tissues/organs, whereas *SDG718* was more expressed in leaves than other tested tissues/organs (Figure 1A). Because *OsEMF2b* and PRC2-interacting PHD domain protein genes are involved in photoperiod regulation of flowering time in rice (Luo et al., 2009; Wang et al., 2013; Yang et al., 2013), we studied whether the rice E(z) genes were photoperiod-responsive. We analyzed mRNA isolated from leaves of 6 week-old SD (10 h light/14 h dark)- and 8 week-old LD (14 h light/10 h dark)- grown plant leaves harvested at intervals of 4 h during a 36 h period. The analysis revealed that *SDG711* mRNA levels were higher in LD than in SD, whereas that of *SDG718* were higher in SD than LD (Figure 1B).

SDG711 IS INVOLVED IN LD REPRESSION OF RICE FLOWERING

To study the function of *SDG711*, we produced transgenic rice plants in DJ which contains functional *Hd1* (Naranjo et al., 2014) to knockdown the gene by RNAi and to over-express the gene by using the maize ubiquitin gene promoter (Sun and Zhou, 2008). Analysis of the transgenic plants revealed several lines with reduced expression and six lines with increased expression of the gene (Figure 2A). To check the protein level of *SDG711*, we performed Western blot analysis of protein extracts from the leaves of the WT and transgenic plants grown in LD by using antibodies generated against *E. coli*-produced *SDG711* protein. The analysis confirmed the OX and RNAi of the gene in the transgenic lines (Figure 2B). Three single copy T3 homozygous lines (offspring of single insertion T2 lines that did not segregate transgene-negative

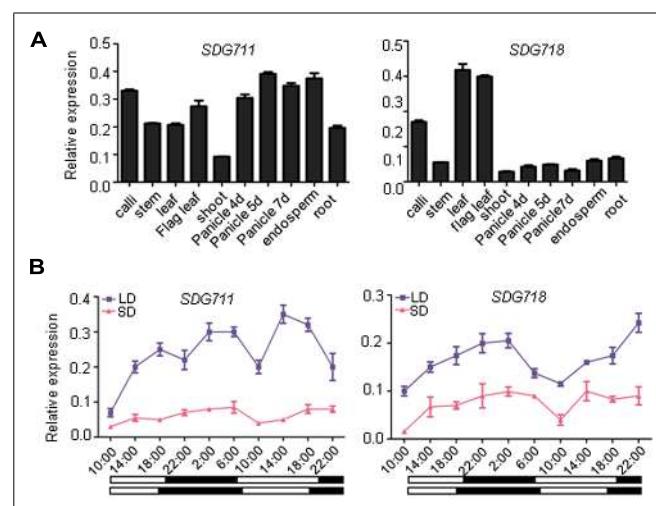
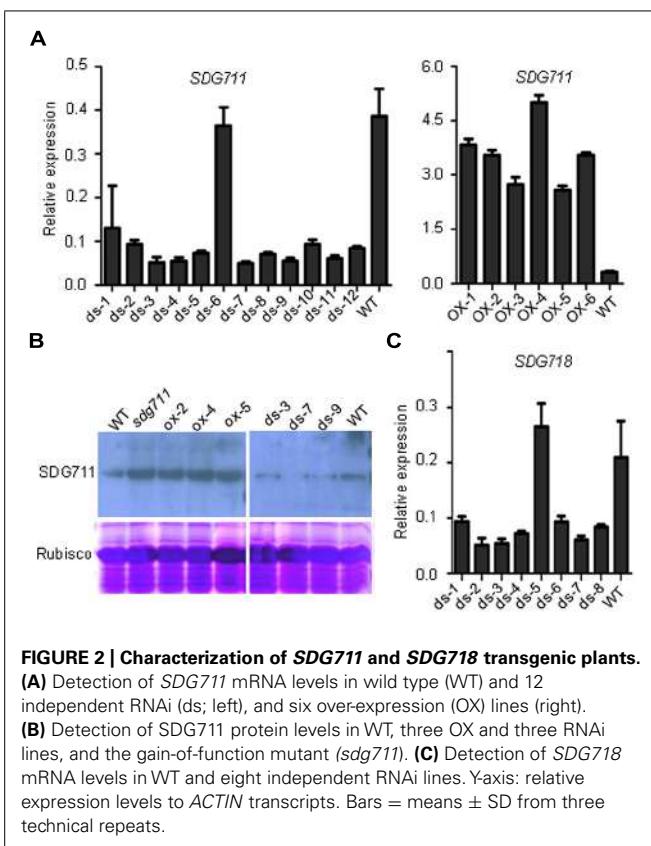
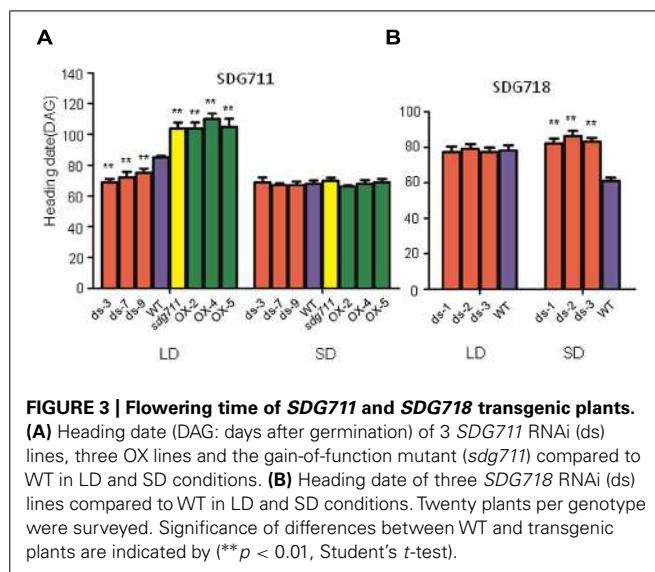


FIGURE 1 | Expression profiles of *SDG711* and *SDG718*. (A) Real-time PCR detection of transcript levels of the genes in indicated organs or developmental stages. (B) transcript levels of the two genes in young leaves harvested at intervals of 4 h during a 36 h period from plants grown under long day (LD) or short day (SD) conditions. Dark periods are indicated by black bars. Bars = mean \pm SD from two biological repeats. Values are shown as relative to *ACTIN* transcript levels.



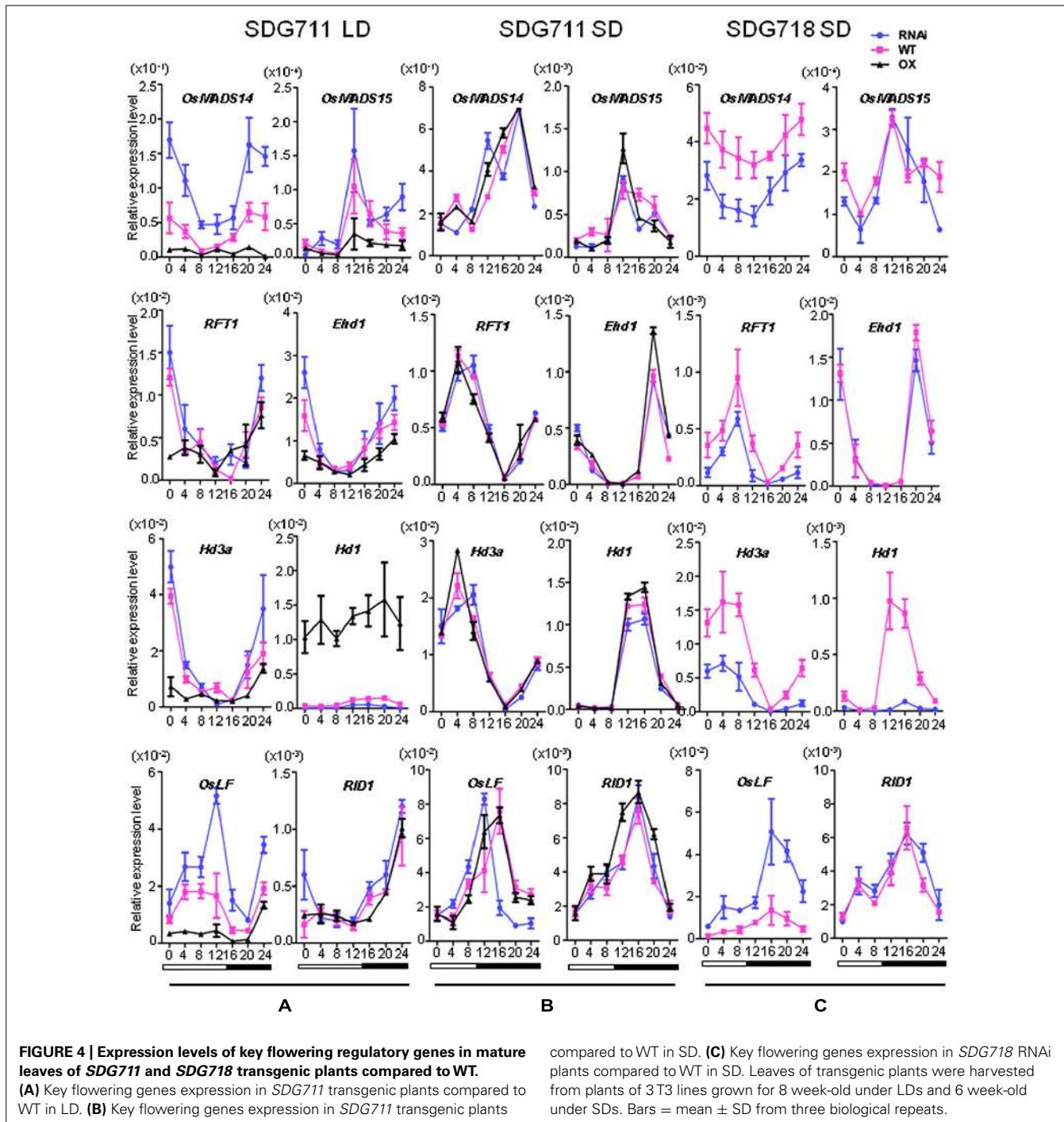
individuals) per transgene were selected for phenotypic analysis. During vegetative growth the transgenic plants did not display any visible morphological defects but exhibited altered HD (or flowering time) compared to the WT plants. In LD (14 h light/10 h dark), the HD of the OX plants was largely delayed (20–25 days, $p < 0.001$, student's *t*-test), while that of the RNAi plants was significantly earlier (10–16 days, $p < 0.001$, student's *t*-test) than WT (Figure 3). However, in SD (10 h light/14 h dark) the HD of *SDG711* OX and RNAi plants was not significantly different from WT (Figure 3). These data suggested that *SDG711* may have a function to repress flowering in LD. In addition to the effect on flowering time, the OX plants produced a higher number of stamens (Figure S2). The pollen viability was reduced in both the OX and RNAi plants of *SDG711* (Figure S2). These observations suggested that *SDG711* might play a role in fertility. To confirm the transgenic results, we characterized a T-DNA mutant that had the insertion located in the 5'-UTR of the gene (Figures S3A,B). One single insertion was identified by Southern blotting (Figure S3C). Real-time PCR revealed a higher level of transcripts of the gene (Figure S3D). Northern blotting experiments revealed that the increased transcripts were about the same size as in WT (Figure S3E). The mutant displayed the same phenotype as that of the OX plants (Figure 3). After three back-crosses the phenotype co-segregated with the presence of the insertion, indicating that the mutation was a gain-of-function mutation.

To study the effect of *SDG711* OX or RNAi on histone methylation, we performed Western blotting analysis of histones isolated



from leaves of the gain-of-function mutant, an RNAi and an OX line. The analysis revealed that compared to WT the levels of H3K27me3 were lower in the RNAi line, but higher in the gain-of-function mutant and OX plants (Figure S4), suggesting that *SDG711* was required for the overall H3K27me2/3 in rice.

The flowering time phenotype suggested that *SDG711* had a function to suppress flowering in LD while without a clear effect in SD. We therefore analyzed the expression of flowering regulatory genes in both pathways. mRNA were isolated from 8 week-old LD or 6 week-old SD plant leaves at intervals of 4 h during a 24 h period. In LD, the mRNA levels of LD flowering activators including *Ehd1*, *Hd3a*, *MADS14*, and *MADS15* were clearly decreased in the OX, but increased in the RNAi plants compared to WT (Figure 4A). By contrast, the transcript levels of the LD flowering repressor *Ghd7* were not clearly altered in the transgenic plants (Figure S5). The expression of *RID1* and *OsMADS50* also appeared unchanged in the transgenic plants (Figure 4A; Figure S5). Interestingly, *Hd1* that acts as a repressor of *Hd3a* in LD was highly induced by *SDG711* OX and repressed by *SDG711* RNAi (Figure 4A), while the expression level of *OsGI* that activates *Hd1* was not changed (Figure S5). Because E(z) proteins are supposed to be transcriptional repressors, the activation of *Hd1* in *SDG711* OX plants might be due to an indirect effect. Recent data have shown that *OsLF*, a bHLH protein that directly represses *Hd1* (Wang et al., 2013). Examination of *OsLF* transcripts in the transgenic plants indicated that *OsLF* was repressed by *SDG711* OX, but activated by *SDG711* RNAi in LD (Figure 4A). The expression of flowering regulatory genes supported the flowering time phenotype observed in the transgenic plants (Figure 3). Analysis of the gain-of-function mutant confirmed the above observed effects of *SDG711* OX on flowering time gene expression (Figure S6). In SD, the expression levels of the flowering genes were not clearly affected by *SDG711* OX or RNAi, except the peak expression of *OsLF* in the RNAi plants was about 4 h earlier than the WT (Figure 4B). These results suggested that *SDG711* is mostly involved in LD repression of rice flowering time.

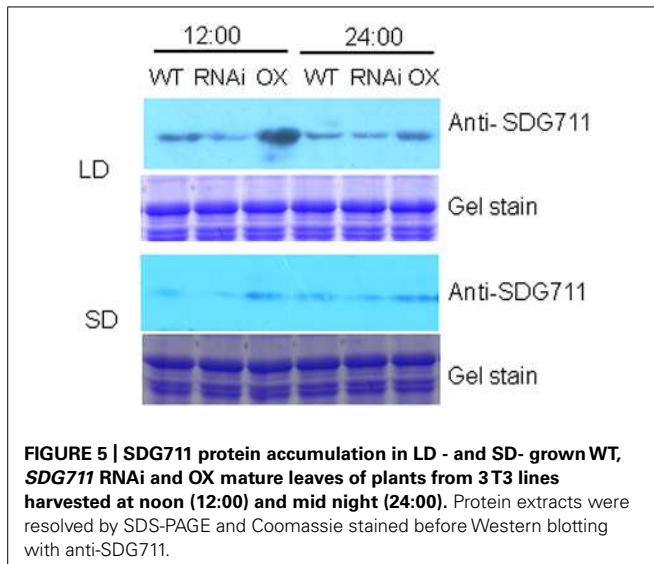


To study whether SDG711 protein levels were differentially regulated in LD and SD, Western blotting analysis of protein extracts from WT, RNAi and OX plants grown in LD and SD harvested at 12:00 (mid day, after 7 h in light for LD condition and 5 h in light for SD condition) and 24:00 am (mid night, after 5 h in dark for LD condition and 7 h in dark for SD condition) was performed by using anti-SDG711. The results shown in Figure 5 revealed that SDG711 protein levels were lower in SD compared in LD and that the high SDG711 level of the OX line was reduced in SD and in

the dark, suggesting that the stability of SDG711 protein may be regulated by day length and light/dark conditions.

SDG718 PROMOTES FLOWERING IN SD

To study whether SDG718 had a function in flowering time control, we obtained several RNAi lines in ZH11 variety (Figure 2C), which also has a functional Hd1 (Luan et al., 2009; Naranjo et al., 2014). These plants showed a similar HD as the WT in LD, but flowered later (>20 days) than WT in SD (Figure 3B). Analysis



of flowering time gene expression revealed that *SDG718* RNAi clearly induced *OsLF*, but repressed the SD activators including *Hd1*, *Hd3a*, *OsMADS14*, and *RFT1* (Figure 4C), suggesting that *SDG718* played a role in promoting flowering in SD. However, the *SDG718* RNAi plants did not show any clear defect in flower organ and pollen viability (Figure S2).

SDG711 AND SDG718 REGULATE H3K27me3 ON FLOWERING GENES

To study whether knockdown and OX of *SDG711* altered chromatin modification of flowering regulatory genes, we analyzed H3K27me3 on *RID1*, *Ehd1*, *RFT1*, *Hd3a*, *MADS14*, *MADS15*, *Hd1*, and *OsLF* in WT, OX and RNAi plants grown under LD conditions by ChIP assays. The 8 week-old plant leaves were harvested at the end of the darkness period under LD conditions. Because H3K27me3 is located in gene body with enrichment on the 5' end of the gene in rice (Hu et al., 2012; Li et al., 2013), we analyzed the ChIP by real-time PCR using two primer sets, one corresponding to the 5' region, the other to the gene body (Figure 6). Among these genes, *Hd1* appeared to be unmodified by H3K27me3 in either the 5' region or the gene body, further supporting an indirect effect of *SDG711* on its expression. *RID* displayed a very low level of H3K27me3 (Figure 6A), consistent with the observation that the expression of the gene was not affected in the *SDG711* transgenic and the gain-of-function mutant plants (Figure 4; Figures S5 and S6). The remaining genes displayed H3K27me3 in the 5' region and/or gene body, suggesting that regulation of these genes might involve PRC2 function. H3K27me3 on these genes (except the gene body of *RFT1* which displayed a relatively weak level of H3K27me3) was clearly reduced in the RNAi but increased in the OX plants (Figure 6A), which reversely correlated with their expression changes in the transgenic plants. These data suggested that *SDG711*-mediated H3K27me3 was involved in the regulation of the genes.

Because H3K27me3 is antagonistic to H3K4me3 on gene activity, we therefore analyzed whether alteration of H3K27me3 affected H3K4me3 on the flowering genes in the transgenic plants.

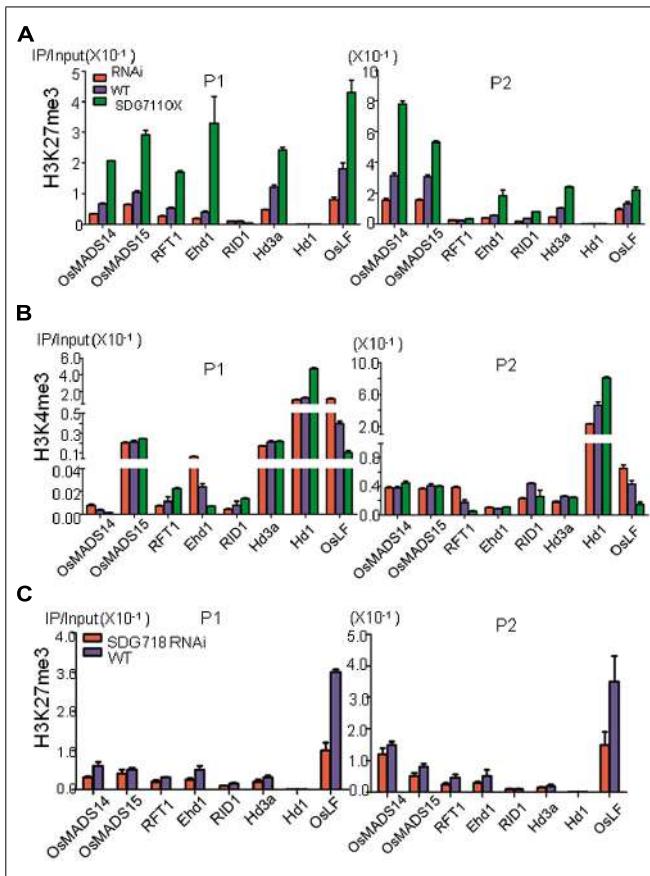


FIGURE 6 | SDG711 and SDG718 function in H3K27me3 and H3K4me3 on key flowering genes. **(A)** chromatin immunoprecipitation (ChIP) analysis of H3K27me3 on the indicated flowering genes in WT, SDG711 RNAi and OX plants (mature leaves pooled from 3 T3 lines). **(B)** ChIP analysis of H3K4me3 on the indicated flowering genes in WT, SDG711 RNAi and OX plants (pooled from 3 T3 lines). H3K27me3 and H3K4me3 enrichments on the 5' end (P1) and the coding region (P2) of the flowering genes were detected by quantitative PCR. Three biological repeats were performed, one repetition is shown. One other biological replicate is shown in Figure S7. Bars = mean \pm SD from three technical repeats. **(C)** ChIP analysis of H3K27me3 on the indicated flowering genes in WT and SDG718 RNAi plants pooled from 3 T3 lines. Leaves of SDG711 transgenic plants were harvested from 8 week-old LD plants grown, and leaves of SDG718 transgenic plants were harvested from 6 week-old SD plants grown.

The analysis revealed relatively higher levels of H3K4me3 in *Hd1*, *Hd3a*, *MADS15*, *Ehd1*, and *OsLF* than in *MADS14*, *RFT1*, and *RID* in WT plants (Figure 6B). H3K4me3 levels in *Hd1*, *MADS15*, and *Hd3a* were not affected by the *SDG711* transgenes except some increases on *Hd1* in the OX plants (Figure 6B). The increased H3K4me3 on *Hd1* may be due to increased expression of the gene in the OX plants, as H3K4me3 is thought to be associated with active genes (Hu et al., 2011). However, H3K4me3 on *Ehd1* and *OsLF* was increased in *SDG711* RNAi but decreased in *SDG711* OX plants (Figure 6B), which conversely correlated to that of H3K27me3 and suggested that *SDG711*-mediated H3K27me3 might affect H3K4me3 in the two loci, which may be linked to the repression of the genes. Analysis of *SDG718* RNAi plants also revealed a clear decrease of H3K27me3 in *OsLF* in SD (Figure 6C).

SDG711 BINDS TO Ehd1 AND OsLF LOCI

To further assess the function of *SDG711* on the flowering gene regulation, we performed anti-*SDG711* ChIP assays and analyzed by real-time PCR using the same primer sets as for the histone methylation ChIP. Non-immunized serum was used as control. The analysis revealed that in LD-grown WT plants *SDG711* binding was clearly enriched in the 5' end and the gene body of *Ehd1* and *OsLF* compared to the other tested genes (**Figure 7A**). However, some enrichment was also observed in the gene body of *RID1*. The *SDG711* enrichment on the three genes was sensibly reduced in RNAi and clearly enhanced in the OX plants (**Figure 7A**), suggesting that *SDG711* may directly target to the genes in LD. In SD-grown plants, the enrichment of *SDG711* on the three genes was much weaker than that in SD (**Figure 7B**), which was consistent with lower accumulation of *SDG711* in SD (**Figure 5**) and suggested that *SDG711* enrichment in the flowering genes was regulated by day length.

DISCUSSION

Our data suggest that *SDG711* and *SDG718* repress *OsLF* in LD and SD, respectively, leading to the activation of *Hd1* that inhibits *Hd3a* and flowering in LD but activates *Hd3a* and *RFT1* and flowering in SD (**Figure 8**). Therefore, the two E(z) genes likely contribute to the accurate photoperiod control of flowering in rice. The increased level of *SDG711* expression in LD and that of *SDG718* in SD may be critical for *OsLF* repression. The SD-induced *SDG718*

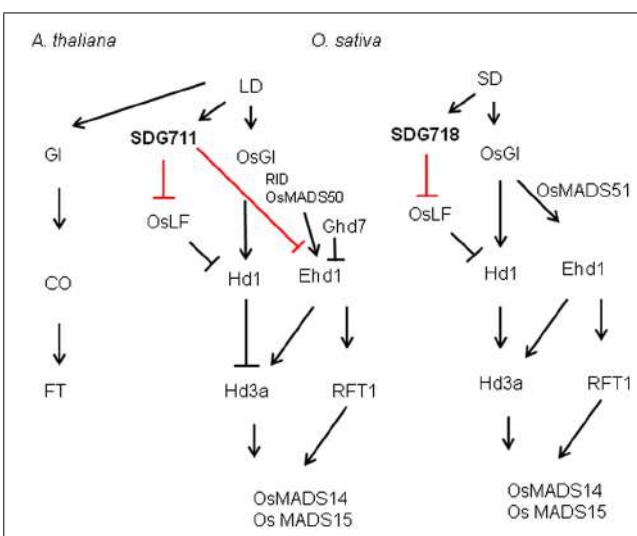


FIGURE 8 | Summary of *SDG711* and *SDG718* function in LD and SD pathways of rice flowering control compared to LD activated flowering in *Arabidopsis*.

expression is consistent with the results showing that *OsVIL* genes that promote flowering in SD are also induced in SD (Wang et al., 2013). The *SDG711*-mediated LD repression of flowering is supported by previous results showing that mutants of other PRC2

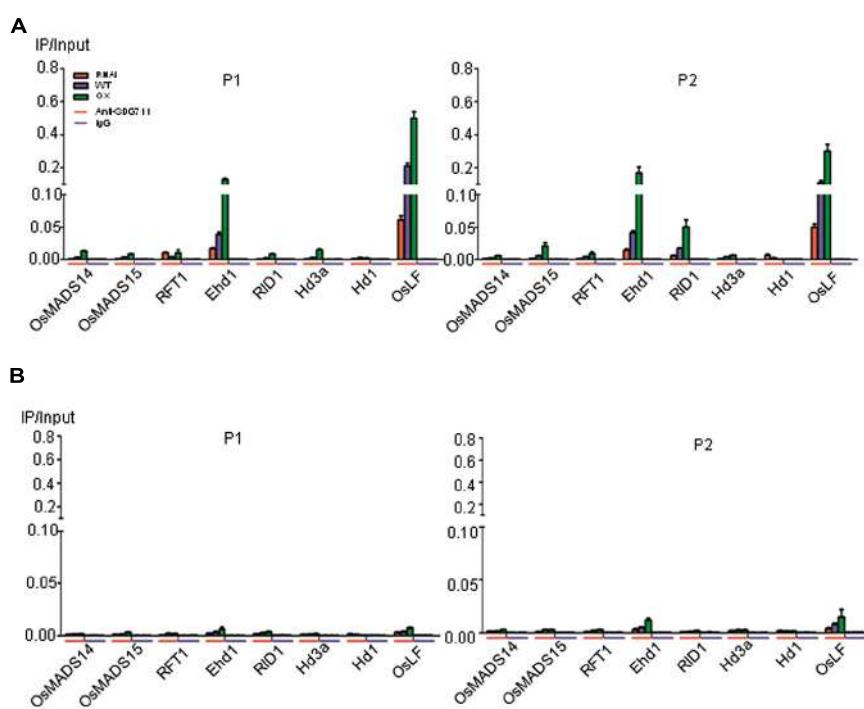


FIGURE 7 | Direct association of *SDG711* protein with flowering genes. **(A)** *SDG711* protein enrichment on the indicated flowering genes in LD-grown WT, *SDG711* RNAi and OX seedlings tested by ChIP with anti-*SDG711*. Non-immunized rabbit serum (IgG) was used as control. Three biological repeats were performed, one repetition is

shown. The two other repeats are shown in Figure S8. Bars = means \pm SD from three technical repeats. **(B)** *SDG711* protein enrichment on the indicated flowering genes in SD-grown plants. Leaves were harvested from plants grown for 8 week-old LD and 6 week-old SD plants grown.

genes such as *osfie2* and *osemf2b* also display an early flowering phenotype in LD (Luo et al., 2009). The observations that the *SDG711* OX only enhanced LD repression of flowering but did not affect flowering in SD (**Figure 3**), suggest that *SDG711* may be also regulated at posttranscriptional levels by day length. This hypothesis is supported by the observation that accumulation of *SDG711* protein in the OX lines in LD was reduced in SD and that *SDG711* binding to key flowering genes was largely reduced in SD (**Figure 7B**). This day length-dependent stability of the protein may allow *SDG711* to regulate flowering only in LD. The changes of H3K27me3 levels on the *OsLF* locus in *SDG711* transgenic plants imply that PRC2-mediated repression may involve the deposition of H3K27me3 on the gene. As *SDG711* was shown to be directly associated with the *OsLF* locus in LD (**Figure 7**), *OsLF* may be a primary target for rice PRC2-mediated LD-repression of flowering. The results are in agreement with recent data showing directly repression of *OsLF* by LC2 that interacts with the *OsEMF2b* protein (Wang et al., 2013; Yang et al., 2013). However, it is not excluded that *SDG711* or *SDG718* represses other genes that mediate *Hd1* repression.

Previous studies have shown that the flowering promoter *Ehd1* and the flowering repressor *Ghd7* could enable manipulation of slight differences in day lengths to control *Hd3a* transcription with a critical day length threshold (Itoh et al., 2010). *Ehd1* and *Ghd7* provide a gating mechanism to set critical day length for *Hd3a* expression in SD, in which *Ehd1* is repressed by the morning activation of *Ghd7* in LD (Itoh et al., 2010; Tsuji et al., 2013). The observations that *SDG711* transgenic and gain-of-function mutant plants affected the expression of *Ehd1* but not that of its upstream regulators (i.e., *Ghd7*, *MADS50* or *RID1*) and that *SDG711* was associated with the *Ehd1* locus (**Figure 6**; Figures S5 and S6), indicate that *SDG711* is a direct repressor of *Ehd1* in LD. In addition, H3K27me3 on the *Ehd1* locus was modulated by the expression level of *SDG711* (**Figure 6A**). Therefore, besides *Ghd7* and *OsMADS50*, *SDG711* represents an additional LD repressor of *Ehd1* (**Figure 8**). The observations that *Ehd1* expression was not clearly affected by down-regulation of *SDG718* and *SDG711* or up-regulation of *SDG711* in SD (**Figures 4B,C**), suggest that PRC2 may mainly target to the *OsLF-Hd1* pathway instead of that of *Ehd1* in SD. Alternatively, the SD-induced expression of *Ehd1* (mainly due to the repression of *Ghd7* in SD) may be overwhelming, which may mask the effect of *SDG718* RNAi. In addition, the chromatin analysis data indicated that key flowering genes displayed different histone modification patterns (**Figure 6**). The changes of H3K27me3 levels on the marked genes caused by *SDG711* RNAi and OX and *SDG718* RNAi, which are correlated with their expression change in the transgenic plants in LD or SD, suggest that *SDG711/SDG718* may be also involved in the deposition of the mark on these loci. Collectively, the data demonstrating that the *SDG711* and *SDG718* are involved respectively, in the LD and SD signaling to promote LD repression and SD activation of flowering, suggest the involvement of PRC2 in the accurate photoperiod control of flowering in rice.

SDG711 and *SDG718* are closely related to *Arabidopsis CLF* and *SWN*, respectively. It is suggested that *CLF* and *SWN* act redundantly to regulate vegetative growth. No vegetative phenotype observed in *SDG711* and *SDG718* transgenic plants support

the idea that the two rice genes may also have a redundant function in the vegetative growth. *Arabidopsis* genome contains a third E(z) gene, *MEA*, which is mainly involved in the regulation of gene imprinting and embryo and endosperm development. The counterpart of *MEA* is not found in rice. Therefore the question arises as that whether either *SDG711* or *SDG718* plays a role in reproductive development. Our data showing that besides flowering time, *SDG711* RNAi and OX affects stamen number, pollen viability, and fertility (Figure S2) suggests that this E(z) gene may be involved in reproductive development in addition to flowering time control in rice.

AUTHOR CONTRIBUTIONS

Xiaoyun Liu and Dao-Xiu Zhou designed the experiments and wrote the manuscript. Xiaoyun Liu, Chao Zhou, and Yu Zhao performed the experiments. Shaoli Zhou performed some images processing. Wentao Wang performed some materials cultivation. All authors have read 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.2014.00591/abstract>

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An efficient method for miRNA detection and localization in crop plants

Flor de Fátima Rosas-Cárdenas^{1,2}, Rocío Escobar-Guzmán¹, Andrés Cruz-Hernández³, Nayelli Marsch-Martínez⁴ and Stefan de Folter^{1*}

¹ Laboratorio Nacional de Genómica para la Biodiversidad, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Guanajuato, México

² Centro de Investigación en Biotecnología Aplicada del Instituto Politécnico Nacional, Tlaxcala, México

³ Facultad de Ingeniería, Universidad Autónoma de Querétaro, Querétaro, México

⁴ Departamento de Biotecnología y Bioquímica, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Guanajuato, México

Edited by:

Clelia De-la-Peña, Centro de Investigación Científica de Yucatán, Mexico

Reviewed by:

Serena Varotto, University of Padova, Italy

Norberto Daniel Iusem, Universidad de Buenos Aires, Argentina

***Correspondence:**

Stefan de Folter, Laboratorio Nacional de Genómica para la Biodiversidad, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Km. 9.6 Libramiento Norte, Carretera Irapuato-León, CP 36821 Irapuato, Guanajuato, México
e-mail: sdfolter@langebio.cinvestav.mx

microRNAs are a class of non-coding small RNAs (sRNAs) that are important regulators of gene expression at the post-transcriptional level by mRNA cleavage or translation inhibition. Another class of sRNAs are siRNAs, which also regulate gene expression but by causing DNA methylation. This epigenetic regulatory role has been observed for some miRNAs as well. The use of sRNAs allows the development of biotechnological applications in plants. To develop these types of applications, and to better understand the natural roles they play, it is important to be able to detect and to localize these sRNAs at the plant tissue level. Sometimes, in crop plants this can be challenging. Therefore, we developed a tissue printing hybridization protocol for easy and efficient detection of sRNAs and demonstrate this by the analysis of the spatio-temporal expression patterns of the miRNAs miR159 and miR164 in fruits of various crop plants. Moreover, we show the possibility to also detect the expression of miRNAs in fruit juice using a dot blot hybridization approach.

Keywords: tissue-printing, miRNA detection, hybridization, fruits, miR164

INTRODUCTION

microRNAs (miRNAs) are a class of small RNAs (sRNAs) that are fundamental regulatory elements of eukaryotic genomes (Voinnet, 2009) and their widespread conservation and divergence in the plant kingdom has been demonstrated (Chavez Montes et al., 2014). Detection of the spatio-temporal expression of miRNAs is critical to understand their function (van Rooij, 2011). Several techniques such as Northern-blot hybridization (Pall and Hamilton, 2008), microarray analysis (Yin et al., 2008), stem-loop RT-PCR analysis (Chen et al., 2005), or sRNA-seq analysis (Chavez Montes et al., 2014) permit the identification of miRNA expression patterns, which may suggest their involvement in certain biological processes. Besides these advantages, a drawback of these techniques is the lack of information about the actual localization of the miRNA in the tissue or organ itself, which is important for understanding the biological function of the miRNA.

A technique overcoming this drawback is *in situ* hybridization, which also allows the detection of miRNAs in thin tissue sections using a labeled complementary probe against the miRNA of interest (Kidner and Timmermans, 2006; Várallyay and Havelda, 2011). Nevertheless, the generation of thin tissue sections from large tissues using paraffin-embedding or cryosections may be challenging. Tissue printing in nitrocellulose or nylon membranes is a technique employed to study the localization of proteins, nucleic acids, and soluble metabolites from freshly cut tissue slices. Tissue printing has been defined as ‘the art and science of visualizing cellular material and information that are transferred to

a receptive surface when the cut surfaces of section of tissues or organs are pressed against such a surface’ (Varner and Ye, 1994). This technique does not require RNA extraction or the preparation of thin tissue sections, and it allows the simultaneous analysis of many samples. Therefore, this technique is especially convenient for large tissues or organs, such as fleshy fruits that are often difficult to section for *in situ* hybridizations. Tissue printing in combination with hybridization has been used successfully to determine mRNA and protein localization in several studies (Holland et al., 2000; Qu et al., 2003; Jolie et al., 2010; Pluskota et al., 2011; Esteves et al., 2013; Mochizuki and Ohki, 2015). However, to date almost no examples have been reported for the detection of miRNAs in plants using tissue printing (e.g., Rosas-Cárdenas et al., 2015).

Here we report a protocol for tissue printing combined with hybridization in order to detect and to localize known miRNAs at the tissue level in different species. Although we focused mainly on fruits, this protocol may be used for other fleshy tissues as we demonstrate here by detecting miRNAs in floral buds. Moreover, we show also the possibility to detect the expression of miRNAs in fruit juice using a dot blot hybridization approach.

MATERIALS AND METHODS

PLANT MATERIAL

Fleshy fruits were purchased at the local market. Agave buds (*Agave atrovirens*) were collected at the Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional

(CINVESTAV-IPN), Irapuato. The tissues were washed and dried at room temperature.

REAGENTS

Nylon membrane (Hybond NX; Amersham/Pharmacia, cat. no. RPN303T)
 3MM Whatman® chromatography paper
 1-Methylimidazole (Sigma-Aldrich)
 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma-Aldrich)
 Rapid-hyb Buffer (GE Healthcare)
 EasyTides® Adenosine 5'-triphosphate, (γ -³²P)-6000 Ci/mmol, 10 mCi/ml (370 mBq/ml), 50 mM Tricine (pH 7.6) (Perkin Elmer)

BUFFERS AND SOLUTIONS FOR HYBRIDIZATION ANALYSIS

- EDC fixation solution (24 ml)
 - 245 μ l of 12.5 M methylimidazole, pH 8.0
 - 0.5 g EDC
- Wash solution
 - 2x SSC
 - 0.1% SDS

TISSUE PRINT

The samples were washed and dried at room temperature. The samples were cut in longitudinal and/or transverse sections. After cutting the samples, they were immediately placed with the cut surface face down on the membrane (Amersham Hybond-N; GE Healthcare). The different sections were firmly pressed on the nylon membrane for 30 s, subsequently the tissue was carefully removed and the membrane was dried (around 5–20 min) at room temperature.

FIXATION OF THE MEMBRANE

The EDC fixation solution was prepared as described Pall and Hamilton (2008) with some modifications. Briefly, 0.753 g of EDC was dissolved in 10 ml of water, 245 μ l of 12.5 M 1-methylimidazole was added and finally 150 μ l of 1 M HCl was added to obtain a pH of 8. This solution was prepared fresh before use. The membranes were incubated in this fixation solution for 1 h at 65°C, and then rinsed twice with water. The membranes were dried at room temperature, and stored at -20°C till further use. miRNA detection was carried out as the hybridization analysis.

HYBRIDIZATION ANALYSIS

To prepare the probes we used the following synthesized oligonucleotides, which sequences are complementary to each 21 nucleotide mature miRNA of interest: 5'-AGGGGCCATGCTAA TCTTCTC-3', 5'-AAGAGCT CCCTTCAATCCAAA-3', 5'-UGGA GAAGCAGGGCACCGUGCA-3', and 5'-TGCACGTGCCCTGCTT CTCCA-3', to detect the small nucleolar RNA U6 (positive control), miR159a, miR164, and miR164* sense (negative control), respectively. The small nucleolar RNA U6 is often used as a loading control or used for signal normalization (e.g., Kou et al., 2012; Rosas-Cárdenas et al., 2015). To prepare the probes the oligonucleotides were labeled as follows: 4 μ l of oligonucleotide 100 μ M, 1 μ l of T4 Kinase (10 U/ μ l), 1 μ l [γ -³²P] ATP (10 mCi/ml), 4 μ l

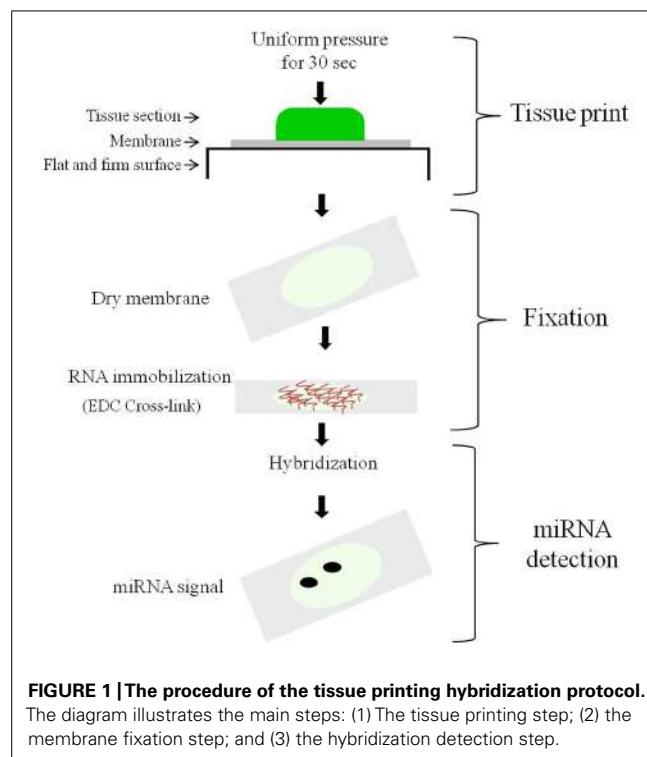
of forward buffer and 10 μ l of water; the reaction solutions were incubated at 37°C for 1 h and subsequently added to the membranes. The hybridization was made as we described before in Rosas-Cárdenas et al. (2011) with some modifications. In summary, the used hybridization solution was the Rapid-hyb buffer (GE healthcare), which contains chemical blocking agents and therefore does not require heterologous DNA to control non-specific binding of probes to the membrane. The membranes were pre-hybridized with 15 ml hybridization solution for 1 h at 42°C with constant agitation, followed by adding the labeled probe of interest, and then incubated for 24 h at 42°C with constant agitation. Membranes were washed with wash solution (2x SSC, 0.1% SDS), first for 4 min, and afterwards for 2 min at room temperature, followed by exposure to a storage phosphor screen for 24 h and/or 48 h at room temperature (highly expressed miRNAs can be detected already at 12 h). Finally, the storage phosphor screen was scanned in a Storm 860 Gel and Blot Imaging System (Amersham Biosciences). This protocol is not yet tested with the use of non-radioactive probes, but we expect it to work.

SUMMARY: TISSUE PRINTING HYBRIDIZATION PROTOCOL

1. Cut a membrane to the appropriate size.
2. Cut the tissue and carefully place a longitudinal and/or a transverse section(s) onto the membrane with the cut surface down and firmly press the tissue for 30 s.
3. Carefully remove the tissue from the membrane.
4. Dry the membrane at room temperature.
5. Fix the membrane with EDC solution for 1 h at 65°C.
6. Rinse the membrane with water and then dry it at room temperature.
7. Pre-hybridize the membrane with 15 ml hybridization solution for 1 h at 42°C with constant agitation.
8. Add the labeled probe of interest and incubate for 3–24 h at 42°C with constant agitation.
9. Discard the hybridization solution and wash the membrane with wash solution for 4 min at room temperature.
10. Discard the wash solution and wash the membrane again for 2 min at room temperature.
11. Expose the membrane to a storage phosphor screen for 24 h at room temperature and then scan the screen and analyze the signal.

RESULTS AND DISCUSSION

In this work we aimed to develop an easy protocol that would allow the *in situ* detection of miRNAs in relatively large plant tissues or organs. For this we used the combination of tissue printing and hybridization. Tissue printing is a simple procedure by which the uppermost layer of cells and the surrounding extracellular matrix of the cut surface from plant tissue are transferred to a membrane after physical contact with the membrane surface generating a bidimensional anatomical image on which the molecule of interest can be visualized by hybridization. The procedure is depicted in **Figure 1** as a flow diagram consisting of three steps: tissue print, membrane fixation, and miRNA detection. The detailed procedure is described in Section “Materials and Methods.” We used this tissue printing hybridization protocol to observe the



spatio-temporal expression of miR159 and miR164 in different tissues (Figures 2–4 and Supplementary Figure S1), as described below.

DETECTION OF miRNAs BY TISSUE PRINTING HYBRIDIZATION

We first tried the protocol with a green tomato fruit (Figure 2A). A transverse cut of the tomato fruit was made (Figure 2B), which was then firmly pressed on the membrane, leaving a faint tissue print behind on the membrane (Figure 2C). The membrane was then hybridized with a ^{32}P labeled probe against miR164 (Figure 2D) and an independently printed membrane hybridized with a probe against the nucleolar U6 (positive control) (Figure 2E), and with a miR164 sense probe (negative control; Supplementary Figure S2). A strong miR164 signal was observed in seeds, a signal in the exocarp and/or mesocarp, and a moderate signal in the endocarp, in line with observed expression in complete tomato fruits (Moxon et al., 2008). The U6 signal was also clearly observed in the seeds and a lower signal in the pericarp tissues. Notably, the nucleolar U6 signal may also change during development, as previously reported (e.g., Kou et al., 2012).

Subsequently, we repeated the miR164 detection in different fruit crops, now in strawberry (botanically speaking not a berry), guava, and again tomato, but for each two different developmental stages were used (Figure 3). Again, the strongest hybridization signal can be observed in seeds and a lower expression in pericarp tissues. Notably, a clear signal can be seen from the seeds that are on the outside in the case of strawberry (Figure 3).

Furthermore, we also tried floral buds to see if we could detect signal for miR164 and for miR159. We used transverse cuts of two developmental stages of floral buds from agave to make the tissue prints (Figure 4). We observed signal for both miRNAs, for miR164 mostly in placenta/transmitting tract tissue and for miR159 mostly in ovules.

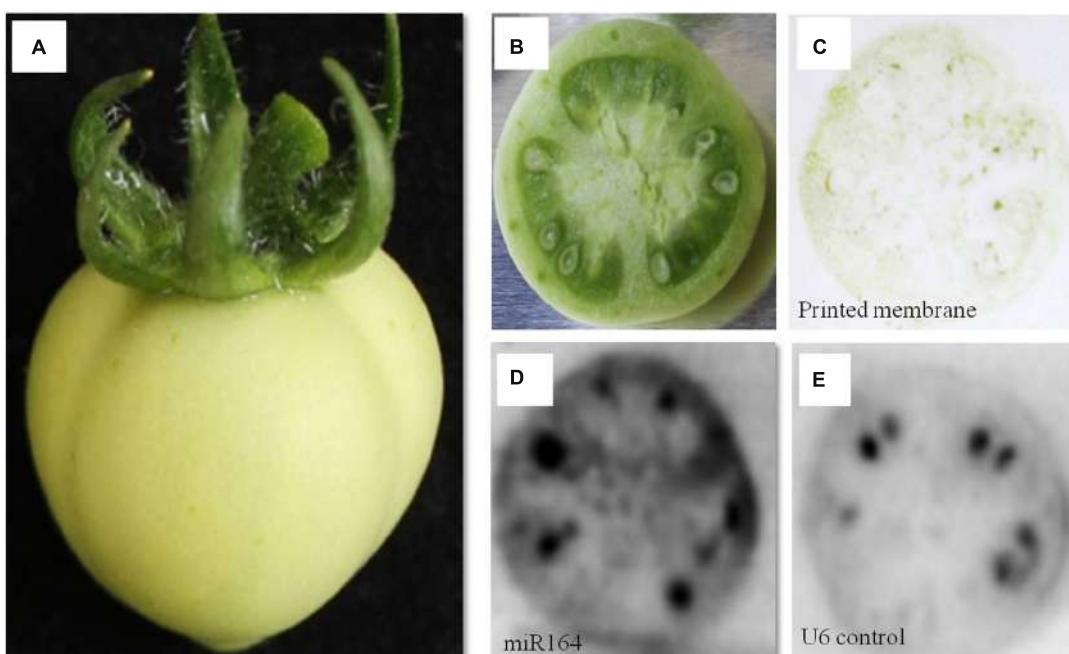
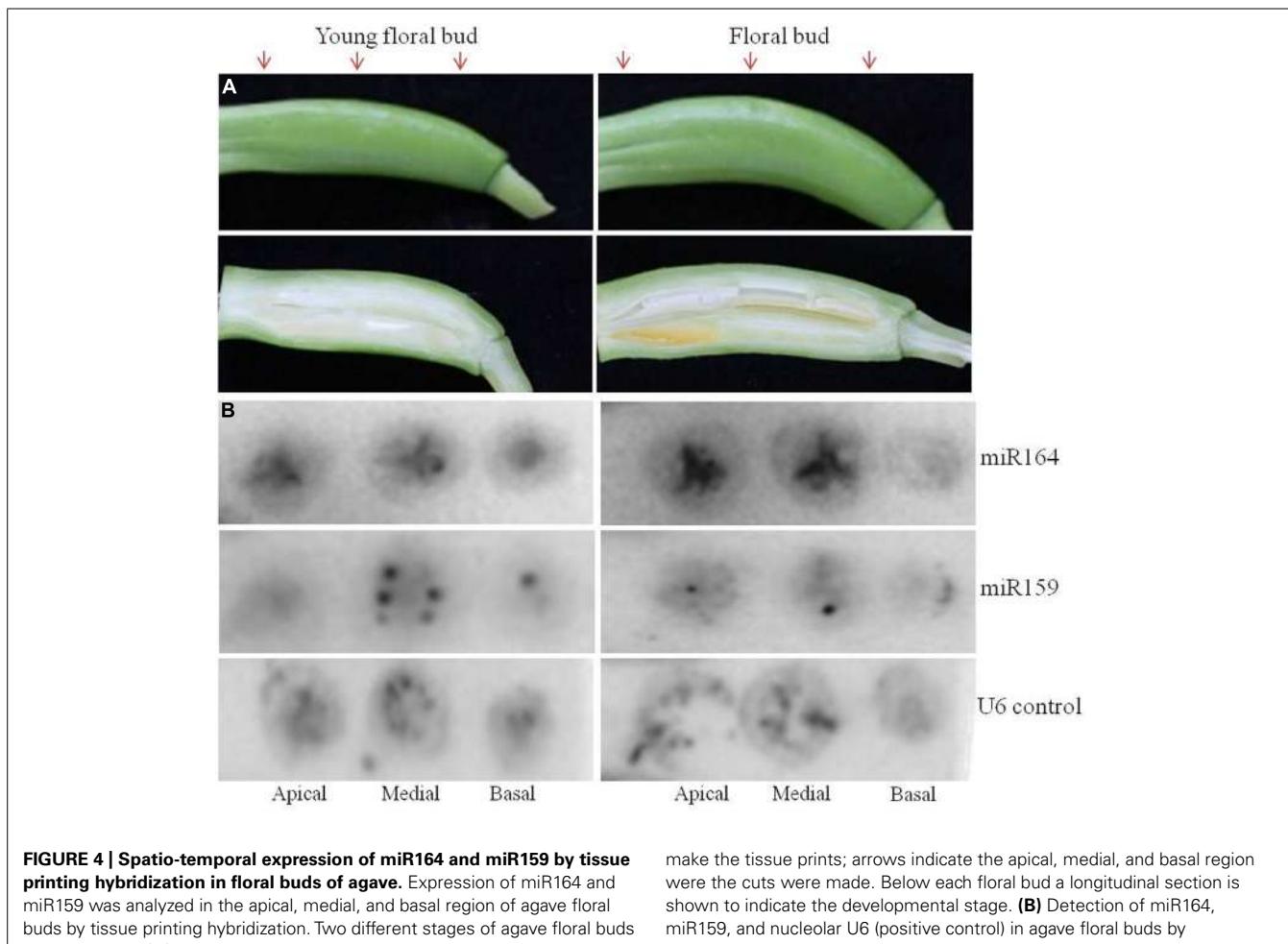
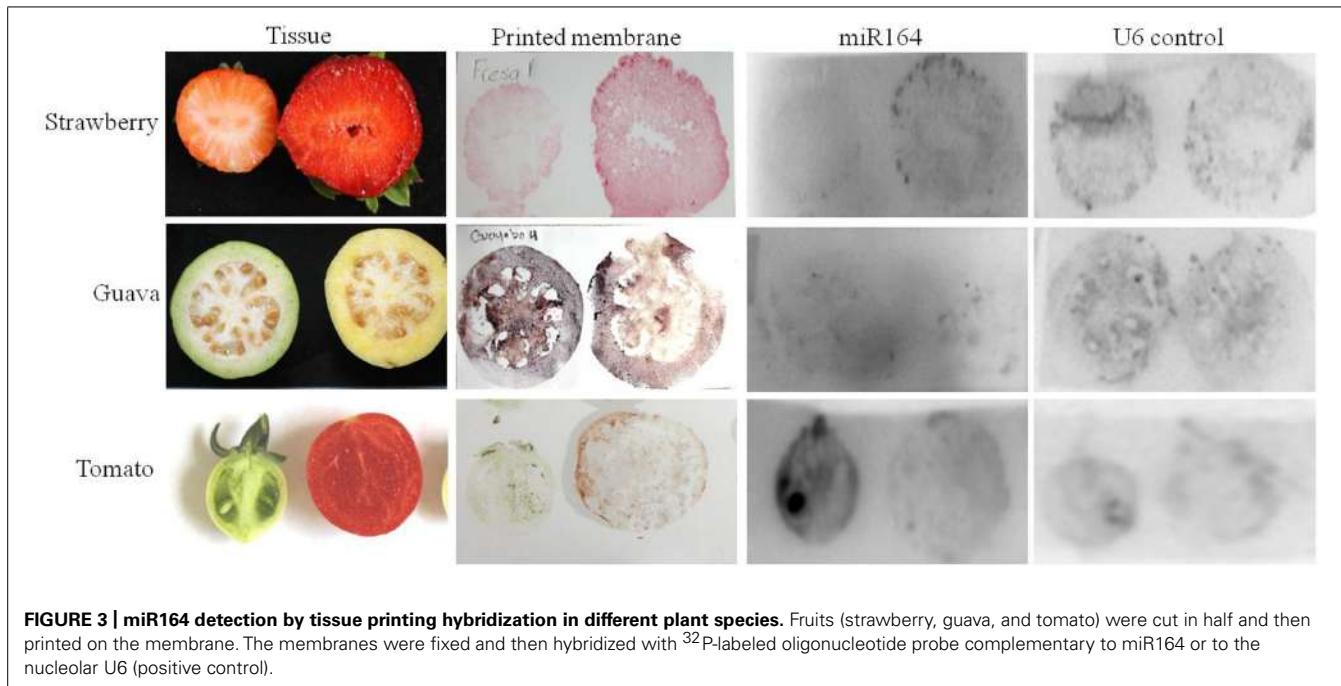


FIGURE 2 | miR164 detection by tissue printing hybridization in tomato fruit. (A) Tomato fruit; (B) Transverse cut tomato fruit to be used for the tissue printing; (C) Tissue print on the membrane made from a transverse cut tomato fruit; (D) miR164 detection by hybridization; (E) nucleolar U6 (positive control) detection by hybridization.



The observed hybridization patterns in the different experiments for miR164, miR159, and nucleolar U6 are different, indicating that the observed signals are not background. Notably, when we used banana fruit for the tissue print, which left a lot of tissue behind on the membrane, we observed signal for the probe against miR164, but also for the sense miR164 negative control (Supplementary Figure S1). For the tissue prints of the other fruits no signal was observed with the miR164 sense probe (Supplementary Figure S2). So, caution should be taken with making conclusions when printing very soft tissues. However, we cannot exclude the possibility that miR164* in banana fruit is stable and detectable, as the detection of some other miRNA*’s has been reported (Zhang et al., 2011; Manavella et al., 2013). A possibility is to use another miRNA sense probe to distinguish between background and real signal.

DETECTION OF miRNAs IN FRUIT JUICE BY DOT BLOT HYBRIDIZATION

Sometimes it might not be important to know exactly where in the tissue the miRNA is expressed, but for instance just presence or absence, which could be useful to analyze genetically modified crops. We reasoned that it should be possible to detect miRNA

expression in fruit juice (or crude extract). For this we prepared membranes with 5 µl fruit juice of lemon, mandarin, prickly pear cactus fruit, and tomato fruit. After applying the juice to the membrane, the membranes were fixed and then hybridized with the probe against miR164 (**Figure 5A**). Indeed, a clear signal was observed for miR164 as well as for the positive control U6. Furthermore, we tried fruit juice of three different developmental stages of tomato fruits and hybridized it with the probe against miR159 (**Figure 5B**). Also for miR159 a clear signal was observed, which was the highest in the youngest fruit stage, suggesting that miR159 is more expressed at that stage. In summary, this is an alternative way to quickly detect the expression of a miRNA.

CONCLUSION

Here we provide a simple, rapid, and useful protocol to detect miRNAs from different tissues and organs of plant species. Using this tissue printing hybridization protocol we were able to determine the spatial-temporal expression pattern of miR159 and miR164 in different tissues. Moreover, we showed the possibility to detect the expression of miRNAs in fruit juice using a dot blot hybridization approach. This allows for instance the semi-high throughput

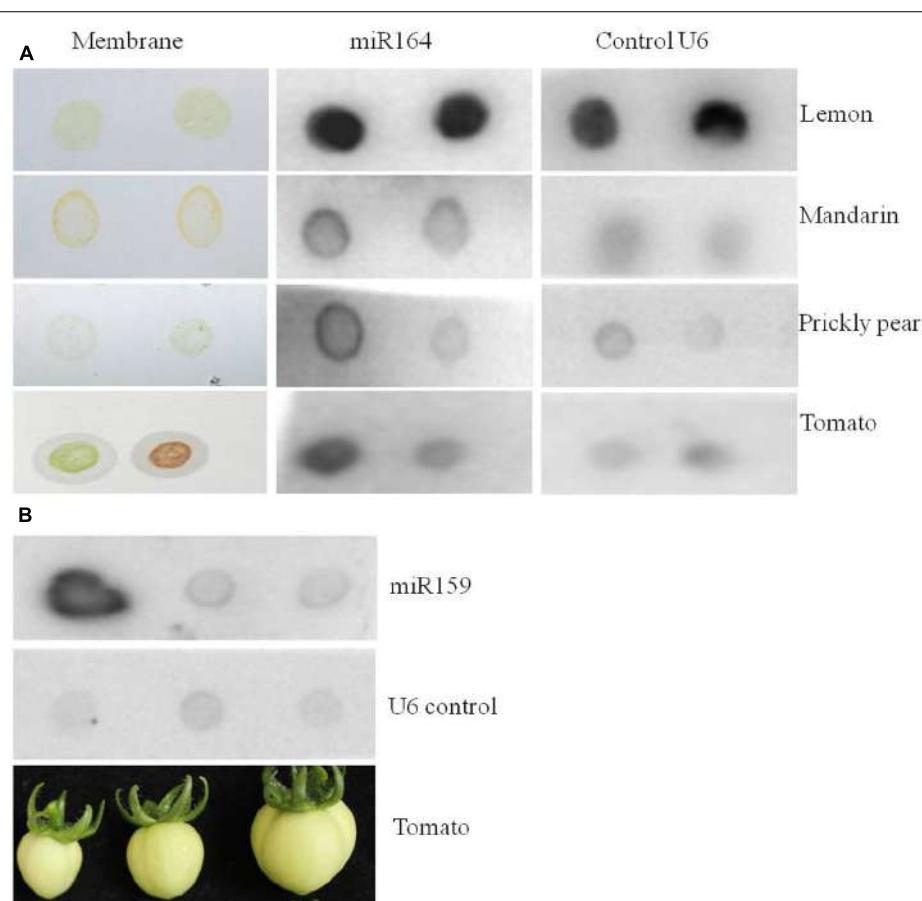


FIGURE 5 | miR164 and miR159 detection in fruit juices of different plant species by dot blot hybridization. Five microliter of fruit juice were spotted on membranes and the miRNAs were detected by hybridization assays. **(A)** Expression of miR164 in lemon and mandarin

juice, and in crude extract of green and ripe stages of prickly pear cactus and tomato fruits. **(B)** Expression of miR159 in different developmental stages of tomato crude extracts. The tomato fruit stages used for the crude extracts are shown below the membrane.

screening for the presence of a miRNA in fruits when no tissue localization is required.

AUTHOR CONTRIBUTIONS

FFRC did the major experimental work. REG contributed to the hybridization assays. FFRC, ACH, NMM, and SDF conceived the project and designed the experiments. FFRC and SDF drafted 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.2015.00099/abstract>

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miRNA regulation of nutrient homeostasis in plants

Soumitra Paul, Swapna K. Datta and Karabi Datta*

Translational Research Laboratory of Transgenic Rice, Department of Botany, University of Calcutta, Kolkata, India

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***Correspondence:**
Karabi Datta,

Translational Research Laboratory
of Transgenic Rice, Department
of Botany, University of Calcutta, 35
Ballygunge Circular Road,
Kolkata-700019, WB, India
krbdatta@yahoo.com

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Small RNAs including micro RNAs (miRNA) play an indispensable role in cell signaling mechanisms. Generally, miRNAs that are 20–24 nucleotides long bind to specific complementary transcripts, attenuating gene expression at the post-transcriptional level or via translational inhibition. In plants, miRNAs have emerged as the principal regulator of various stress responses, including low nutrient availability. It has been reported that miRNAs are vital for maintaining nutrient homeostasis in plants by regulating the expression of transporters that are involved in nutrient uptake and mobilization. The present review highlights the role of various miRNAs in several macro- or micronutrient deficiencies in plants. Understanding the regulation of different transporters by miRNAs will aid in elucidating the underlying molecular signal transduction mechanisms during nutritional stress. Recent findings regarding nutrient related-miRNAs and their gene regulation machinery may delineate a novel platform for improving the nutritional status of cereal grains or crop biofortification programs in the future.

Keywords: miRNA, plant, micronutrient, macronutrient, transporters, nutrient homeostasis

Introduction

Plants acquire mineral ions from the soil, which they allocate to various compartments following long- or short-distance transport. The underlying molecular mechanisms of uptake, transport, and loading of mineral ions into storage organs depend on the differential expression of various transporters based on mineral availability (Sperotto et al., 2014). The deficiency of mineral ions in the soil elicits signaling responses in plants, and interactions among signaling molecules and transporter or carrier genes can facilitate ion transport (Vigani et al., 2013a,b). If a plant is under nutrient starvation, the information is immediately transmitted to the genetic material via signaling molecules to maintain nutrient homeostasis. The scarcity of nutrients activates transporters or alters the architecture and growth of roots to enhance mineral uptake (Jung and McCouch, 2013). Signaling molecules interact with specific nucleotide sequences of mRNA to alter gene expression.

Small regulatory RNAs are considered the most ubiquitous signaling molecules that regulate post-transcriptional gene expression. These small RNAs bind with specific mRNA that exhibit their perfect complementary bases and attenuate gene expression (Bartel, 2004). In plants, two major types of small RNAs, small interfering RNAs (siRNAs) and microRNAs (miRNAs), are likely associated with the silencing of gene expression. siRNAs, which are small 20- to 24-nucleotide regulatory RNAs, are primarily derived from endogenous genomic regions or exogenously supplied duplexes of nucleic acid, as used in RNAi-mediated gene silencing technology (Jamalkandi and Masoudi-Nejad, 2009; Khraiwesh et al., 2012). Dicer-like enzymes (DCL in *Arabidopsis*) cleave the double-stranded duplex (with hairpin loop) to form short, perfect duplexes, which are loaded onto the RNA-induced silencing complex (RISC). The sense strand of the RISC complex recognizes the

target gene or transcript and binds to the perfect complementary nucleotide sequences and regulates the gene expression level at post transcriptional or transcriptional levels (Schauer et al., 2002). However, miRNAs, 20–24 nucleotides in length, are derived from endogenous primary transcripts upon processing. The processed mature miRNA is incorporated into the RISC complex and directs the RISC complex toward degradation or the translational inhibition of target mRNAs (Lee et al., 2004; Vazquez et al., 2010).

In plants, miRNAs are primarily associated with several physiological phenomena, such as growth, development, organogenesis, and responses to various biotic and abiotic stresses (Chen, 2004; Sunkar and Zhu, 2004; Kidner and Martienssen, 2005; Lu et al., 2008; Zhang et al., 2008; Kruszka et al., 2012). Recently, several novel miRNAs were reported for the uptake and transport of nutrient minerals in plants (Jones-Rhoades and Bartel, 2004; Fischer et al., 2013; Kehr, 2013). In the present review, current knowledge of the different classes of nutrient responsive-miRNAs and of their functions in nutrient homeostasis was extensively studied, which may aid in understanding the role of miRNAs as master regulators of nutrient loading in plants. The regulation of the differential expression of transporter genes by miRNAs may reveal a novel paradigm for crop biofortification or mineral bioavailability in cereal grains in the future.

miRNAs in Nutrient Homeostasis

miRNA and Phosphorus Nutrition

Inorganic phosphate (Pi), an essential mineral for plant growth and development is often a limiting factor in plant nutrition. Despite the adequate amount of phosphorus (P) in cultivated land, plants can uptake very small amounts of phosphorus due to its low availability. Plants synthesize several classes of nucleases and phosphatases to solubilize external Pi or to release Pi from organic substances and to upregulate the gene expression of certain exporter/importer transport proteins to acclimatize to phosphate starvation (Poirier and Bucher, 2002; Abel, 2011). Recent studies have identified the involvement of small RNAs, particularly miRNAs, in the differential regulation of phosphate-related gene expression in *Arabidopsis*, rice, wheat, barley, maize, soybean, white lupin, and tomato, etc. (Table 1; Pant et al., 2009; Gu et al., 2010; Lundmark et al., 2010; Zhu et al., 2010; Hackenberg et al., 2013; Pei et al., 2013; Xu et al., 2013; Zhao et al., 2013a). In *Arabidopsis*, the deprivation of Pi is rapidly transmitted to the shoots and induces the synthesis of miR399a-f families. miR399 binds to the five complementary sites of the phosphate over accumulator2 (*PHO2*) transcript, which is in the 5' untranslated region (UTR), 200–400 bp upstream and induces mRNA cleavage (Pant et al., 2008). *PHO2*, an essential transporter for phosphate mobilization, is associated with E2 ubiquitin conjugating enzyme. The reduction of *PHO2* helps to accumulate high amounts of Pi in the shoots. The role of miR399 and its biosynthesis regulation have been well demonstrated. The myeloblastosis (MYB) transcription factors like phosphate starvation response1 (PHR1) binding sites, such as *P1BS*, *PHO*, and *PHO-like*, are

present upstream of the promoter region of miR399 (Bari et al., 2006; Zeng et al., 2010; Xu et al., 2013). The expression of miR399f is also regulated by the binding of MYB2 transcription factor at the *cis*-acting elements of the MIR399f promoter, as reported in *Arabidopsis* (Baek et al., 2013). Notably, long non-coding RNA, another signaling molecule, which is induced by phosphate starvation1 (IPS1), plays an inhibitory role by protecting the *PHO2* transcript and interferes with miR399-mediated *PHO2* gene regulation (Franco-Zorrilla et al., 2007; Hu et al., 2011; Huang et al., 2011).

In *Arabidopsis*, phosphate limitation also increases the amounts of three other miRNAs: miR778, miR827, and miR2111. Intriguingly, within 3 h of Pi re-addition, the abundance of miR2111, and miR778 rapidly reduce by approximately twofold. Two copies of miR2111 are present in the *Arabidopsis* genome, and both loci show similar phosphate response activities (Pant et al., 2009). Recently, the dual responses of miR827, miR399, miR2111, and miR827 have been elucidated. The activity of miR2111 is reversed under nitrogen (N)-starvation compared to phosphate (P)-starvation (Liang et al., 2012). The miR827 and miR399 have also been identified to function in N-starvation by targeting the *nitrogen limitation adaptation (NLA)* gene and are thought to increase the expression of the *PHO2* transporter. In lower abundances of nitrates, the *nla* mutant has been found to play an important role in phosphorus homeostasis by accumulating excessive Pi. A similar condition has been observed for miR827-overexpressing *Arabidopsis* plants and supports the miR827-mediated gene regulation of phosphate transporters (Kant et al., 2011). In rice, Pi accumulation followed by a perturbation of phosphorus mobilization in old leaves is also manifested by miR827 (Wang et al., 2012). Notably, the expression of miR398a is regulated by P, carbon (C) and N limitation, indicating a more general role in mineral homeostasis. Repression due to C limitation was also shown to be correlated with the induction by sucrose (Dugas and Bartel, 2008). Furthermore, soybean roots, miR159a has been found to be up regulated during P deficiency, whereas down regulation of miR319a, miR396a, miR398b, miR1507a has been shown. The differential regulation of miRNAs during P-starvation depends on the frequency of phosphorus responsive motifs (P responsive motifs) in the promoter of the miRNA genes. The number of P-responsive motifs in the *cis*-acting region of miRNA genes is reported to be higher than those of non-responding miRNA genes (Zeng et al., 2010).

miRNAs in Nitrogen Uptake and Transport

Nitrogen, an essential constituent for nucleic acids, protein, chlorophyll, etc., plays a crucial role in plant growth and development. N transport depends on the external acquisition of N by roots from the soil, except the mechanism of biological N fixation in legumes. Plants can adapt in N-limiting soil conditions by up- or down-regulating a specific group of exporter or importer proteins. The differential regulation of transporters is selectively controlled by several small miRNA families. In *Arabidopsis*, depending on the occurrence at N-starvation, the miRNA-responsive populations can be categorized into two groups by Solexa high-throughput sequencing. N-starvation-induced (NSI) miRNA

TABLE 1 | Differentially expressed miRNAs reported under different nutrient/metal (N, P, S, Cu, Mn, Fe, Zn) deprivation in various plant species.

micro RNA (miRNA)	Phosphorus (P)	Nitrogen (N)	Sulfur (S)	Copper (Cu)	Manganese (Mn)	Iron (Fe)	Zinc (Zn)
miR156	↑ (White lupin)	↑ (<i>Arabidopsis</i>)	↑ (<i>Brassica napus</i>)		↑ (<i>Phaseolus vulgaris</i>)		
miR156h		↑ (<i>Arabidopsis</i>)					
miR157					↑ (<i>P. vulgaris</i>)		
miR158	↑ (Tomato)					↑ (<i>Arabidopsis</i>)	
miR159	↑ (White lupin)					↑ (<i>Arabidopsis</i>)	
miR159a	↑ (Soybean)						
miR159b	↑ (Wheat)						
miR160	↑ (White lupin)	↑ (<i>Arabidopsis</i> , Maize)	↑ (<i>B. napus</i>)				
miR164	↑ (White lupin)	↑ (Maize leaves)	↑ (<i>B. napus</i>)		↑ (<i>P. vulgaris</i>)	↑ (<i>Arabidopsis</i>)	
miR166	↑ (White lupin)				↑ (<i>P. vulgaris</i>)		↑ (<i>Sorghum bicolor</i>)
miR167	↑ (Wheat)	↓ (<i>Arabidopsis</i> , ↑ maize)	↑ (<i>B. napus</i>)		↑ (<i>P. vulgaris</i>)		
miR168	↑ (White lupin)	↑ (maize roots)	↑ (<i>B. napus</i>)				
miR169		↑ (<i>Arabidopsis</i> , <i>Medicago truncatula</i> , maize)			↑ (<i>P. vulgaris</i>)		
miR169 (a-c)		↓ <i>Arabidopsis</i>					
miR169 (d-g)	↑ (miR189g in Tomato)	↑ Soybean					
miR169 (h-n)		↓ Maize					
miR170					↑ (<i>P. vulgaris</i>)		
miR171		↑ (<i>Arabidopsis</i>)					↑ (<i>S. bicolor</i>)
miR172	↑ (Tomato)	↑ (<i>Arabidopsis</i> , maize)			↑ (<i>P. vulgaris</i>)	↑ (<i>Arabidopsis</i>)	↑ (<i>S. bicolor</i>)
miR172b	↑ (Tomato)						
miR173						↑ (<i>Arabidopsis</i>)	
miR319	↑ (Tomato, White lupin)	↑ (<i>Arabidopsis</i> , maize)			↑ (<i>P. vulgaris</i>)		↑ (<i>S. bicolor</i>)
miR319a	↓ (Soybean)						
miR390	↓ (White lupin)				↑ (<i>P. vulgaris</i>)		
miR394			↑ (<i>B. napus</i>)			↑ (<i>Arabidopsis</i>)	
miR395	↓ (White lupin)	↓↑ (<i>Arabidopsis</i> , maize)	↑ (<i>B. napus</i>)		↑ (<i>P. vulgaris</i>)		

(Continued)

TABLE 1 | Continued

micro RNA (miRNA)	Phosphorus (P)	Nitrogen (N)	Sulfur (S)	Copper (Cu)	Manganese (Mn)	Iron (Fe)	Zinc (Zn)
miR396a	↑ (White lupin) ↓ (Soybean)				↑ (<i>P. vulgaris</i>)		
miR397		↑ (Maize)		↑ (<i>Arabidopsis</i>)		↓ (<i>Arabidopsis</i>)	
miR398a	↑ (<i>Arabidopsis</i> , Tomato)	↑ (<i>Arabidopsis</i> , maize)		↑ (<i>Arabidopsis</i>)		↓ (<i>Arabidopsis</i>)	↑↓ (<i>S. bicolor</i>)
miR398b	↓ (Soybean)					↓ (<i>Arabidopsis</i>)	
miR398c						↓ (<i>Arabidopsis</i>)	
miR398s						↓ (<i>Arabidopsis</i>)	
miR399(a-f)	↑ (<i>Arabidopsis</i> , Wheat, Tomato)	↓↑ (<i>Arabidopsis</i> , maize)	↑ (<i>B. napus</i>)			↓ (<i>Arabidopsis</i>)	↑ (<i>S. bicolor</i>)
miR408	↓ (Wheat)	↑ (Maize)		↑ (<i>Arabidopsis</i>)		↓ (<i>Arabidopsis</i>)	
miR437	↑ (White lupin)						
miR447	↓ (White lupin)						
miR528		↑ (Maize)					↓ (<i>S. bicolor</i>)
miR771	↓ (Tomato)						
miR775	↓ (Tomato)						
miR778	↑ (<i>Arabidopsis</i>)						
miR826		↑ (<i>Arabidopsis</i>)					
miR827	↑ (<i>Arabidopsis</i>)	↓ (Rice, maize)					
miR829		↑ (<i>Arabidopsis</i>)					
miR830	↑ (White lupin)						
miR837-3p	↑ (Tomato)						
miR839		↑ (<i>Arabidopsis</i>)					
miR846		↑ (<i>Arabidopsis</i>)					
miR850		↓ (<i>Arabidopsis</i>)					
miR857	↑ (White lupin)	↓ (<i>Arabidopsis</i>)		↑ (<i>Arabidopsis</i>)			
miR863		↓ (<i>Arabidopsis</i>)					
miR896	↑ (White lupin)						

(Continued)

TABLE 1 | Continued

micro RNA (miRNA)	Phosphorus (P)	Nitrogen (N)	Sulfur (S)	Copper (Cu)	Manganese (Mn)	Iron (Fe)	Zinc (Zn)
miR1122	↑ (Wheat)						
miR1125	↑ (Wheat)						
miR1135	↑ (Wheat)						
miR1136	↑ (Wheat)						
miR1211	↓ (White lupin)						
miR1222	↑ (White Lupin)						
miR1507a	↓ (Soybean)						
miR2111	↑ (Arabidopsis)	↓ (Arabidopsis)		↑ (Arabidopsis)		↓ (Arabidopsis)	

families include several members, such as miR156, miR169, miR171, miR160, miR319, miR826, miR829, miR839, and miR846, whereas miR167, miR172, miR399, miR395, miR850, miR857, miR863, and miR827 are recognized as N-starvation-suppressed (NSS) miRNA group members (Liang et al., 2012; Table 1). Furthermore, 15 and 14 miRNA families have been identified to be responsive in N-limiting conditions in rice and maize, respectively (Xu et al., 2011; Nischal et al., 2012). The miR156 family in *Arabidopsis* has been found at the highest abundance, and miR156h is thought to be the most important among the three members of the miR156 family. On the contrary, miR172 is negatively regulated by miR156 and inhibits the reproductive phase by prolonging the juvenile period. In N-starvation, the induction of miR160 inhibits lateral root development, whereas miR170 hastens the growth of primary roots by targeting auxin response factor (ARF16/17) and SCL6 regulatory proteins, respectively (Liang et al., 2012). In contrast, the perturbation of miR167 biogenesis in N-limiting condition attenuates the expression of ARF6/8, which in turn facilitates the development of lateral and adventitious roots (Jones-Rhoades and Bartel, 2004; Gifford et al., 2008).

In *Medicago truncatula*, miR169 and miR172 play a pivotal role in nodule development by regulating the expression of the *HAP2* and *AP2* genes. The lower abundances of miR169 during N-limitation upregulates *HAP2* gene expression and the subsequent differentiation of nodule primordial by maintaining low N in the roots (Pant et al., 2009). However, different members of the miR169 family, such as miR169a, miR169bc, miR169d-g, and miR169h-n, have been reported in different plant species of *Arabidopsis*, soybean, and maize, etc. They are actively associated with the up-regulation of nitrate transporters during N-starvation (Xu et al., 2011; Zhao et al., 2011, 2012, 2013b; Liang et al., 2012; Wang et al., 2013b). Under low N availability, the expression of miR169d-g has been shown to be increased. miR169d-g exhibits a similar pattern of expression during P and sulfur (S) deficiencies, whereas other members are expressed differently (Liang et al., 2012). Very recently,

miR172 was found to be exclusively expressed in the nodules of soybeans, targeting *Arabidopsis* homologous gene *APETALA2*-related transcription factors in response to P-starvation (Yan et al., 2013).

miRNAs in Sulfur Homeostasis

Sulfur (S), an indispensable inorganic mineral, is mainly taken up by roots in the form of sulfate from the soil. The S is assimilated into cysteine, methionine, glutathione, glucosinolate compounds, and various Fe-S proteins, cofactors, and lipoic acids, which are associated with both primary and secondary metabolism during stress (Rausch and Wachter, 2005). In *Arabidopsis*, sulfate is transported through xylem or phloem via cell-specific transporters such as sulfate transporters1;1 (SULTR1;1), SULTR2;1, and SULTR2;2. The expression of the transporters is predominantly regulated by miR395 depending on S starvation or abundance (Kawashima et al., 2009). Intriguingly, sulfate limitation induces the expression of miR395 and its low affinity sulfate transporter SULTR2;1, in contrast to the inhibitory effect of sulfate deficiency. SULTR2;1 is primarily confined to the xylem parenchyma, whereas miR395 is highly abundant in the phloem parenchyma and plays crucial role in sulfate remobilization between leaves during sulfate deficiency (Liang and Yu, 2010). The restriction of SULTR2;1 expression by miR395 in the xylem parenchyma facilitates the translocation of sulfate ions from the roots to the shoots. In addition, S deficiency leads to the elevated synthesis of SULFUR LIMITATION1 (SLIM1) protein in the roots, which in turn activates various sulfate transporters to enhance S uptake (Liang et al., 2010; Kawashima et al., 2011). The role of miR395 has also been elucidated in S assimilation by suppressing the expression of ATP sulfurylase genes, such as *APS1*, *APS3*, and *APS4*, which catalyze the first step of S assimilation (Matthewman et al., 2012). The expression levels of miR156, miR160, miR164, miR167, miR168, and miR394 are also modulated by S deprivation, as observed in *Brassica napus* (Huang et al., 2010).

miRNAs in Copper Homeostasis

Copper (Cu) is an essential micronutrient that serves primarily as a cofactor of metabolic enzymes and protein complexes in the electron transport chain. It is an integral member of plastocyanin, which actively participates in the electron transport of chloroplast grana during photosynthesis. Cu plays an important role against oxidative stress responses by acting as a cofactor of Copper/Zinc superoxide dismutase (CSD). During Cu limitation, the induction of miR398 down regulates *CSD1*, *CSD2*, and Cu chaperones for superoxide dismutase *SOD1* (*CCS1*) gene expression (Sunkar et al., 2006; Beauclair et al., 2010). *CCS1* is a chaperone protein that delivers the Cu ions to *CSD1* and *CSD2* apoprotein. Under Cu-deficient conditions, Cu/Zn superoxide function is replaced by iron (Fe) superoxide dismutase due to the low availability of Cu. The subunit of cytochrome C oxidase, the inner membrane protein of mitochondria encoded by *CYCOLOOXYGENASE* is also repressed by miR398 expression in *Arabidopsis* (Abdel-Ghany and Pilon, 2008). Other families of miRNAs, such as miR397, miR408, and miR857, have been found to be up regulated during Cu starvation, which in turn suppresses the expression of laccase and plastocyanin genes. The three laccase genes (*LAC*), such as *LAC3*, *LAC12*, and *LAC13*, are down regulated by miR408, and the mRNA of *LAC2*, *LAC4*, and *LAC17* are degraded by miR397. miR857 is primarily responsible for targeting *LAC7* transcripts. Notably, miRNAs related to Cu homeostasis facilitate plastocyanin biosynthesis by reducing the biosynthesis of non-essential Cu enzymes, thus ensuring Cu homeostasis by altering Cu availability among various groups of proteins (Gifford et al., 2008).

miRNA in Other Mineral Homeostasis

Manganese (Mn), Fe, and Zinc (Zn) are essential minerals for plant growth and nutrition. Several miRNAs have been found to be up regulated during Mn starvation and are also associated with other mineral stresses. In *Phaseolus vulgaris*, miR319, miR169, miR396, miR170, miR164, miR390, miR395, miR166, miR172, miR157, miR156, and miR167 are up regulated during Mn toxicity and attenuate the expression of a wide group of genes, including various transcription factors such as *TEOSINTE-LIKE1*, *CYCLOIDEA*, *PROLIFERATING CELL FACTOR1* (*TCP*), *HAPLESS* (*HAP2*), *SCARECROW-LIKE*, *NO APICAL MERISTEM* (*NAC*), *Arabidopsis* transcription activation factor, *CUP SHAPED COTYLEDON*, serine threonine protein kinase, and *APETALA2*, etc. (Valdes-Lopez et al., 2010).

Iron and Zn are indispensable micronutrients for plants, as they are the major cofactors for several key metabolic enzymes, including Fe-S cluster proteins and ferredoxin molecules (Couturier et al., 2013; Forieri et al., 2013). Furthermore, the inadequate amounts of Fe and Zn that are stored in the edible parts of cereal grains play immense roles in human nutrition. The bioavailability of minerals in grains is directly associated with the uptake, transport, and loading of mineral ions (Aung et al., 2013). Recently, the miRNA-mediated regulation of Fe-related transporters and storage proteins were elucidated in *Arabidopsis*. miR398, one of the key activators for *CSD* gene expression during Cu deficiency, is also regulated by Fe deficiency but in an

opposite manner. Fe deficiency reduces the expression of miR397, miR398a, miR398b, miR398c, miR398s, miR399, miR408, and miR2111, whereas Cu deficiency increases their expression and in turn regulates the expression of *CSD1* and *CSD2* (Buhtz et al., 2010; Waters et al., 2012). Therefore, the Cu-Fe interrelationship is another novel finding regarding the study of gene expression during Fe homeostasis. In addition, eight miRNAs from five families, including miR159, miR164, miR172, miR173, and miR394, were previously identified as Fe-responsive families from the small RNA library population in *Arabidopsis*. Intriguingly, the Fe deficiency responsive *cis*-acting elements1 and 2 (*IDE1/IDE2*) were found within the promoters of twenty-four miRNA genes in *Arabidopsis* and resemble the Fe-responsive gene families that are regulated during Fe deficiencies (Kong and Yang, 2010). However, the roles of other miRNAs in Fe transport and storage have not been clearly established.

The Zn deficiency in *Sorghum bicolor* aggravates the upregulation of several miRNA families, such as miR166, miR171, miR172, miR398, miR399, and miR319, which in turn target many gene family members including transporters (Li et al., 2013). Interestingly, two miRNA family members were found to be involved in the regulation of the expression of the *CSD* gene family but in an opposite manner. The upregulation of miR398 reduces the gene expression of *CSD* in the roots, whereas attenuated miR528 elevate the level of *CSD* transcripts in the seeds and the leaves. Furthermore, miRNAs maintain nutrient homeostasis by possessing an endogenous signal for the transport of micro and macronutrients (Liu et al., 2009; Marín-González and Suárez-López, 2012; Kehr, 2013). The up- or downregulation of diverse miRNAs during other metal stresses, such as Al, Cd, Hg, and Cd stress, have also been reported and likely play a role in the adaptive mechanisms of plants by regulating the expression of various stress-related genes (Zeng et al., 2012; Zhou et al., 2012; Zhang et al., 2013).

miRNAs in Systemic Mobility and Long-Distance Transport

After uptake by the roots, nutrients are allocated to the various storage parts of plants following long-distance transport. Xylem and phloem contribute to long-distance transport, and phloem-mediated communication plays an important role during nutrient stress. Phloem not only preserves the source-sink relationship but it also ensures cell-to-cell signal communication during different biotic and abiotic stress responses. Increasing evidence suggests that phloem-specific mRNAs coupled with small RNAs act as signaling molecules in different physiological responses, including nutrient transport under low nutrient conditions in several plant species (Varkonyi-Gasic et al., 2010; Kehr, 2013). Recently, microarrays of *B. napus* have revealed the presence of a specific set of phloem sap-specific miRNAs that are accumulated during S and Cu deficiency and are distinct in the roots, leaves, and inflorescence axis (Buhtz et al., 2010). miR395, which is known as sulfur deficiency responsive-miRNA, accumulates in phloem sap with miR399 and miR2111. miR399

TABLE 2 | Categorization of “conserved” and “unique” miRNAs under different nutrient stress and their predicted target genes.

Nature of miRNA families	miR ID	Frequencies of occurrence under different low/high nutrient conditions	Predicted target genes
Conserved	Highest	miR164	5 (P ¹ , N ² , S ³ , Mn ⁵ , Fe ⁶)
		miR172	5 (P ¹ , N ² , Mn ⁵ , Fe ⁶ , Zn ⁷)
		miR398	5 (P ¹ , N ² , Cu ⁴ , Fe ⁶ , Zn ⁷)
		miR399	5 (P ¹ , N ² , S ³ , Fe ⁶ , Zn ⁷)
	High	miR156, miR167, miR395	4 (P ¹ , N ² , S ³ , Mn ⁵)
		miR319	4 (P ¹ , N ² , Mn ⁵ , Zn ⁷)
		miR408, miR2111	4 (P ¹ , N ² , Cu ⁴ , Fe ⁶)
	Moderate	miR160, miR168	3 (P ¹ , N ² , S ³)
		miR166	3 (P ¹ , N ² , Zn ⁷)
		miR397	3 (N ² , Cu ⁴ , Fe ⁶)
Less		miR857	3 (P ¹ , N ² , Cu ⁴)
		miR158, miR159	2 (P ¹ , Fe ⁶)
		miR169, miR170	2 (N ² , Mn ⁵)
		miR171, miR528	2 (N ² , Zn ⁷)
		miR390, miR396	2 (P ¹ , Mn ⁵)
		miR394	2 (S ³ , Fe ⁶)
		miR827	2 (P ¹ , N ²)
		miR437, miR447, miR771, miR775, miR778, miR830, miR837, miR896, miR1122, miR1125, miR1135, miR1136, miR1211, miR1222, miR1507	P
		miR826, miR829, miR839, miR846, miR850, miR863, miR173	N
			Fe
Unique			PHO2, ARF6, ARF8, AP2
			NLA, ARF6, ARF8, ARF16, ARF18, HAP2, AP2
			Fe–S Cluster Proteins, FERRIDOXIN, Fe transporters

1,2,3,4,5,6,7 denote P, N, S, Cu, Mn, Fe, and Zn-mediated target gene regulation, respectively.

and miR2111, the phosphate starvation responsive miRNAs, have also been found in phloem sap under Cu-deficient conditions (Abdel-Ghany and Pilon, 2008; Pant et al., 2008; Buhtz et al., 2010). miR399d, the member of miRNA399 family, exhibits long-distance transport from the shoots to the roots via phloem, conjugated with small RNA binding proteins, exemplified by *Cucurbita maxima* phloem small RNA binding protein 1 (*CmPSRP1*) and *C. maxima* phloem protein 16 (*CmPP16*; Pant et al., 2009). Furthermore, grafting experiments in the *Arabidopsis* mutant, *hen-1-1*, corroborates the mobility of miR399 and miR395 from the shoots to the roots via phloem, thus transmitting signals during nutrient deficiency. Interestingly, the translocation of miR395 was found to down-regulate only *APS4* but not *APS1* or *AtSULTR2;1*. On the other hand, miR158 in phloem sap appears to play an important role in nutrient transport by targeting lipase and xyloglucan fucosyltransferase genes during Fe deficiency, whereas miR172 was found to play an essential role in tuber

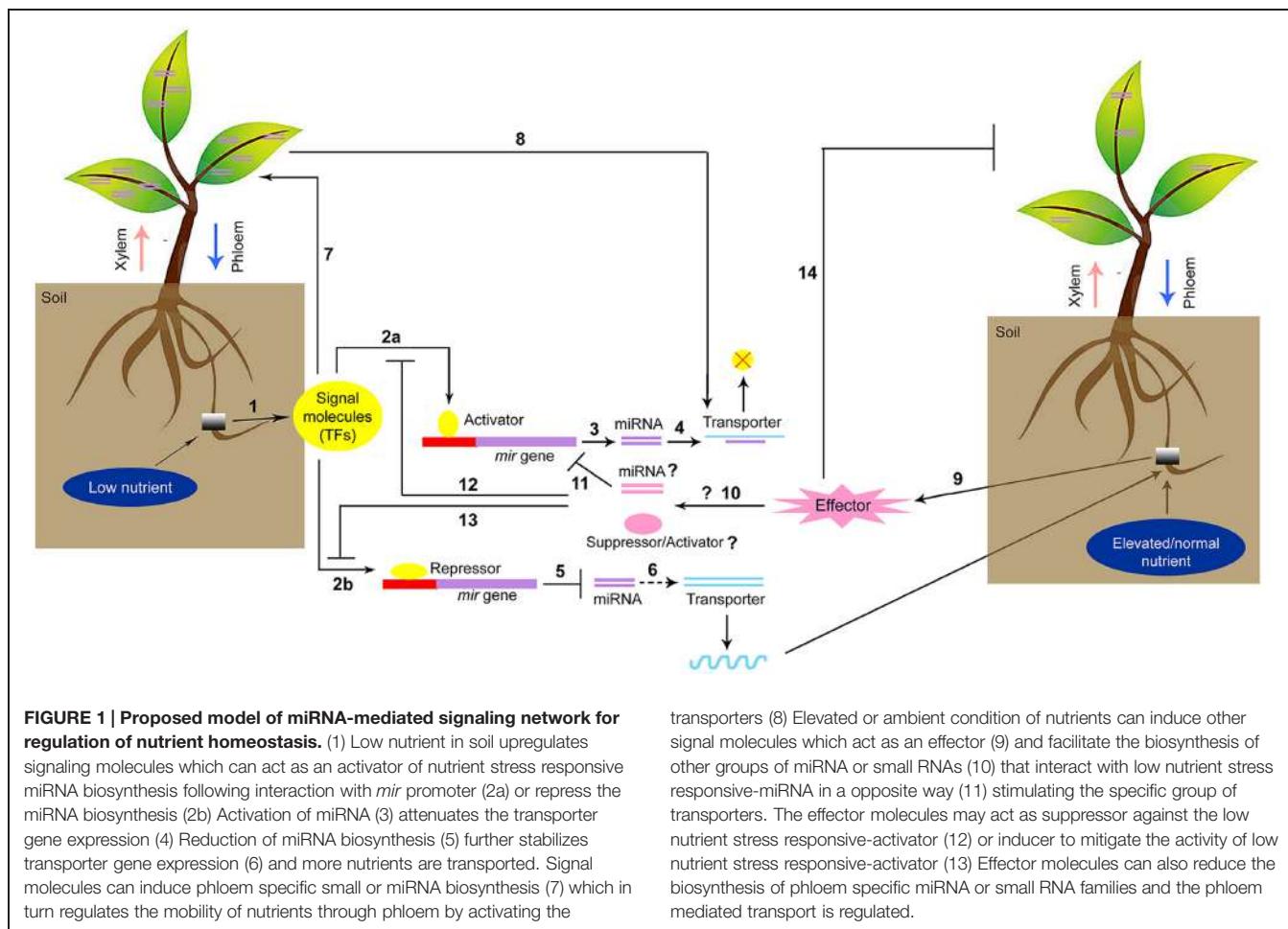
formation and is considered as a phloem-specific signaling intermediate for plant growth and development (Martin et al., 2009; Kasai et al., 2010).

Conserved Groups of miRNAs in Nutrient Homeostasis

microRNAs play a crucial role in nutrient homeostasis by altering gene expression in plants. A single miRNA family has been reported to take part in different nutrient homeostasis conditions, thus playing orchestrated roles as signaling intermediates in several metabolic pathways. For example, two families of miRNAs, the miR169 and miR172 families are exclusively found in nodules and are involved in N, P, and Mn stresses. In addition, elevated levels of miR167 and miR395 during N and S starvation have been reported. These findings can be attributed to the fact that

TABLE 3 | Some probable future strategies for improvement of plant nutrition associated with miRNAs research.

Future strategies	Purpose to be solved
Investigation of novel miRNAs and their role in phytate biosynthesis like regulation of different inositol phosphate kinase genes, alteration of specific miRNA expression by overexpression or genome editing	Alternative approach for combating the phytate barrier in grains to increase the mineral bioavailability
Role of miRNAs in regulation of nitrate transporters and metabolic enzymes such as aspartate amino transferase, glutamine synthase, glutamate dehydrogenase	Improvement of biomass production in crops (since nitrogen and carbon ration is crucial for biomass)
Role of miRNAs in different groups of Fe and Zn transporters from roots to seed, miRNA promoter/ genome editing	Improvement of transport and allocation of Fe and Zn in seeds
Identification of root specific novel miRNAs under nutrient stress and investigation the role of miRNA- mediated miRNA activation or removal of suppressors of transporters	Improvement of nutrient uptake by roots by overexpression of miRNAs
Novel phloem-specific miRNAs under nutrient stress	Studying the signal transduction mechanism during long distance transport, interconnecting relationships among different nutrient transport
Novel miRNA under different nutrient stress	Central signaling role of regulatory network between different metabolic pathways
Role of miRNAs in down-regulation of heavy metal transporters	Development of heavy metals or arsenic tolerant plants by overexpressing specific group of miRNAs



some common transcription factors activated by N and S-stress responses are responsible for the biosynthesis of these two miRNAs. The up-regulation of miR319 and miR396 during N and Mn starvation also supports the hypothesis (Valdes-Lopez et al., 2010). In this review, based on the abundances during various

nutritional stresses, many families of miRNAs can be categorized into four conserved groups (Table 2). In different plant species, miR164, miR172, miR398, and miR399 are involved in the homeostasis of five different nutrients, thus representing the highest conserved group. miR156, miR167, miR395, miR319, miR408,

and miR2111 are classified as highly conserved depending on their up-regulation during the four types of nutrient stress responses. miR160, miR168, miR166, miR397, and miR857 are categorized as moderately conserved (abundance frequencies three times), and miR158, miR159, miR169, miR170, miR171, miR528, miR390, miR396, miR394, and miR827 belong to the least conserved group (twice abundance frequencies). A particular miRNA can regulate different nutritional homeostasis conditions by up- or down-regulating the expression of various target genes (**Table 2**), which suggests a common signaling role of miRNAs in the regulation of diverse nutritional stress responses. Other unique miRNAs also have been identified in some plant species during particular nutrient stress responses, and their roles in the homeostasis of other nutrients should be investigated in the future.

Concluding Remarks and Future Perspectives

Major research endeavors have focused on the genetic regulation of P and N transporters under the respective nutrient stress conditions. Future approaches for miRNA-mediated regulation of nutrient transporters and other metabolic enzymes and their implementation in future biotechnological research are summarized in **Table 3**. Because phytate is an important source of inorganic phosphorus, the role of miRNAs in phosphate metabolism, including inositol phosphate or phytic acid biosynthesis is a promising arena for future research. Nitrate metabolism and the N to C ratio determine the biomass of cereals. The roles of miRNA in the regulation of nitrate transporters and metabolism, including several enzymes such as aspartate amino transferase, glutamine synthase, and glutamate dehydrogenase, should be more extensively investigated. Regarding metal homeostasis, future investigations on differentially expressed-miRNAs and their regulatory roles in various Fe and Zn transporters may aid in the development of a novel platform for Fe and Zn loading in cereal grains. Fe and Zn, two important dietary nutrients, are found only in small amount in the consumable parts of cereal

grains. To increase the content of these metals in milled grain, various biotechnological strategies have been utilized (Paul et al., 2013; Wang et al., 2013a; Borrill et al., 2014; Khan et al., 2014). However, the role of miRNAs in regulating specific transporters or transcription factors in Fe nutrition has not been studied extensively to date. The Fe-related gene regulation mechanism is important for understanding Fe nutrition and may elucidate the clear scenario of gene regulation during nutrient homeostasis.

microRNAs-mediated signal transduction during low/high nutrient stress is a fascinating topic of plant nutrition research. The alteration of nutrient levels in soil can trigger specific signaling molecules that act as repressors of target nutrient responsive-miRNAs. The decreased accumulation of miRNAs subsequently stabilizes the expression of transporters (**Figure 1**). On contrary, the optimal conditions or higher amounts of nutrients can trigger a specific group of miRNAs/small RNAs that directly affect the transporter (as exemplified by the phosphate transporter) or induce other miRNAs that suppress the expression of repressor genes. Therefore, the differential expression of miRNAs and their regulation under nutrient stress provide valuable information. The discovery of phloem-specific novel miRNAs during nutrient starvation and their cell-to-cell transmission will lead to a better understanding of the interrelationship among different nutrients. The identification of promoter regions of specific up- or down-regulated miRNAs that are responsive to micronutrient stresses and the subsequent development of knock-out mutants by inducing mutation in *cis*-acting elements using targeted genome-editing technologies, such as transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeats -CRISPR-associated 9 (CRISPR-Cas9) techniques, may lead to essential crop-improvement strategies in the future.

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Iron deficiency in barley plants: phytosiderophore release, iron translocation, and DNA methylation

Marika Bocchini¹, Maria Luce Bartucca¹, Simona Ciancaleoni¹, Tanja Mimmo², Stefano Cesco², Youry Pii², Emidio Albertini^{1*} and Daniele Del Buono¹

¹ Department of Agricultural, Food and Environmental Sciences, University of Perugia, Perugia, Italy, ² Faculty of Science and Technology, Free University of Bolzano, Bolzano, Italy

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University of Torino, Italy

***Correspondence:**

Emidio Albertini,
Department of Agriculture, Food and
Environmental Sciences, Borgo XX
Giugno 74, 06121 Perugia, Italy
emidio.albertini@unipg.it

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All living organisms require iron (Fe) to carry out many crucial metabolic pathways. Despite its high concentrations in the geosphere, Fe bio-availability to plant roots can be very scarce. To cope with Fe shortage, plants can activate different strategies. For these reasons, we investigated Fe deficient *Hordeum vulgare* L. plants by monitoring growth, phytosiderophores (PS) release, iron content, and translocation, and DNA methylation, with respect to Fe sufficient ones. Reductions of plant growth, roots to shoots Fe translocation, and increases in PS release were found. Experiments on DNA methylation highlighted significant differences between fully and hemi-methylated sequences in Fe deficient plants, with respect to Fe sufficient plants. Eleven DNA bands differently methylated were found in starved plants. Of these, five sequences showed significant alignment to barley genes encoding for a glucosyltransferase, a putative acyl carrier protein, a peroxidase, a β -glucosidase and a transcription factor containing a Homeodomin. A resupply experiment was carried out on starved barley re-fed at 13 days after sowing (DAS), and it showed that plants did not recover after Fe addition. In fact, Fe absorption and root to shoot translocation capacities were impaired. In addition, resupplied barley showed DNA methylation/demethylation patterns very similar to that of barley grown in Fe deprivation. This last finding is very encouraging because it indicates as these variations/modifications could be transmitted to progenies.

Keywords: iron deficiency, barley, phytosiderophores release, DNA methylation, MSAP

Introduction

Iron (Fe) represents an essential nutrient for all organisms, due to its fundamental role in numerous cellular processes and functions. Its deficiency is a very serious problem for human nutrition (Hell and Stephan, 2003). For instance, in 1995 it has been documented that Fe deficiency was affecting almost 4 billion people worldwide, whilst in 2002 it has been estimated that almost 30% of human population was anemic (Hell and Stephan, 2003). The occurrence of Fe deficit in humans is mainly caused by the consumption of food with low Fe levels. An inadequate Fe intake from diet can lead to varying degrees of deficiency (Kobayashi and Nishizawa, 2012). Crops are the major source of Fe to humans and animals (Hell and Stephan, 2003).

Despite its usually high abundance in the geosphere, Fe is characterized by a scarce solubility in soils, which restricts its availability to plant roots (Mimmo et al., 2014). In particular, the Fe(III) solubility decreases strongly at increasing pH values of soils. This is the consequence

of hydrolysis (as Fe-oxohydroxides), polymerization and precipitation reactions (Neilands et al., 1987). These processes can reduce the levels of Fe in soils below those required for an adequate plant growth. The alteration of Fe content of plants is of great importance, since Fe is one of the most yield and quality limiting crop nutrient in the world (Schachtman et al., 1998).

In order to assimilate enough Fe, plants regulate its absorption in response to its availability in soils (Römhild and Marschner, 1986). In fact, *Strategy I* plants improve Fe uptake, acidifying the rhizosphere, by excreting protons by a plasma membrane H⁺-ATPase (Hell and Stephan, 2003).

Thereafter, Fe is reduced at the root surface to Fe(II), through a ferric-chelate reductase, and taken up by a specific Fe transporter (Hell and Stephan, 2003). Differently, *Strategy II* plants, which comprises grasses and graminaceous species (Hell and Stephan, 2003), base their capacity to take up Fe on the release of phytosiderophores (PSs), which are organic compounds with a strong chelation affinity for Fe(III). The Fe-PS complex is then transported into root cells through a high affinity uptake system (Curie et al., 2001; Inoue et al., 2009). PSs belong to the family of mugineic acids (MA), which are biosynthesized starting from the amino acid methionine and then converted into nicotianamine (NA). NA is one of the most important specific Fe chelators operating in the cells. Successively, the deamination of NA leads to oxyMA, which is then hydroxylated to MA (Takahashi et al., 1999). The concentration and kinds of PSs released by plants into the rhizosphere differ between plant species (Mori, 1999). In barley PSs are released with a diurnal trend, showing a peak in the morning (Takagi et al., 1984). Once entered into the cells, Fe is compartmentalized for its uses, but also to avoid an excessive accumulation, which can even lead to cytotoxicity. The highest concentration of Fe is in chloroplasts, for photosynthetic purposes, and in mitochondria in order to carry out the cellular respiration (Mimmo et al., 2014).

Generally, plants modulate gene expression either to cope with environmental changes or in order to counteract biotic and abiotic stresses. The induction of Fe acquisition-related genes under Fe shortage is particularly significant for both *Strategy I* and *II* plants (Hell and Stephan, 2003). Certainly, the purpose of these metabolic regulatory mechanisms is to achieve an adequate supply of this nutrient. Nonetheless, also a number of unexpected proteins and genes, whose exact role is still unknown, respond under Fe deficiency. For example, genes encoding factors that sense intracellular levels of Fe, transcriptional activators for regulating gene expression in response to Fe-deficiency, and components of signaling pathways to monitor Fe status in the environment, have not yet been identified (Negishi et al., 2002).

Although environmental conditions like soil, light, temperature, and microbial activity have been reported to influence Fe uptake and storage (Kokot and Phuong, 1999; Lueders and Friedrich, 2000; Vansuyt et al., 2000), genetic regulatory factors, such as DNA methylation and co-suppression may also play a role (Finnegan et al., 2000; Meins, 2000). In this regard, cytosine methylation may play an integral role in the regulation of gene expression at both the transcriptional and post-transcriptional levels. Specifically, DNA methylation results in the conversion of the cytosine to N4- or N5-methylcytosine or

of the adenine to N6-methyladenine. Changes on the methylation status of these cytosine residues in genomic DNA play a pivotal role in the regulation of genome functions (Causevic et al., 2005). Generally, hypermethylation is correlated with gene silencing, while hypomethylation is connected with active transcription (Paszkowski and Whitham, 2001). In addition, Zhang et al. (2006) suggest that body-methylated genes are constitutively expressed at a higher level, whereas promoter-methylated genes tend to be expressed in a tissue-specific manner. DNA methylation is very sensitive to different stresses and indicate how plants adapt themselves to cope with these situations. Several previous researches have demonstrated that cytosine methylation categorically plays important role in regulating various biotic and abiotic stresses such as low temperature (Steward et al., 2000), water deficiency (Labra et al., 2002), bacteria blight (Sha et al., 2005), ion implantation (Yu et al., 2011), hybridization (Hegarty et al., 2011), heavy metals (Aina et al., 2004), salt stress (Marconi et al., 2013), low nutrients (Kou et al., 2011), and tissue culture (Gao et al., 2010; Dann and Wilson, 2011). Nevertheless, scanty genome-wide DNA methylation information is available for *Hordeum vulgare* L. Barley DNA methylation was investigated by Shan et al. (2012) and Smith et al. (2014), but to the best of our knowledge, there is no information regarding barley methylation related to mineral nutrient deficiency/stress. Therefore, a study on methylation patterns in such an important crop as *H. vulgare* under Fe deficiency stress becomes relevant to support knowledge about general genome methylation in this species and regarding the interaction between change in DNA methylation and Fe stress.

On the basis of these assumptions, in the present study we investigated Fe deficient and sufficient barley plants. We chose this species since it is an important crop, and a *Strategy II* plant, which releases PS under Fe shortage. We investigated the effect of Fe deprivation on plant growth, chlorophyll content, on the concentrations of PS released by roots, the Fe content in shoots and roots and the changes in the DNA methylation status caused by the Fe deprivation. To better evidence the plant responses, the above parameters were also assessed in Fe deficient barley plants resupplied with Fe.

Materials and Methods

Plant Material and Growth Conditions

Barley (*Hordeum vulgare* L. research line Europa) seeds were placed in Petri dishes and added of ultrapure water. Four days later, the seedlings were positioned and grown in continuously aerated hydroponic solutions (12/12 h of light/dark, 23/19°C) composed as follows: 2 mM Ca(NO₃)₂ × 4H₂O, 0.5 mM MgSO₄ × 7H₂O, 0.7 mM K₂SO₄, 0.1 mM KCl, 0.1 mM KH₂PO₄, 1 μM H₃BO₃, 0.5 μM MnSO₄ × H₂O, 0.5 μM CuSO₄, 0.5 μM ZnSO₄ × 7H₂O, 0.01 μM and (NH₄)₆Mo₇O₂₄ × 4H₂O and ± 100 μM Fe-EDTA to carry out experiments ±Fe deprivation. The nutrient solutions, which had a pH of 6.0 independently to the presence of Fe-EDTA, were renewed every 4 days.

Shoots and roots length together with the chlorophyll concentration (SPAD measurements—SPAD-502 Plus, Konica Minolta, Japan), were assessed on seedlings starting from 9 days

after sowing (DAS). SPAD measurements were taken on the first leaf of each plant, 5–10 cm from the bottom, midway between the midrib and the leaf margin. The measurements were then transformed into chlorophyll content (Markwell et al., 1995).

Resupply Experiments

Barley plants were grown in Fe deficiency as described above. At 13 DAS, a subset of Fe deficient plants were subjected to Fe resupply by adding Fe-EDTA (100 µM) to the nutrient solution. Plants were harvested at 2, 4, and 6 days after this treatment for subsequent analyses. Studies on the DNA methylation status were carried out on plants harvested at 2 and 6 days after Fe resupply.

Root Exudates Collection and PS Quantification

Barley plants were picked up at different times after sowing and submitted to the procedure for the determination of the amount of PS released (Shenker et al., 1995). Briefly, root exudates were collected from plants in the morning, 2 h after the beginning of the photoperiod. After accurate washing of the roots, 3 plants per sample were placed into beakers containing 20 mL of ultrapure water. Roots exudates were collected for 5 h under continuous aeration. The concentration of PS exuded was then quantified colorimetrically using the Cu-CAS assay (Shenker et al., 1995).

Determination of Shoot and Root Fe Concentration

Barley plants were harvested and then roots and shoots were separated, weighed, and oven-dried at 60°C until constant weight was reached. Plant tissues were microwave digested with concentrated nitric acid (65% v/v, Carlo Erba) using a single reaction chamber (SRC, Ultra WAVE, Milestone Inc, Shelton, CT, USA). Fe concentration was then determined by Inductively Coupled Plasma—Optical Emission Spectroscopy (ICP-OES, SpectroCirosCCD, Spectro, Germany).

DNA Extraction and Methylation Sensitive Amplified Polymorphism (MSAP) Analysis

The MSAP protocol was applied according to Marconi et al. (2013). Briefly, genomic DNA was extracted from three Fe-sufficient and three Fe-deficient barley samples, collected at 9, 13, 15, and 19 DAS, as well as from three Fe resupplied samples collected at 2 and 6 days after the nutrient addition, (hereafter named as 15R and 19R) using the DNeasy Plant Mini Kit (Qiagen). For each sample, 300 ng of the genomic DNA was incubated for 4 h at 37°C in a 45 µl mix containing 5 units EcoRI, and 5 units *Hpa*II, 1X Restriction-Ligation buffer (1X NEB Buffer 4, New England Biolabs, added with 0.1 M DTT and 250 ng BSA), 50 pmol *Hpa*II adapter, 50 pmol EcoRI adapter, 10 mmol ATP, and 1 unit T4 DNA Ligase. The reaction was stopped by incubation at 65°C for 10 min and then diluted 10 times in 0.1X TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8). The second digestion/ligation reaction was carried out in the same way, except that *Msp*I was used instead of *Hpa*II. Two consecutive PCRs were performed to selectively amplify the EcoRI-*Hpa*II and EcoRI-*Msp*I fragments. The pre-selective amplification was performed using 5 µl of the above-mentioned diluted mixture, which was added to a 45 µl mix.

Selective amplifications of the diluted pre-selective amplified products was carried out. For each reaction, 5 µl of 1:10 diluted pre-selective amplified samples was added to 15 µl selective amplification mix, using a total of 8 primer combinations (Table S1) in a final volume of 20 µl, using the same temperature profile used for the pre-selection step. One µl of each amplified sample was added to 10 µl of formamide and to 0.3 µl of size standard (Genescan LIZ 500, Life technologies). After denaturation (94°C for 5 min) amplified fragments were separated with the ABI 3130xl Genetic Analyzer (Life Technologies).

As described in Marconi et al. (2013) amplified fragments were divided into four types based on the presence or absence of bands, which resulted from the differential sensitivity of the fragments to digestion by *Msp*I and *Hpa*II.

Silver Staining and DNA Sequences of Fe-stress-related Fragments

Some samples, which were chosen on the basis of interesting polymorphisms, were run on acrylamide gels and silver stained with the aim of isolating and sequencing the selected bands. Following Marconi et al. (2013), 2.5 µL of selected samples were added to 1X formamide dye and denatured. After denaturation, samples were loaded onto a 5% denaturing polyacrylamide gel, and run for 4–4:30 h at 55 W at 38°C. Gels were then silver stained. The gels were fixed in 10% acetic acid, washed three times with ultrapure water for 2 min, transferred to a silver solution (1.5 g/L AgNO₃, 0.056% formaldehyde) for 30 min, and then rinsed 1 time with ultrapure water. Image development was carried out with agitation for 8–10 min in developer solution. To stop the development and to fix the gel, 10% acetic acid was added directly to the developing solution and incubated with shaking for 3–5 min. The gel was dried at room temperature.

A total of 16 interesting polymorphic bands were excised from gels, rehydrated with 200 µL of ultrapure water o/n at 4°C. Tubes were centrifuged at 10,000 g for 10 min, and the supernatant transferred into a fresh tube. Aliquots of 6 µl were used as template for re-amplification by PCR in a 16 µL reaction volume using Type-it Microsatellite PCR Kit (Qiagen). All PCR reactions were carried out with the same primer combinations used in pre-selective amplification step with the following profile: 94°C for 1 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, ending with a 20 min extension step at 72°C.

One µl of the re-amplified DNA was cloned into a pCR4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen). Three plasmid DNAs for each transformation were purified from 5 ml of overnight cultures of *Escherichia coli* in LB medium using the GenElute Plasmid miniprep kit (Sigma). The sequences of both strands of each plasmid were determined after running sequencing reactions (obtained with BigDye Terminator v3.1 Cycle Sequencing Kit, Life Technologies) on an ABI 3130xl Genetic Analyzer sequencer.

Statistical Analysis

Each reported value represents the mean ± standard deviation (SD) of data from four independent experiments on at least three biological replicates per experiment. Shoot and root length

and plant weights were assessed using 20 replicates. Statistical analyses of data were carried out by ANOVA tests and significant differences were established by Duncan's tests at $P < 0.05$. Finally, chi-square was used to test the independence between methylation level and Fe deprivation (stress) condition, using SAS Version 9.2 (SAS Institute, Cary, NC). Student's *t*-test was also performed using SAS software.

Results

Length and Weight of Shoots and Roots, and Chlorophyll Content in Barley Plants \pm Fe

Iron shortage affected the growth of seedlings of barley starting from 15 DAS. In fact, significant reductions in both shoots and roots length and weight were observed (Table 1). In particular, at 15 and 19 DAS, the shoot length of the Fe deficient barley plants was reduced by 12.0 and 20.7%, respectively, with respect to the Fe sufficient samples. Also shoot fresh weight (FW) was significantly affected by Fe shortage. In fact, reductions in shoot FW of 22.2 and 36.0% were found at 15 and 19 DAS, respectively. A similar negative effect occurred to the root length that was reduced by 17.2%, at 19 DAS. Finally, the nutrient privation also caused a 14.2% reduction in the root FW at 19 days after the beginning of the Fe shortage treatment.

Changes in chlorophyll contents in response to the Fe starvation were assessed in Fe deficient leaves of barley by a SPAD meter (Figure 1). The leaf chlorophyll level was significantly reduced in the Fe deficient plants at 13, 15, and 19 days after this nutrient deprivation and the reductions were of 16.3, 27.4, and 34.5%, respectively.

Phytosiderophores Release

Root exudates were collected from barley plants grown under Fe deficiency in hydroponic solutions. The quantification of exudates was started 8 DAS, but, at this time, the concentration

of these organic compounds was scarce and far below the limit of detection of the method ($30 \mu\text{M}$). Starting from 9 DAS, Fe deficient plants began to exude higher concentrations of PSs, and this exudation pattern was found to be quite constant until 12 DAS (Figure 2). On the other hand, starting from 13 DAS the amount of extruded PSs by the plants strongly increased. In particular, at 13, 15, and 19 DAS Fe deficient barley exuded 1.03, 1.00, and $1.10 \mu\text{mol g}^{-1}$ RFW, respectively.

Fe Concentration in Barley Plants

Iron concentration was determined in both shoots and roots of barley plants grown in either a complete or Fe-free hydroponic nutrient solution (Table 2). As expected, Fe deprivation strongly affected the Fe content in barley and its distribution between shoots and roots (Table 2). The differences between Fe content deficient and sufficient plants were found to be significant already at 8 DAS. In particular, Fe sufficient barley showed a total Fe content (shoots + roots) of $19.49 \mu\text{g g}^{-1}$ FW, while Fe deficient barley samples evidenced a total Fe content of $14.37 \mu\text{g g}^{-1}$ FW. The Fe root to shoot ratio was found to be significantly higher in Fe fed barley and reached values of 2.15, 3.55, 9.48, 9.70, and 16.92, at 8, 9, 13, 15, and 19 DAS, respectively. This ratio was found to be decreased in Fe deficient barley plants already at 8 DAS (Table 2), if compared to Fe sufficient plants, and reached the values of 1.13, 1.08, 1.09, 1.61, and 2.00 at 9, 13, 15, and 19 DAS, respectively (Table 2).

Chlorophyll and Fe Concentrations in Barley Shoots and Roots after Fe Resupply

Iron deficient barley plants were resupplied with $100 \mu\text{M}$ Fe-EDTA at 13 DAS. The monitoring of chlorophylls evidenced that leaves of Fe-resupplied plants reached the SPAD values recorded in control plants over a period of 6 days (Figure 3). In particular, the chlorophyll recovery became evident at 4 days after the resupply (Figure 3).

TABLE 1 | Length and fresh weight (FW) of shoots and roots of barley plants grown under Fe sufficiency (+Fe) and Fe-deficiency conditions (-Fe), at 9, 12, 13, 15, and 19 DAS.

	DAS (days after sowing)				
	9	12	13	15	19
SHOOT LENGTH (CM)					
+Fe	$15.30 \pm 1.36\text{a}$	$18.63 \pm 1.32\text{a}$	$20.20 \pm 1.74\text{a}$	$24.87 \pm 1.70\text{a}$	$32.07 \pm 2.24\text{a}$
-Fe	$14.47 \pm 1.52\text{a}$	$17.43 \pm 1.50\text{a}$	$18.75 \pm 1.57\text{a}$	$21.87 \pm 1.38\text{b}$	$25.42 \pm 2.06\text{b}$
SHOOT FW (G)					
+Fe	$0.18 \pm 0.03\text{a}$	$0.25 \pm 0.04\text{a}$	$0.24 \pm 0.03\text{a}$	$0.27 \pm 0.03\text{a}$	$0.39 \pm 0.08\text{a}$
-Fe	$0.16 \pm 0.04\text{a}$	$0.19 \pm 0.06\text{a}$	$0.21 \pm 0.02\text{a}$	$0.21 \pm 0.03\text{b}$	$0.25 \pm 0.03\text{b}$
ROOT LENGTH (CM)					
+Fe	$13.00 \pm 1.41\text{a}$	$17.30 \pm 2.84\text{a}$	$18.65 \pm 3.16\text{a}$	$19.07 \pm 2.18\text{a}$	$24.13 \pm 2.29\text{a}$
-Fe	$11.30 \pm 2.16\text{a}$	$14.57 \pm 2.68\text{a}$	$15.55 \pm 2.64\text{a}$	$17.67 \pm 2.17\text{a}$	$19.97 \pm 2.41\text{b}$
ROOT FW (G)					
+Fe	$0.11 \pm 0.02\text{a}$	$0.12 \pm 0.01\text{a}$	$0.11 \pm 0.02\text{a}$	$0.12 \pm 0.01\text{a}$	$0.14 \pm 0.01\text{a}$
-Fe	$0.09 \pm 0.01\text{a}$	$0.11 \pm 0.01\text{a}$	$0.11 \pm 0.01\text{a}$	$0.12 \pm 0.01\text{a}$	$0.12 \pm 0.01\text{b}$

Data are means \pm SD ($n \leq 20$). For each column, means followed by different letters are significantly different at $P = 0.05$.

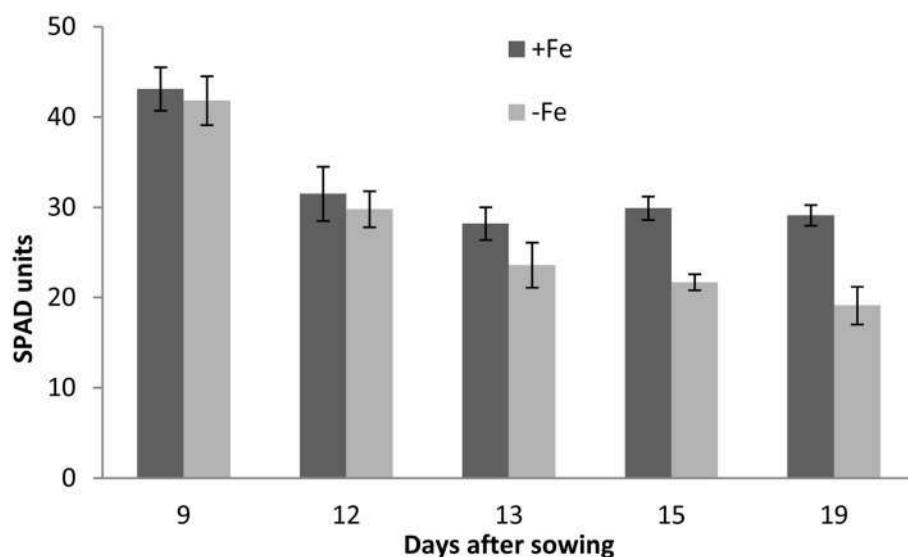


FIGURE 1 | Chlorophyll concentration in barley shoots grown under Fe sufficiency (+Fe) and Fe-deficiency (-Fe) conditions, at 9, 12, 13, 15, and 19 DAS. At 9 DAS the SPAD was measured at the first leaves, thereafter, it was recorded on the second leaves.

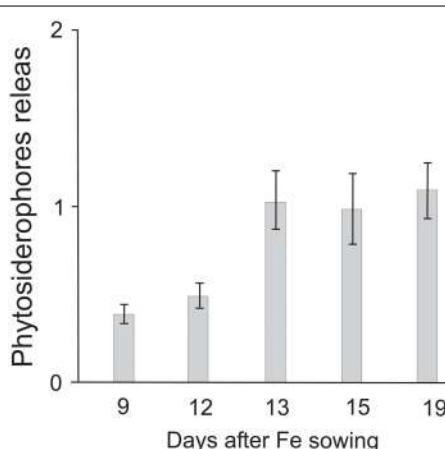


FIGURE 2 | Phytosiderophores release ($\mu\text{mol g}^{-1}$ RFW) from barley plants grown under Fe-deficiency at 9, 12, 13, 15, and 19 DAS.

Regarding the Fe concentrations, the resupply experiments (**Table 3**) evidenced, at 2 days after this nutrient addition, some increases in the Fe root to shoot ratio (2.59). The recovery of Fe resupplied plants continued until 6 days after this nutrient addition, when the root to shoot ratio reached the value of 8.87 (**Table 3**). Nonetheless, these samples did not reach the values exhibited by Fe sufficient controls (**Table 2**).

Extent and Pattern of DNA Methylation under Control Conditions and Fe Deficiency

Eight primer combinations (Table S1) were used to assay cytosine methylation at 5'-CCGG-3' sequences in the Europa research line of *H. vulgare*, at different times after sowing, in

Fe deficient/sufficient plants. Samples were collected from three plants grown \pm Fe at 7, 9, 13, 15, and 19 DAS. In addition, for the MSAP analysis, other samples were collected at 2 and 6 days after Fe-EDTA100 μM resupply (hereafter named 15R and 19R). 7 DAS samples were collected before treatment and therefore there are not stressed samples. They represented our T0.

A total of 563 clear and reproducible bands were amplified from plants growth \pm Fe. Under the 7, 9, 13, 15, and 19 DAS control conditions, the total methylation of CCGG sequences averaged 61.28, 62.34, 60.92, 62.17, and 62.88% respectively, while the extent of DNA methylation ranged from 59.33 (9 DAS) to 61.99% (13 DAS) in stressed samples (**Table 4**). In particular, when compared with the respective Fe well-fed controls, DNA methylation levels in Fe deficient samples decreased (-3.02% in 9 DAS, -2.66% in 15 DAS and -1.24% in 19 DAS); the only exception was at 13 DAS when an increase of 1.07% in DNA methylation was observed.

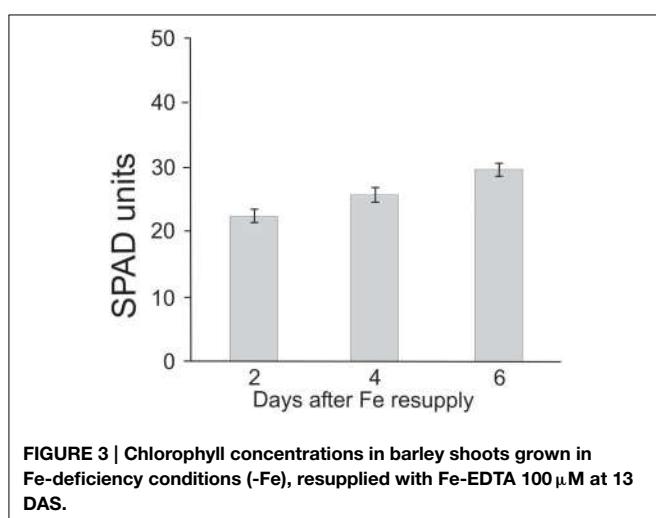
In addition, all samples showed a level of full methylation higher of hemi-methylation (**Table 4**). Fe deficiency caused a general decrease of fully methylated bands (-3.20 , -0.53 , -1.24 , and -4.26% at 9, 13, 15, and 19 DAS, respectively) with respect to control samples. In contrast the level of hemi-methylated amplicons in Fe-deficient samples increased ($+0.18$, $+1.6$, and $+3.02\%$) at 9, 13, and 19 DAS, respectively when compared with Fe sufficient samples. The only exception was at 15 DAS when a decrease of 1.42% was observed.

The average level of DNA methylation (60.2%) after the Fe resupply was comparable to that of samples grown under Fe deficiency. The relative abundances of fully methylated bands did not change during the recovery, if compared with -Fe sufficient samples (average of 46.35 and 46.53% in 15R and 19R vs. 47.25% in 15S and 47.07% in 19S).

TABLE 2 | Iron concentration in shoots and roots of barley plants grown in iron sufficiency (+Fe) and iron deficiency (-Fe) conditions, at 8, 13, 15, and 19 DAS.

DAS	[Fe] _{shoots} ($\mu\text{g g}^{-1}$ FW)		[Fe] _{roots} ($\mu\text{g g}^{-1}$ FW)		[Fe] _{roots/shoots} (ratio)	
	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe
8	6.18 ± 0.43a	6.45 ± 0.65a	13.31 ± 1.23a	7.92 ± 1.64b	2.15	1.13
9	6.05 ± 0.33a	5.24 ± 0.48a	21.52 ± 2.67a	5.65 ± 1.18b	3.55	1.08
13	6.19 ± 0.62a	4.28 ± 0.52b	58.71 ± 5.55a	4.68 ± 0.25b	9.48	1.09
15	5.79 ± 1.39a	3.33 ± 0.32b	56.18 ± 2.55a	5.37 ± 0.50b	9.70	1.61
19	5.50 ± 1.125a	2.81 ± 0.50b	93.07 ± 4.30a	5.46 ± 0.28b	16.92	2.00

Data are means ± SD ($n = 20$). Means within the same row followed by the same letter are not significantly different from the relative +Fe controls.

**FIGURE 3 | Chlorophyll concentrations in barley shoots grown in Fe-deficiency conditions (-Fe), resupplied with Fe-EDTA 100 μM at 13 DAS.****TABLE 3 | Iron concentration in shoots and roots of barley plants grown in iron deficiency (-Fe) conditions, resupplied with 100 μM Fe-EDTA at 13 DAS.**

Days after resupply	[Fe] _{shoots} ($\mu\text{g g}^{-1}$ FW)	[Fe] _{roots} ($\mu\text{g g}^{-1}$ FW)	[Fe] _{roots/shoots} (ratio)
2	3.41 ± 0.16	8.82 ± 0.15	2.59
4	3.49 ± 0.13	23.73 ± 3.25	6.80
6	3.88 ± 0.19	34.42 ± 2.74	8.87

Data are means ± SD ($n = 3$).

Effect of Fe Deficiency on the Level of Methylation in Barley

Consistently with the approach used by Marconi et al. (2013), all possible banding patterns between control and Fe deprived barley plants at 9, 13, 15, and 19 DAS were compared for identifying the changes in cytosine methylation patterns under Fe shortage. Sixteen banding patterns were apparent from the MSAP analysis (Table 5). Patterns A–D represent monomorphic classes in which the methylation pattern is the same following either the control or the Fe-deprived samples. Patterns E–J are indicative of cytosine demethylation, whereas possible cytosine methylation events induced by Fe deficiency are represented by patterns K–P.

Methylation at 462 (82.06% of total sites), 510 (90.59% of total sites), 493 (87.57% of total sites), and 506 (89.88% of total sites) CCGG sites remained unchanged after the imposition of Fe deficiency at 9, 13, 15, and 19 DAS, respectively (Table 5).

Under Fe shortage, demethylation was observed for 74 (13.1%) 27 (4.8%), 43 (7.6%), and 43 (7.6%) CCGG sites at 9, 13, 15, and 19 DAS, respectively (Table 5), highlighting a higher level of DNA demethylation soon after the Fe deprivation (Table 5). Methylation due to Fe shortage was generally less abundant accounting for 27 (4.8%), 26 (4.62%), 27 (2.49%), and 14 (4.8%) at 9, 13, 15, and 19 DAS, respectively (Table 5). Therefore, Fe deficiency caused more DNA demethylation rather than DNA methylation (Table 5).

Alteration of DNA Methylation Pattern under Fe Deprivation and after Subsequent Resupply

To identify the DNA methylation changes (i.e., demethylation or methylation under Fe deprivation and subsequent resupply), we classified all differentially methylated DNA fragments into various classes. As indicated in Table 6, the a, b, and c classes include bands with DNA demethylation induced by Fe deficiency; the d, e, and f classes comprise methylated DNA fragments induced by Fe shortage; and the g and h classes included DNA fragments for which Fe stress had no effect on their methylation status. The majority of bands (83.65 and 84.66%) remained unchanged (class h) in the Fe resupplied barley plants at 2 (15R) and 6 (19R) days after the Fe addition. As many as 24 (61.54%) and 27 (69.23%) out of 39 demethylated DNA bands remained hypomethylated (class b), whereas 13 (33.3%) and 12 (30.77%) out of 39 demethylated DNA bands were remethylated (class a) after resupply, at 2 (15R) and 6 (19R) days after the Fe addition, respectively (Table 6). Only few bands (2 and 0 at 15R and 19R samples) were found to belong to class c (demethylated by Fe deprivation but re-methylated with a different pattern after resupply).

A similar behavior was observed in 15R and 19R samples in terms of the methylated DNA fragments induced by Fe deficiency (Table 6). For both samples, resupply affected the methylation status of few of the fragments subject to Fe-induced DNA methylation. In fact, only 9 (33.33%) and 4 (30.77%) out of 27 and 13 bands were found to belong to d class (i.e., methylated by Fe deprivation, but demethylated after

TABLE 4 | DNA methylation changes at 9, 13, 15, and 19 DAS of barley under Fe deprivation.

MSAP band type	Control					Fe deprived				Fe resupplied	
	7	9	13	15	19	9	13	15	19	15R	19R
I	218	212	220	213	209	229	214	228	216	223	225
II	62	71	71	77	65	72	80	69	82	79	76
III	209	210	228	223	234	222	233	224	232	224	219
IV	74	70	44	50	55	40	36	42	33	37	43
Tot. Amplified bands	563	563	563	563	563	563	563	563	563	563	563
Tot. methylated bands ^a	345	351	343	350	354	334	349	335	347	340	338
Fully methylated bands ^b	283	280	272	273	289	262	269	266	265	261	262
Hemi-methylated bands ^c	62	71	71	77	65	72	80	69	82	79	76
MSAP (%) ^d	61.3	62.3	60.9	62.2	62.9	59.3	62.0	59.5	61.6	60.4	60.0
Fully methylated ratio (%) ^e	50.3	49.7	48.3	48.5	51.33	46.5	47.8	47.2	47.1	46.4	46.5
Hemi-methylated ratio (%) ^f	11.0	12.6	12.6	13.7	11.5	12.8	14.2	12.3	14.6	14.0	13.5

^a(I+III+IV).^b(III+IV).^c(II).^dMSAP (%) = [(II+III+IV)/(I+II+III+IV)] × 100.^eFully methylated ratio (%) = [(III+IV)/(I+II+III+IV)] × 100.^fHemi-methylated bands (%) = [(II)/(I+II+III+IV)] × 100.

Type I indicated absence of methylation due to the presence of bands in both EcoRI/HpaII and EcoRI/MspI digest; type II bands appeared only in EcoRI/HpaII digestion but not in the EcoRI/MspI digest; type III generated bands obtained in EcoRI/MspI digest but not in the EcoRI/HpaII digest; and type IV represents the absence of band in both enzyme combinations.

TABLE 5 | Analysis of DNA methylation patterns in barley plants under Fe deprivation compared with plants well-fed with iron.

Pattern ^a	Class	H ₂ O		Fe-/Resup		Fe deprived				Fe resupplied	
		HpaII	MspI	HpaII	MspI	9	13	15	19	15R	19R
No change	A	1	0	1	0	52	63	56	62	60	55
	B	0	1	0	1	187	217	207	217	210	209
	C	1	1	1	1	196	205	207	197	205	198
	D	0	0	0	0	27	25	23	30	19	29
	Total					462 (82.1%)	510 (90.6%)	493 (87.6%)	506 (89.9%)	494 (87.7%)	491 (87.2%)
Demethylation	E	1	0	1	1	10	0	4	2	3	6
	F	0	1	1	1	19	8	12	15	9	16
	G	0	0	1	1	4	1	5	2	6	5
	H	0	1	1	0	2	0	0	1	0	1
	I	0	0	1	0	13	15	12	18	19	19
	J	0	0	0	1	26	3	10	5	6	2
Methylation	Total					74 (13.1%)	27 (4.8%)	43 (7.6%)	43 (7.6%)	43 (7.6%)	49 (8.7%)
	K	1	1	1	0	5	2	1	1	0	1
	L	1	1	0	1	8	12	5	10	8	8
	M	1	1	0	0	3	1	0	1	0	2
	N	1	0	0	1	1	1	2	0	0	0
	O	1	0	0	0	8	7	15	1	14	4
	P	0	1	0	0	2	3	4	1	4	8
	Total					27 (4.8%)	26 (4.6%)	27 (4.8%)	14 (2.5%)	26 (4.6%)	23 (4.1%)

^a1, band present in all stages; 0, band absent in all stages.

resupply) at 15R and 19R, whereas the vast majority of them remained unchanged after resupply from Fe deprivation (62.96% and 61.53%, class e). Only one band of class f (methylated

by Fe deprivation and demethylated with a different pattern after resupply) for was observed for both resupplied samples (15R and 19R).

TABLE 6 | Summary of the changes in the DNA methylation patterns in barley after 2 (15R) and 6 (19R) days of resupply.

Band class*	a	b	c	a+b+c	d	e	f	d+e+f	g	h	i	Total
Recovery 15R	13	24	2	39	9	17	1	27	22	471	4	563
Recovery 19R	12	27	0	39	4	8	1	13	31	475	5	563

*(a) demethylated by Fe deprivation, but remethylated after recovery; (b) demethylated by Fe deprivation, and remaining hypomethylated after recovery; (c) demethylated by Fe deprivation but re-methylated in a different pattern after recovery; (d) methylated by Fe deprivation, but demethylated after recovery; (e) methylated by Fe deprivation, and remaining methylated after recovery; (f) methylated by Fe deprivation, but demethylated in a different pattern after recovery; (g) DNA methylation pattern remained unchanged under Fe deprivation, but changed after recovery; (h) DNA methylation pattern was unchanged under Fe deprivation, and remained unchanged after recovery; (i) others.

Sequencing and Bioinformatics Analysis of Methylated DNA Fragments

Sixteen differentially methylated DNA bands were cloned and sequenced. The resulting sequences were blasted against the databases at NCBI, IPK, Gramene and Uniprot websites. Five fragments' sequences were too short (between 40 and 70 bp) and resulted in no similarities. As shown in **Table 7**, the remaining 11 sequences (82–340 bp, with an average of 220 bp) scored when aligned to plant databases.

Six sequences aligned well with at least one plant database sequence (*e*-value lower than 0.05) (i.e., Hv_01, Hv_03, Hv_06, Hv_07, Hv_09, and Hv_11); among these, five sequences were significantly associated with *H. vulgare* genes; (i) one (accession number AK373414) encodes for a glucosyltransferase; (ii) one (Uniprot accession number MOX2R2) encodes for an acyl carrier protein; (iii) a third one (accession number AK375528) gives a peroxidase protein; (iv) one (accession number EF067844.1) encodes a Homeobox-leucine zipper protein HOX1; and (v) the fifth sequence score well with a β -glucosidase protein (accession number AK250128). It is worth noting that also the other 4 sequences resulted in similarities with either barley or *Arabidopsis* genes even if with no significant *e*-values.

Discussion

Iron deficiency is a widespread problem affecting more and more cropping systems and thus causing negative impacts on crop productions. Specific soil characteristics are the main cause for low Fe availability to plant roots (Römhild and Marschner, 1986); for instance, one third of the cultivated areas is considered Fe-deficient (Mori, 1999). For these reasons, this study was aimed at investigating in barley, on a wide temporal scale, the effects of Fe deficiency on growth, phytosiderophores (PSs) release, Fe content and on methylation status of DNA. To the best of our knowledge, there is no literature on barley describing the effects of Fe deficiency on DNA methylation status.

Results of our experiments indicate that, as expected, shoot and root biomass production was significantly reduced by Fe starvation (**Table 1**). In particular, shoot length and weight were affected at 15 DAS, while root length and weight were decreased at 19 DAS. The magnitude of these negative effects was found to be of more consistent entities on the aerial biomass (**Table 1**). Van der Werf and Nagel (1996) documented that plants grown in sufficient availability of nutrients invest more energy in constructing aerial biomass than roots. *Viceversa*, in situations of

low nutrient availability, plants respond to this stress by reducing preferably aerial biomass than roots (Van der Werf and Nagel, 1996).

Regarding Fe content, we found that plant ±Fe showed significant differences in this nutrient content already at 8 DAS. At this date, it can be reasonably assumed that seeds Fe content was already exhausted (Yousfi et al., 2009). Consequently, the early chlorosis symptoms were now depending on the rate of leaves expansion (Yousfi et al., 2009). In addition, Fe deficient barley seemed to slightly decrease the rate of Fe translocation to leaves. As indicated in **Table 2**, the root to shoot ratios varied from 1.13 at 8 DAS to 2.00 at 19 DAS. Fe redistributions were observed also in other plants, and this trait is considered an aptitude of plants to guarantee an adequate Fe transport to their leaves (Yousfi et al., 2009).

Chlorophylls concentrations, expressed as SPAD index, was strongly reduced by Fe starvation (**Figure 1**). In fact, decreases were continuous and progressive from 13 to 19 DAS (**Figure 1**). The establishment of chlorosis was found to be quite successive to the alteration in the Fe content in starved barley (**Table 2**). This asynchrony is due to the chlorophylls dilution in leaves growing at normal rates in Fe deficiency (Abadía et al., 2000).

The release of PS is known to be depending on the Fe content of plants (Mimmo et al., 2014). In fact, the presence/absence of Fe in tissues deactivate/activate biosynthesis and release of these organic ligands (Mimmo et al., 2014). Our experiments showed that plants started to release PSs at 9 DAS. The concentration of exuded ligands more than doubled at 13 DAS (**Figure 2**). In addition, data of **Table 2** and **Figure 2** indicate that PS release started at 9 DAS, when the Fe concentration in the roots of Fe deficient plants was found significantly lower than that of Fe sufficient roots (**Table 2**). This result indicates a correlation between the roots Fe contents and the beginning of PS exudation. On the other hand, shoots of Fe deficient plants started 13 DAS to show lower Fe concentrations than well fed ones. At this time, the PSs release resulted more than doubled (**Figure 2**). These findings would be discussed by considering that the control of the Fe status in plants is regulated by sensors which are localized both in leaves and roots (Schmidt, 2003). In particular, the sensor allocated in shoots would promote the synthesis and translocation to roots, thorough phloem, of signal molecules. Then, plants would biosynthesize and release PSs into rhizosphere (Schmidt, 2003). Differently, the sensor in roots would modulate the signals received by shoots, which is in turn regulated by the Fe content of apoplast (Schmidt, 2003). Our experiments indicate that the low initial amount of exuded PS

TABLE 7 | Functional association of the methylated fragments.

MSAP band	Size (bp)	Chr. Location	Accession number, putative function, and Blast score
Hv_01	290		I2GL33*; oxidoreductase domain protein [<i>Fibrisoma limi</i> gen. nov., sp. nov.]; 3.5e-36
Hv_02	118	4	AT4G30660; proteolipid membrane potential modulator [<i>Arabidopsis thaliana</i> L.]; 7.5
Hv_03	120	7	AK373414; glucosyltransferase [<i>Hordeum vulgare</i> L.], 0.004
Hv_04	300		AK364780; cysteine protease family protein putative [<i>Hordeum vulgare</i> L.]; 1.4
Hv_05	115		AK364906; unknown; [<i>Hordeum vulgare</i> L.]
Hv_06	340	2	MOX2R2* acyl_carrier_prot-like; [<i>Hordeum vulgare</i> L.]; 1.9e-164
Hv_07	290		AK375528; peroxidase-like superfamily [<i>Hordeum vulgare</i> L.]; 3e-34
Hv_08	82		AAV80394; nucleic acid binding; [<i>Hordeum vulgare</i> L.]; 7.9
Hv_09	83		EF067844.1 vrs1 locus and Hox1 gene; [<i>Hordeum vulgare</i> L.]; 0.003
Hv_10	259		AK370298; Uncharacterized protein- GHMP-kinase C terminal domain [<i>Hordeum vulgare</i> L.]; 8.9
Hv_11	260		AK250128; beta-glucosydase [<i>Hordeum vulgare</i> L.]; 5e-11

Resulting sequences were blasted against the databases at NCBI, IPK, Gramene, and Uniprot websites. *UniProt ID.

(from 9 to 12 DAS), was mainly correlated with the alteration in the Fe-content in the roots (**Table 2**). In fact, Fe deficient barley roots showed at 9 DAS a Fe content much lower than the Fe sufficient plants (5.65 vs. 21.52 $\mu\text{g g}^{-1}$ FW). At 13 DAS, a significant decrease in the Fe content was found in the shoots of the starved plants (4.28 $\mu\text{g g}^{-1}$ FW), with respect to those of Fe sufficient samples (6.19 $\mu\text{g g}^{-1}$ FW). This finding indicates that the massive release of PSs at 13 DAS was mainly correlated to the reduced Fe concentration in the shoots.

Resupply studies conducted after the addition to starved plants 13 days old of Fe-EDTA 100 μM , evidenced some interesting trends. In particular, the chlorophylls estimation (**Figure 3**) indicate that this variety of barley reached a normal SPAD index in 6 days after Fe addition. Relatively to Fe contents (**Table 3**), resupplied plants immediately started to increase Fe concentration at roots level, while the translocation to the aerial parts seemed to be very slow, without achieving, after 6 days of resupply, values comparable to those of unstressed controls. Finally, it is to be mentioned that PS exudation of resupplied plants (data not shown) did not substantially change from that of Fe deficient plants (**Figure 2**). Just some slight decreases were ascertained at 6 days after Fe addition. This trend seems to confirm that Fe concentration in shoots plays the major role in controlling biosynthesis and exudation of these compounds.

In this paper, the MSAP technique was employed in order to determine the level of DNA methylation in *H. vulgare* and to ascertain its changes in result of Fe deficiency. The overall rate of methylation observed (60%) is higher than that found by Smith et al. (2014) and similar to that found by Shan et al. (2012) in the same species. In addition our results indicate that in barley the level of methylation is higher than that of *Gossypium hirsutum* (37%), *Triticum aestivum* (38%), *Trifolium repens* (28%), and *Cannabis sativa* (23%) (Aina et al., 2004; Zhong et al., 2009; Cao et al., 2011) and similar to that of *Brassica oleracea* (Salmon et al., 2008), *Oryza sativa* L. (Karan et al., 2012), and *Brassica napus* var. *oleifera* (Marconi et al., 2013).

Our data highlighted important differences between the amount of fully and hemi-methylated bands. In particular, in control samples, fully, and hemi-methylated bands represented

the 49.6 and 12.3% of the total bands, respectively, while in the Fe deprived samples these ratios were 47.15 and 13.5%, respectively. These results show a small decrease of fully methylated bands and a small increase of hemi-methylated bands in Fe deprived samples, when compared with Fe well-fed ones. These percentages are similar to those seen by Marconi et al. (2013) in a salt tolerant cultivar of rapeseed and, limited to hemi-methylated bands, in cotton (Cao et al., 2011). Lower values were found in rice genotypes under salt stress by Wang et al. (2011) and Karan et al. (2012), in a salt-sensitive cultivar of rapeseed by Marconi et al. (2013), and in both white clover and industrial hemp by Aina et al. (2004). All together, these studies suggests that the amount of fully and hemi-methylated bands depends both on the species, and on differences between genotypes belonging to the same species.

In our samples, we observed a very small variation between stages either for Fe well-fed samples or in Fe-deprived and resupplied samples for each class of fragment (**Table 5**) and this was also confirmed by a Chi-square Test (data not shown). Our results showed that, although cytosine methylation levels remained unchanged or similar between both control and Fe-deprived staged samples, this level varied when the same stages (DAS) of Fe well-fed samples were compared with Fe-deprived ones (**Table 5**).

Methylation changes due to Fe deficiency in this experiment occurred very rapidly. In fact (**Table 5**) the demethylation ratio in samples at 9 DAS (13.1%) was much higher than the methylation one (4.8%). At 19 DAS the amount of cytosine demethylation (7.6%) was alike that recorded for the previous stage, while methylation decreased from 4.8% at 17 DAS to 2.5% at 19 DAS. The level of methylation/demethylation recorded in this study confirm results obtained with the same technique in other plant species subjected to environmental changes (i.e., *Arabidopsis*, clover, hemp, tobacco, and wheat; Lízal and Relichová, 2001; Aina et al., 2004; Choi and Sano, 2007; Zhong et al., 2009). In addition, the results reported in this study suggests that Fe-deprivation induced more DNA demethylation than DNA methylation; these results are consistent with previous studies that showed that

abiotic stresses tends to demethylate genomic DNA (Aina et al., 2004; Choi and Sano, 2007; Zhong et al., 2009; Cao et al., 2011; Wang et al., 2011).

Finally, a large amount cytosine methylated/demethylated during Fe-deprivation remained methylated/demethylated after resupply (i.e., 77% at 15R and 61.5% at 19R and 61% at 15R and 69% at 19R, respectively, **Table 6**). This suggested that variation in methylation/demethylation was maintained even once the deficiency was removed and could likely be transmitted to progeny.

Six nucleotide sequences were significantly associated with barley genes (**Table 7**). Hv_03 displayed sequence homology with a gene encoding for a glucosyltransferase. These enzymes catalyze glucosylation reactions, by forming a glucosydic bond (Liu and Mushegian, 2003), and are involved in plant stress responses to harmful metabolites and toxic environmental compounds (Edwards et al., 2005). Regarding to Fe starvation, a gene encoding for an UDP-glucosyltransferase protein was found to be up-regulated in *Glycine max* grown in Fe deficiency conditions (O'Rourke et al., 2007).

Hv_06 sequence was very similar a *H. vulgare* putative acyl carrier protein (ACP), which is believed to play a central role in fatty acid biosynthesis in all living organisms, carrying the acyl chains through the various steps in fatty acid biosynthesis (Chan and Vogel, 2010). Recently, it has been shown that in *Chlamydomonas reinhardtii* Fe deficiency causes the formation of intracellular lipid droplets and a quick down-regulation of genes involved in the *de novo* synthesis of fatty acid, among which ACP1/2 encoding for the acyl carrier protein 1 (Urzica et al., 2013). Hv_07 nucleotide sequence showed a significant sequence similarity with a barley gene that encodes for a protein belonging to the peroxidase superfamily, which are ubiquitous enzymes involved in many physiological processes pivotal for plant life (Passardi et al., 2005). Peroxidases are mainly implicated in the cellular regulation of reactive oxygen species (ROS) and hydrogen peroxide (Passardi et al., 2005). Since Fe is a cofactor for many antioxidant enzymes, Fe deficient plants can be expected to be more sensitive to oxidative stress. To date, very few attention has been paid to the question wheatear Fe starvation can impair antioxidant defenses in plants. However, some evidence were reported on the correlation between low Fe availability and reduced peroxidases and superoxide dismutase activities, in sunflower and *A. thaliana* (Ranieri et al., 2001; Ramírez et al., 2013).

The six-rowed spike 1 (*vrs1*) gene, was the best match for Hv_09 DNA. The six-rowed phenotype is genetically determined by homozygosity for the recessive allele at the *vrs1* locus, which has been identified as a homeobox gene (*HvHox1*) (Sakuma et al., 2009). This gene encodes for a transcription factor containing a homeodomain (HD). HD is a 60 amino acids motif present in a number of eukaryotic transcription factors, frequently involved in developmental processes (Gago et al., 2002). Recently, this gene has been associated to the regulation of plant stress responses correlated with changes in environmental conditions and water deficit (Elhitia and Stasolla, 2009). The sequence

of Hv_11 DNA led to the identification of a *H. vulgare* gene encoding for a putative β -glucosidase, an enzyme involved in the mobilization of complex carbohydrates, and this is in line with the observation that Fe deficient plants generally display higher sugar concentrations than Fe sufficient ones (López-Millán et al., 2000). In addition, an extracellular β -glucosidase, isolated from barley leaves, was found to be able to catalyze the de-esterification of glucosyl-abscisic acid to generate ABA (Dietz et al., 2000). It has been proposed that this activity was involved in the modulation of stress signals and gene expression in cells (Dietz et al., 2000). Finally, Zamioudis et al. (2014) showed that a β -glucosidase from *A. thaliana* was responsive to Fe starvation and involved in the secretion of Fe-mobilizing phenolic metabolites, in the close proximity of the roots.

Since agriculture is strictly depending on mineral nutrition, and most of the nutrients are present in the soil in unavailable forms, the knowledge of how plants mobilize them, by releasing organic compounds, is of pivotal importance. Consequently, the understanding of how the plants modulate gene expression to cope with nutrients fluctuations or deficiency appears even more important. Concerning our study, we ascertained that barley plants responded to iron starvation by activating some physiological and biochemical changes, in order to cope with this adverse nutritional situation. In particular, Fe starved barley plants exuded huge amounts of organic ligands in correlation with the aerial and radical Fe contents. Finally, the MSAP technique was employed to ascertain the level of DNA methylation/demethylation of barley genome, and its variations in Fe deficiency. Not only the results demonstrated a clear effects of Fe starvation on the status of fully and hemi-methylated bands, but also the resupply experiments suggested, at least, that the methylation/demethylation status could be maintained even once the Fe deficiency is removed. The relevance of this last finding is supported by the fact that such modifications could likely be transmitted to progeny.

Author Contributions

Conceived and designed the experiments: EA, DD, MB, MLB, YP, TM, SCe. Performed the experiments: MB, MLB, YP, SC. Analyzed the data: EA, DD, MB, MLB, TM, SC, YP. Contributed reagents/materials/analysis tools: EA, DD, SCe. Wrote the paper: EA, DD, MB, TM, SC.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00514>

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Isolation and characterization of an osmotic stress and ABA induced histone deacetylase in *Arachis hypogaea*

Liang-Chen Su [†], Bin Deng [†], Shuai Liu, Li-Mei Li, Bo Hu, Yu-Ting Zhong and Ling Li ^{*}

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Raúl Alvarez-Venegas,
Centro de Investigación y de Estudios
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Reviewed by:

Gong-yin Ye,
Zhejiang University, China
Keqiang Wu,
National Taiwan University, Taiwan
Xuncheng Liu,
South China Botanical Garden,
Chinese Academy of Sciences, China

*Correspondence:

Ling Li,
Guangdong Provincial Key Laboratory
of Biotechnology for Plant
Development, School of Life Sciences,
South China Normal University,
No. 55, Zhongshan Avenue West,
Tianhe District, Guangzhou 510631,
China
liling502@126.com

[†]These authors have contributed
equally to this work.

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Histone acetylation, which together with histone methylation regulates gene activity in response to stress, is an important epigenetic modification. There is an increasing research focus on histone acetylation in crops, but there is no information to date in peanut (*Arachis hypogaea*). We showed that osmotic stress and ABA affect the acetylation of histone H3 loci in peanut seedlings by immunoblotting experiments. Using RNA-seq data for peanut, we found a RPD3/HDA1-like superfamily histone deacetylase (HDAC), termed AhHDA1, whose gene is up-regulated by PEG-induced water limitation and ABA signaling. We isolated and characterized AhHDA1 from *A. hypogaea*, showing that AhHDA1 is very similar to an *Arabidopsis* HDAC (AtHDA6) and, in recombinant form, possesses HDAC activity. To understand whether and how osmotic stress and ABA mediate the peanut stress response by epigenetics, the expression of AhHDA1 and stress-responsive genes following treatment with PEG, ABA, and the specific HDAC inhibitor trichostatin A (TSA) were analyzed. AhHDA1 transcript levels were enhanced by all three treatments, as was expression of peanut transcription factor genes, indicating that AhHDA1 might be involved in the epigenetic regulation of stress resistance genes that comprise the responses to osmotic stress and ABA.

Keywords: epigenetics, ABA, osmotic stress, acetylation, HDAC, RNA-seq, TSA

Introduction

Plants respond to various abiotic stresses by altering the expression of many genes. Drought is one of the most significant of such abiotic stresses because it limits cell growth and development; consequently, plants have developed diverse strategies to cope with limited water availability (Jung et al., 2005). One such strategy is epigenetic modification of chromatin structure through post-translational modification of histones, for example by acetylation and ubiquitination of lysine residues, methylation of arginine, and phosphorylation of serine or threonine (Henderson and Jacobsen, 2007; Kim et al., 2010). This regulates the expression of genes within the modified chromatin, thereby affecting plant growth and development (Lopez-Gonzalez et al., 2014; Zhang et al., 2014).

Histone acetylation is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDAs or HDACs). In general, HATs transfer acetyl groups to core histone tails, thereby promoting transcription of target genes, whereas HDACs remove acetyl groups from the Lys residues of

histone tails, resulting in the repression of gene transcription (Kurdustani and Grunstein, 2003). Plant HDACs are classified into three distinct families, namely RPD3/HDA1-like HDAs, SIR2-like HDAs, and HD2 proteins, based on sequence similarity, substrate specificity, and cofactor requirement (Pandey et al., 2002; Loidl, 2004; Fong et al., 2006; Zhong et al., 2013). *Arabidopsis thaliana* has 12 RPD3/HDA1 subfamily genes (*HDA2*, *HDA5*, *HDA6*, *HDA7*, *HDA8*, *HDA9*, *HDA10*, *HDA14*, *HDA15*, *HDA17*, *HDA18*, and *HDA19*) among 18 putative HDAC family genes (Ma et al., 2013). Of these, *HDA6* has been reported to participate in jasmonic acid-mediated plant defense responses and to be involved in transgene silencing and the regulation of rRNA transcription (Murfett et al., 2001; Devoto et al., 2002; Tanaka et al., 2008); *HDA19* is involved in jasmonic acid and ethylene signaling during the response to pathogens, and redundantly with *HDA6* regulates the repression of embryonic properties during germination (Zhou et al., 2005; Tanaka et al., 2008); both up-regulation and down-regulation of *HDA7* and *HDA18* in *Arabidopsis* cause growth delays at different developmental stages (Cigliano et al., 2013; Liu et al., 2013); *HDA9*, which acts to oppose the effect of its homologs *HDA6* and *HDA19*, is a negative regulator of germination in seedlings (van Zanten et al., 2014). Thus, these HDAs respond to environmental stress or participate in plant development.

HDAs function on various histone loci within chromatin and these can be detected by Western blot, chromatin immunoprecipitation (ChIP) assays or immunocytochemistry. Research in plants has focused on modifications of histones H3 and H4, which are involved in cell development, flowering, transposon repression and abiotic stress response (Zhao et al., 2014). In *Arabidopsis*, there is region-specific enrichment of H3K23ac and H3K27ac in the coding regions of the drought-responsive genes *RD29B*, *RD20*, and *RAP2.4*, while enrichment of H3K4me3 and H3K9ac correlates with *RD29A*, *RD29B*, *RD20*, and *RAP2.4* gene activation in response to drought stress (Kim et al., 2008). DREB1 (dehydration responsive element binding 1) proteins, have been shown to play an important role in the response of plants to low-temperature stress (Liu et al., 1998). During cold stress in rice, histone H3K9 acetylation is increased throughout the 800 bp region of *OsDREB1b*, whereas H3K14 and K27 acetylation is biased more toward the core promoter and upstream region, respectively (Roy et al., 2014). Immunoblotting analysis shows that H3K9ac, H3K18ac, H3K27ac, and H4K5ac levels increase with the expression of HATs in response to drought treatment in rice leaves (Fang et al., 2014). In maize, H3K9ac, H4K5ac, and H4ac levels in the *ZmICE1* and *ZmCOR413* promoter and coding regions increase with *ZmDREB1* up-regulation on cold treatment (Hu et al., 2011). Thus, modifications in histone acetylation patterns in plants during stress treatment are associated with the expression of stress response genes.

Drought is one of the most growth-limiting factors for crops. In our previous research on the molecular consequences of environmental stress and abscisic acid (ABA) action in peanut (*Arachis hypogaea*), an economically important oil- and protein-rich crop plant, we analyzed the role of drought-related genes under conditions of water limitation. *AhNCED1*

(9-cis-epoxycarotenoid dioxygenase) protein catalyzes the rate-limiting step in the ABA biosynthetic pathway in peanut, and its expression is up-regulated by dehydration and ABA; furthermore, heterologous expression of *AhNCED1* increases drought resistance in *Arabidopsis* (Wan and Li, 2005, 2006). In addition, we also found that *AhAREB1*, a gene which encodes a transcription factor (TF), was induced by ABA or drought (Li et al., 2013). Genes encoding stress-combative dehydrins, i.e., *AhDHNs*, were also upregulated by ABA and PEG (which imposes osmotic stress) in peanut leaves (Su et al., 2012). RNA-seq results show that other TF-like genes (*MYB92*-like and *WRKY33*-like) participate in the early stages of the peanut response to ABA and osmotic stress (Li et al., 2014). However, whether any of these genes are involved in epigenetic regulation, specifically with respect to the osmotic stress response, is still unknown and there are very few reports of the relationship between osmotic stress, ABA signals and plant deacetylation in crops.

In this paper, histone acetylation status in peanut was found to be modified as part of the response to both ABA and PEG treatment. By reference to an RNA-seq database for peanut, we discovered a histone deacetylase 6-like gene that was up-regulated by water deficit and ABA (Li et al., 2014), a result we confirmed by quantitative real-time PCR (qRT-PCR). This histone deacetylase sequence, termed *AhHDA1*, was isolated and its expression was analyzed to determine transcripts abundance in different tissues of peanut. The expression of *AhHDA1* was compared to that of various drought resistance genes during osmotic stress and ABA treatment to attempt to understand the role of *AhHDA1* under these conditions.

Materials and Methods

Peanut Plants and Growth Conditions

Seeds of peanut (*Arachis hypogaea* L. cv Yueyou 7) (Fang et al., 2007) were sown in pots with a potting mixture of vermiculite, perlite and soil (1: 1: 1), and grown in a illumination incubator with 16 h of light from fluorescent and incandescent lamps ($200 \mu\text{mol m}^{-2}\text{s}^{-1}$) at 26°C followed by 8 h of darkness at 22°C . Plants were watered with half-strength Murashige and Skoog nutrient solution every other day (Wan and Li, 2005).

Abiotic Stress and Hormone Treatments of Peanut Plants

Four-leaf stage peanut seedlings were treated with PEG6000 (Roche) to simulate osmotic stress conditions, and ABA (Roche) and trichostatin A (TSA, Roche) were also applied exogenously for other treatments. After water had been removed by filter paper, the seedlings were harvested, rinsed with deionized water, and placed in beakers containing different solutions of PEG, ABA, or TSA in deionized water. The seedlings were transferred to an illumination incubator (26°C , 60% moisture) under continuous light. PEG and ABA were applied at a concentration of 20% (w/v) and $100 \mu\text{M}$, respectively (Wan et al., 2011). TSA was applied at a concentration of $1 \mu\text{M}$. Control plants were planted in soil but not treated. Mock plants were placed in an equivalent volume of deionized water as experimental plants

instead of ABA and TSA solutions. Control group and treated groups were also used for qRT-PCR and immunoblotting (see below for details). Peanut leaf samples (100 mg) were taken at 0, 1, 2, 5, and 8 h and were maintained at -70°C until further use.

Protein Gel Electrophoresis and Immunoblotting

Peanut leaves (800 mg) were ground to a powder in liquid nitrogen and mixed to homogeneity in 1 ml ice-cold extraction buffer 1 (10 mM potassium phosphate, pH 7.0, 0.1 M NaCl, 10 mM beta-mercaptoethanol, 1 M hexylene glycol) with protease

inhibitor (Roche, catalog No. 06538304001). The extract was centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant was discarded. The pellet was resuspended gently in 0.5 ml pre-cooled buffer 2 (10 mM potassium phosphate, pH 7.0, 0.1 M NaCl, 10 mM beta-mercaptoethanol, 1 M hexylene glycol, 10 mM MgCl₂, 0.5% Triton X-100) with protease inhibitor, centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant was discarded. The buffer 2 step was repeated until the supernatant after centrifugation was light green. Then the pellet was resuspended gently in 1 ml pre-cooled buffer 3 (10 mM potassium phosphate, pH 7.0, 0.1 M NaCl, 10 mM beta-mercaptoethanol) with protease inhibitor, centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant was discarded. The nuclear pellet was resuspended gently in 0.5 ml pre-cooled sonication buffer (10 mM potassium phosphate, pH 7.0, 0.1 M NaCl, 10 mM EDTA pH 8.0, 0.5% sarkosyl). The resuspended mixture was sonicated for 5 min on ice and sonicated samples were centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was transferred into a new tube and stored at -70°C.

The nuclear extract was suspended in 5 × SDS PAGE loading buffer (0.25 M Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, and 5% 2-mercaptoethanol). The concentration of protein samples was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA), loaded and run on 15% polyacrylamide gels, then gels were blotted onto a 0.22 μm PVDF membrane. The membrane was blocked in Tris-buffered saline with 0.1% Tween 20 (TBST, pH 7.6) containing 5% dry milk overnight and then incubated with 0.01–0.05 mg/mL of anti-histone H3 (Abcam, catalog no. ab1791), anti-acetyl-histone H3 (Abcam, catalog no. ab47915), anti-acetyl-histone H3K9 (Millipore, catalog no. 07-352) and anti-acetyl-histone H3K14 (Millipore, catalog no. 07-353) for 2 h at room temperature. After washing, the primary antibody was detected with secondary goat anti-rabbit alkaline phosphatase-coupled antibody (Millipore, catalog no. AP307A) at room temperature for 45 min. Visualization was achieved using the ECL system (Millipore, catalog no. 345818).

Isolation and Sequence Analysis of AhHDA1 from *Arachis Hypogaea* L.

First-strand cDNA was synthesized by reverse transcription (RT) of 1 μg of total RNA from peanut leaves, either untreated or treated for 5 h with 20% PEG 6000, using 200 units Superscript III Reverse Transcriptase (Invitrogen, catalog No. 18080) and 500 ng oligo-dT primer. The cDNA was used as the template for PCR using specific primers (ORF1-F: AAGTTGAAACCCCAACCT; ORF1-R: CACCAAGCAGACTAAAGCAAAA) for the amplification of *AhHDA1*. These primers were designed to amplify the full length sequence of the *AhHDA1* ORF. RT conditions were: 70°C for 10 min, followed by 42°C for 1 h, followed by 15 min at 70°C. PCR amplification was performed as follows: 94°C for 5 min, then 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 1 min, then finally 72°C for 10 min.

PCR fragments were gel purified with an Agarose Gel DNA Purification Kit (TaKaRa, catalog no. DV805A) and were ligated into the pMD 19-T Vector (TaKaRa, catalog no. 6013). Plasmids were isolated and were sequenced from both strands. Sequence

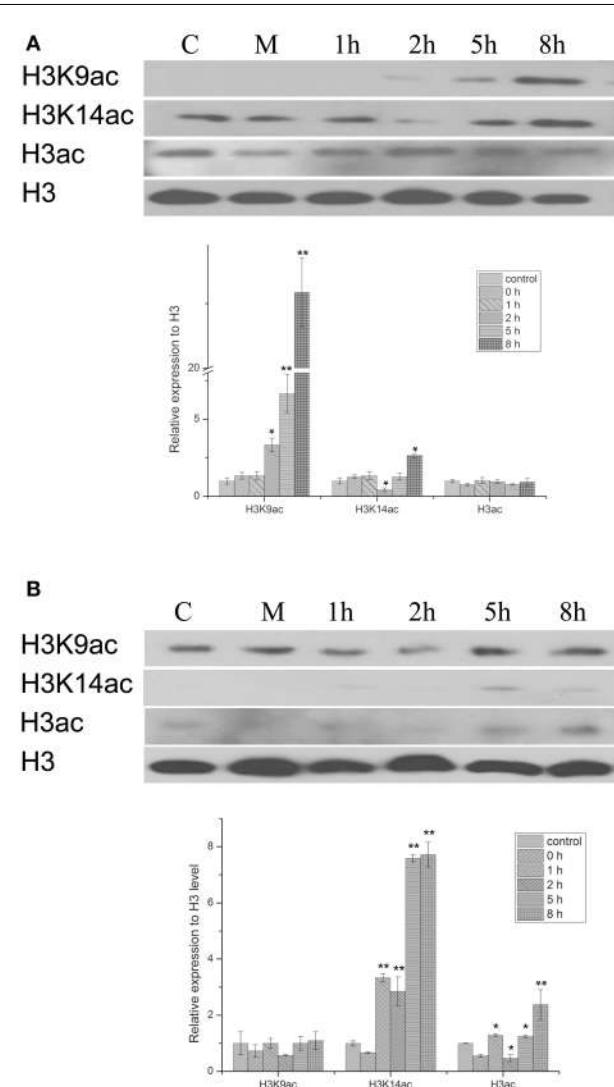


FIGURE 1 | Western blot showing the effect of PEG and HBA on histone H3 acetylation status in nuclear proteins from peanut leaves. (A) H3 acetylation status in peanut leaves treated with 20% (w/v) PEG. (B) H3 acetylation status in peanut leaves treated with 100 μM ABC. C, control group; M, Mock plants were placed in an equivalent volume of deionized water as experimental plants; 1–8 h, time point after treatment. The experiments have been carried out at least three times. Each graph displays the mean and SD of three independent experiments. **, different from control as revealed by t-test, $p < 0.05/0.01$.

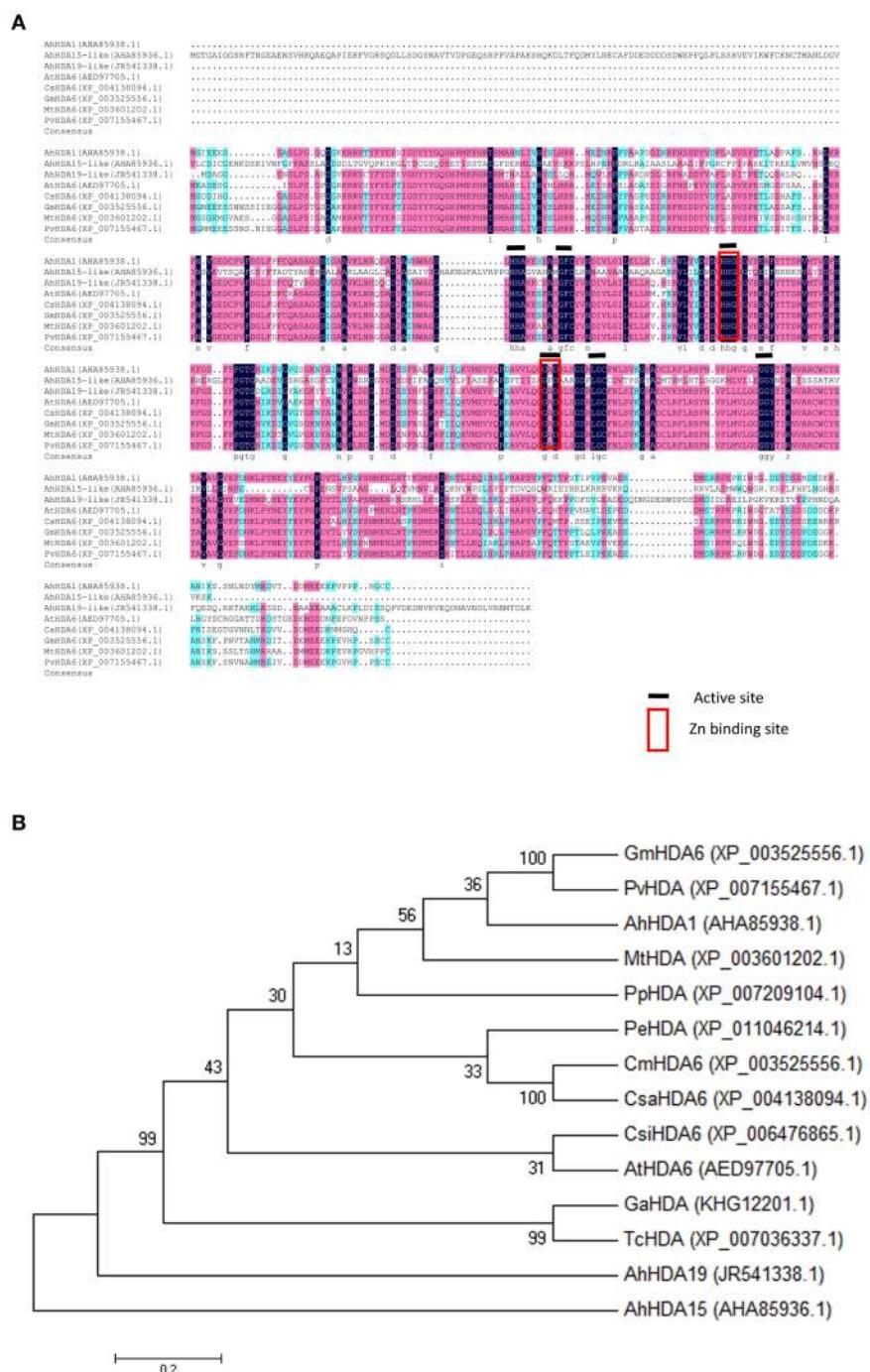


FIGURE 2 | Relatedness of peanut HDACs sequences to counterparts in other plants. (A) Alignment of deduced amino acid sequence of peanut HDACs with other plant HDAC sequences. The degree of similarity of the amino acid residues at each aligned position is shaded black, red, blue, in decreasing order. GenBank accession numbers for each aligned HDAC protein are indicated in parenthesis. **(B)** Phylogenetic analysis of amino acid sequences of AhHDA1 and other plant HDACs. A multiple sequence alignment was performed using Clustal W and the phylogenetic tree was constructed via the Neighbor-Joining method in MEGA 4 software. Bootstrap values from 1000 replicates for each branch are shown. GenBank

accession numbers: *Glycine max* HDA6 (XP_003525556.1), *Phaseolus vulgaris* HAD (XP_007155467.1), *Arachis hypogaea* HDA1 (JR541338.1), *Medicago truncatula* HDA (XP_003601202.1), *Prunus persica* HDA (XP_007209104.1), *Populus euphratica* HDA (XP_011046214.1), *Cucumis melo* HDA6 (XP_00864523.1), *Cucumis sativas* HDA6 (XP_004138094.1), *Citrus sinensis* HDA6 (XP_006476865.1), *Arabidopsis thaliana* HA6 (AED97705.1), *Gossypium arboreum* HDA (KHG12201.1), *Theobroma cacao* HDA (XP_007036337.1), *Arachis hypogaea* HDA19-like (AHA85936.1), *Arachis hypogaea* HDA15 (AHA85936.1). The scale bar is 0.02.

analysis was performed using EditSeq software (Lasergene7.0). Computer analysis of the DNA and amino acid sequences was carried out using the BLAST program at the National Center for Biotechnology Information Services (<http://www.Ncbi.Nlm.Nih.gov/BLAST>). For phylogenetic analysis, we used neighbor-joining (NJ) methods implemented using the full alignment program in DNAMAN software (Wan and Li, 2005). 3D comparative protein structure models of peanut AhHDA1 were generated with the automatic modeling mode of SWISS-MODEL implemented on the SWISS-MODEL Workspace website (<http://swissmodel.expasy.org/>) (Schwede et al., 2003; Arnold et al., 2006).

Quantitative Real-time PCR (qRT-PCR)

RNA extraction was carried out as described by Wan and Li (2005). Three biological replicate RNA samples of each time point and treatment were used for downstream applications. First-strand cDNAs, obtained using the Superscript III reverse transcriptase kit with 0.3 nmol random 15-mers for reverse transcription of 1 µg RNA, were used as templates for qRT-PCR. Aliquots of 1 µl cDNA were then used for each RT-qPCR reaction. Absolute QPCR SYBR Green ROX Mix (ABgene, catalog no. AB-4105) was used according to the manufacturer's instructions for quantification with the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, UK). A melting curve confirmed single product amplification. Analysis of the raw data and calculation of the efficiency (E) for every single well was done using the software PCR Miner (Zhao and Fernald, 2005). Relative expression for each well was calculated as $(1 + E) - CT$ (Muller et al., 2002). Expression data for *A. hypogaea* L. was normalized using the geometric mean (geomean) of the validated

housekeeping genes, ACTIN and ADH3 (Chi et al., 2012; Reddy et al., 2013): the primers ACT11-F and ACT11-R, specific to the peanut ACTIN gene (GenBank accession no. GO339334), were used to amplify a fragment of 108 bp, and the primers ADH3-F and ADH3-R, specific to the peanut ADH3 gene (GenBank accession no. EG529529), were used to amplify a fragment of 143 bp. The mean values shown (\pm SE) were calculated from three biological replicates. Primers are listed in Table S1.

Production of Recombinant AhHDA1 in *E. coli*

The PCR product of AhHDA1 was cloned into an *E. coli* expression vector, pPROEX HT (Invitrogen, catalog No. 10711-018) (Pompon et al., 1996). The resulting plasmid was transformed into *E. coli* strain BL21 (Figure S2). Transformants were grown in LB medium (10 g/L Tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, 50 g/mL ampicillin) at 37°C for 8–10 h. Once the OD600 reached 0.7, 0.1 mM IPTG was added to the LB medium. Then the bacterial suspension was placed in a shaking incubator at 16°C for 20 h. To prepare total proteins, *E. coli* cells were collected and suspended in 0.1 mol/L potassium phosphate buffer (pH 7.6). The lysates of the bacterial cells were centrifuged (at 4°C, 10,000 rpm, 10 min), and the supernatants were subjected to Ni-NTA HisTrap FF crude column chromatography for purification of the recombinant protein. The purified protein was dissolved in phosphate-buffered saline (pH 7.6) to a final concentration of 0.8 mg/mL. The purity of the recombinant AhHDA1 protein was analyzed using SDS-PAGE.

HDAC Enzyme Activity Assay (Colorimetric Detection)

This two-step procedure was performed in a microtiter plate using an HDAC Assay Kit (Millipore, catalog no. 17-374). Each well-contained 10 µl 2X HDAC assay buffer, or 2X HDAC assay buffer containing 4 µM trichostatin A, to which 20 µl test protein sample, or 20 µl HeLa nuclear extract (positive control; supplied with kit) or 20 µl water (negative control) were added; the plate was then equilibrated at the assay temperature (37°C). After adding 10 µl of the 4 mM HDAC assay substrate and mixing thoroughly, the microtiter plate was incubated at 37°C for 60 min. Then 20 µl of the diluted activator solution was added to each well, mixed thoroughly and the microtiter plate was incubated at room temperature for 15 min. The absorbance was read in a plate reader at 405 nm.

Results

PEG and ABA Mediate Alterations of H3K9 and H3K14 Acetylation Status in *Arachis hypogaea* L. Leaves

The histone acetylation status of chromatin was investigated in peanut leaves subjected to PEG-induced osmotic stress or to treatment with the stress-protective hormone ABA. Immunoblotting experiments showed that the H3K9ac level increased with 20% PEG treatment, while the H3K14ac level increased with 100 µM ABA treatment (Figure 1). H3K9ac

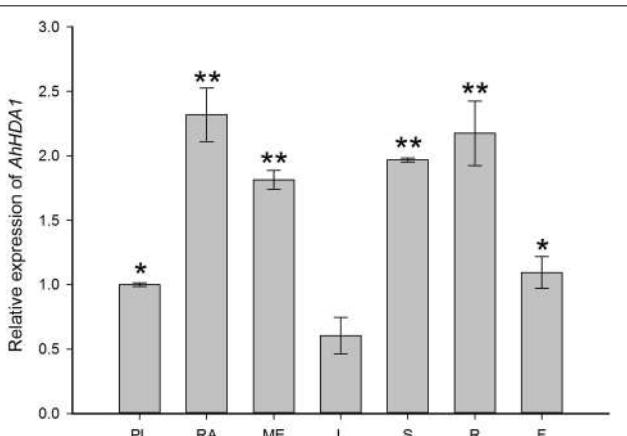


FIGURE 3 | Quantitative RT-PCR validations of AhHDA1 expression in different peanut tissues. Column chart showing expression of AhHDA1 in PL, plumule; RA, radicle; ME, mesocotyl; L, leaf; S, stem; R, root; and F, flower, respectively. Plumules, radicles, and mesocotyls were taken from peanut embryos which had been cultivated for 7 days germination. Leaves stems and roots were taken from four-leaf stage peanut seedlings. Flowers were taken from peanuts during budding. All plants were grown as started in Materials and Methods. All values \pm Standard Error (SE) for $n = 3$ biological replicates. Each graph displays the mean and SD of three independent experiments. */**, different from control as revealed by *t*-test, $p < 0.05/p < 0.01$.

levels following PEG treatment began to increase from 2 h and continued to increase through to the 8 h time point; thus, after 5 h H3K9ac levels showed a significant increase to 7 times that of the control group, and at 8 h had increased further to 23 times control levels. Treatment with PEG produced only a marginal increase in H3K14ac levels at 8 h, but ABA treatment significantly increased the amount of H3K14ac by 5 h to 8 times that of the control group. The results indicate that both PEG and ABA can mediate changes in acetylation at different histone H3 loci; these different patterns of modification suggest that the two treatments result in different gene activation profiles in peanut leaves. At the same time, it has also been proved that the acetylation of H3K9, H3K14, and H3 were increased with 1 μ M TSA treatment from 1 to 8 h (Figure S4).

Isolation and Characterization of the Peanut AhHDA1 Gene

From the above results, it is clear that osmotic stress and ABA affect the acetylation of histone H3. We therefore screened an RNA-seq database which identifies genes that are differentially expressed following PEG and ABA treatment of peanut (<http://www.ncbi.nlm.nih.gov/bioproject/243319>) and found a full length ORF of a sequence (*comp66763_c0*) similar

to the Arabidopsis *HDA6* gene. According to the RNA-seq data, this gene, named *AhHDA1* (GenBank accession No. KC690279), is inducible by PEG and ABA treatment in peanut leaves from four-leaf seedlings.

Specific forward and reverse primers (ORF1-F and ORF1-R) were designed from *comp66763_c0* to isolate an *AhHDA1* cDNA as detailed in Materials and Methods. By sequence alignment, the predicted sequence of the *AhHDA1* protein showed a high degree of similarity with other HDACs in the GenBank DNA database, and *AhHDA1* possessed the same active site and Zn²⁺ binding sites as other plant HDACs (Figure 2A). *AhHDA1* consists of a polypeptide of 467 amino acid residues with a calculated molecular weight of 52.37 kDa and an isoelectric point of 5.28. It can be deduced from Figure 2B that *AhHDA1* is most similar to counterparts in eudicots, especially soybean.

The SWISS-MODEL tool was used to generate 3D structures for the *AtHDA6* (encoded by the Arabidopsis *HDA6* gene) and *AhHDA1* proteins (<http://www.swissmodel.expasy.org>; Figure S1). The 3D structures of both *AhHDA1* and *AtHDA6* were very similar, implying that the *AhHDA1* gene in peanut has a similar function to that of *AtHDA6* in Arabidopsis.

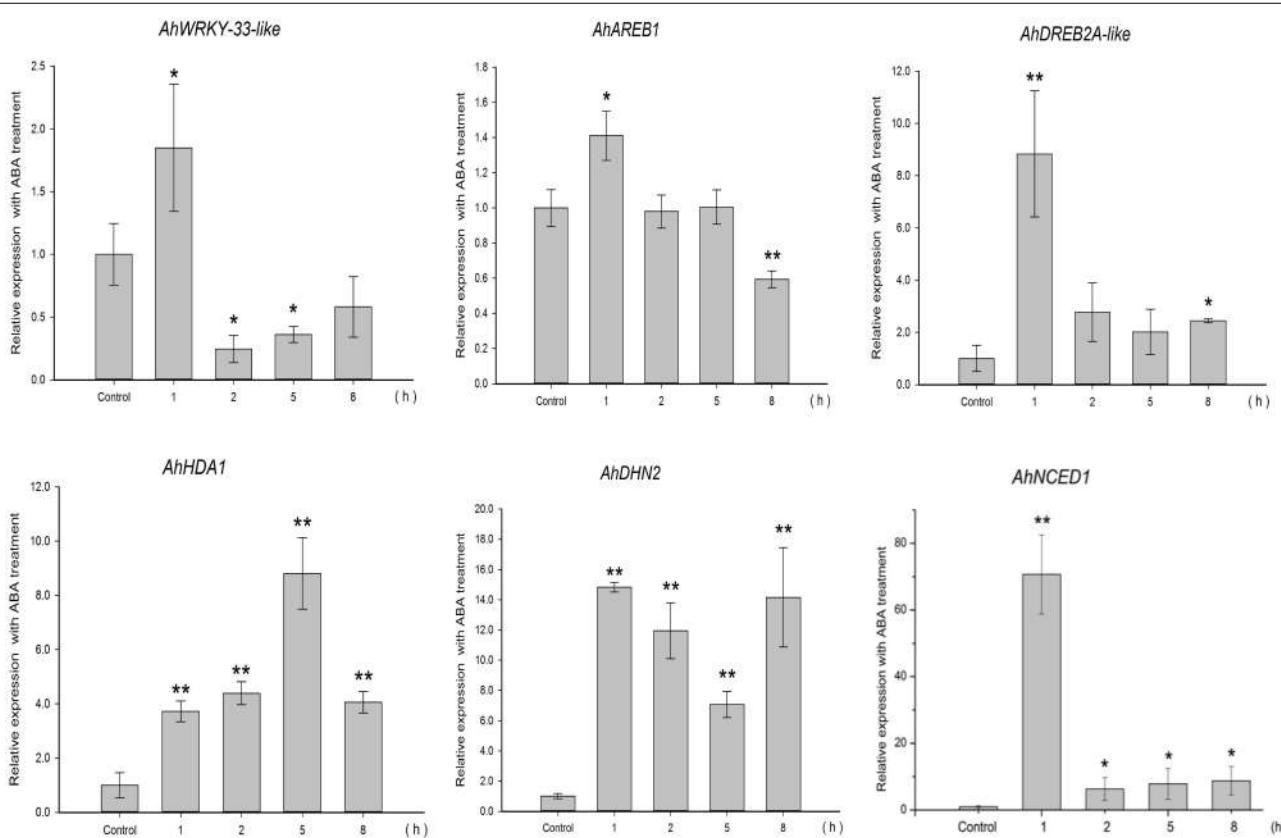


FIGURE 4 | Expression analyses of *AhHDA1* and stress resistance genes following ABA treatment by qRT-PCR. Time points of 1, 2, 5, and 8 h were sampled to observe the changing trend. The untreated group was

used as the control (no chemical treatment). Each graph shows the mean and SD of three independent experiments. */**, different from control as revealed by t-test, $p < 0.05/p < 0.01$.

Differential Expression Analysis in Different Tissues of *Arachis Hypogaea* L.

Quantitative RT-PCR analysis was performed to examine the expression of *AhHDA1* in untreated embryos (plumule, radicle, and mesocotyl) and four-leaf seedlings (leaf, stem, root, and flower) (Figure 3). *AhHDA1* mRNA predominantly accumulates in radicle and mesocotyl of the embryo; similarly, in seedlings, *AhHDA1* mRNA predominantly accumulates in stem and root.

Enhancement of *AhHDA1* Transcript Level in Peanut Leaves from Four-leaf Seedlings in Response to Osmotic Stress, ABA, and Histone Deacetylase Inhibitor

To gain insight into the regulation of *AhHDA1*, qRT-PCR analyses were carried out in peanut leaves from four-leaf seedlings using gene-specific internal primers (Table 1). We investigated the changing trend of *AhHDA1* expression resulting from ABA treatment, as well as during the first rapid phase of water stress resulting from treatment with PEG. At the same time, the specific HDAC inhibitor TSA was used to examine the role of *AhHDA1* in the response to ABA and osmotic stress (Figure S4). Drought resistance genes were also analyzed with all these treatments. By comparison with the control group, we found that the expression of *AhHDA1* was enhanced by all three treatments (Figures 4–6). The *AhHDA1* transcript level increased to 4 times that of the control group at 1 h, and remained at a relatively

high level from 2 to 8 h in ABA-treated plants. PEG and TSA treatments gave an expression profile almost identical to that of ABA-treated seedlings: *AhHDA1* expression in TSA groups increased from 1 h and stayed at a high level throughout the remainder of the experiment, while *AhHDA1* expression in PEG groups increased from 5 h, rather than 1 h, and stayed high at 8 h.

The expression profiles of TF genes (*AhAREB1*, *AhDREB2A-like*, *AhWRKY33-like*) and functional genes (*AhDHN2* and *AhNCED1*) were also determined in all three groups. It is clear from our results (Figures 4–6) that the expression patterns of these TF genes and functional genes in peanut leaves were very similar to that of *AhHDA1*, both of which show an initial increase followed by a decline in expression. More specifically, the expression of *AhAREB1*, *AhDREB2A-like*, and *AhWRKY33-like* began to increase at 1 h in both ABA and TSA groups and stayed at a high level or decreased from 2 h. *AhAREB1* and *AhWRKY33-like* expression began to increase from 5 h during PEG treatment, while the expression of *AhDREB2A-like* increased from 2 h. At the same time, the expression of *AhDHN2* and *AhNCED1*, the functional genes, began to increase from 1 h in all three treatment groups and maintained a high level of expression after 2 h.

Histone Deacetylase Activity of Recombinant *AhHDA1* Protein

Recombinant *AhHDA1* was produced in *E. coli* (Figure S2) as a polypeptide of about 53 kDa (Figure 7A). A total protein

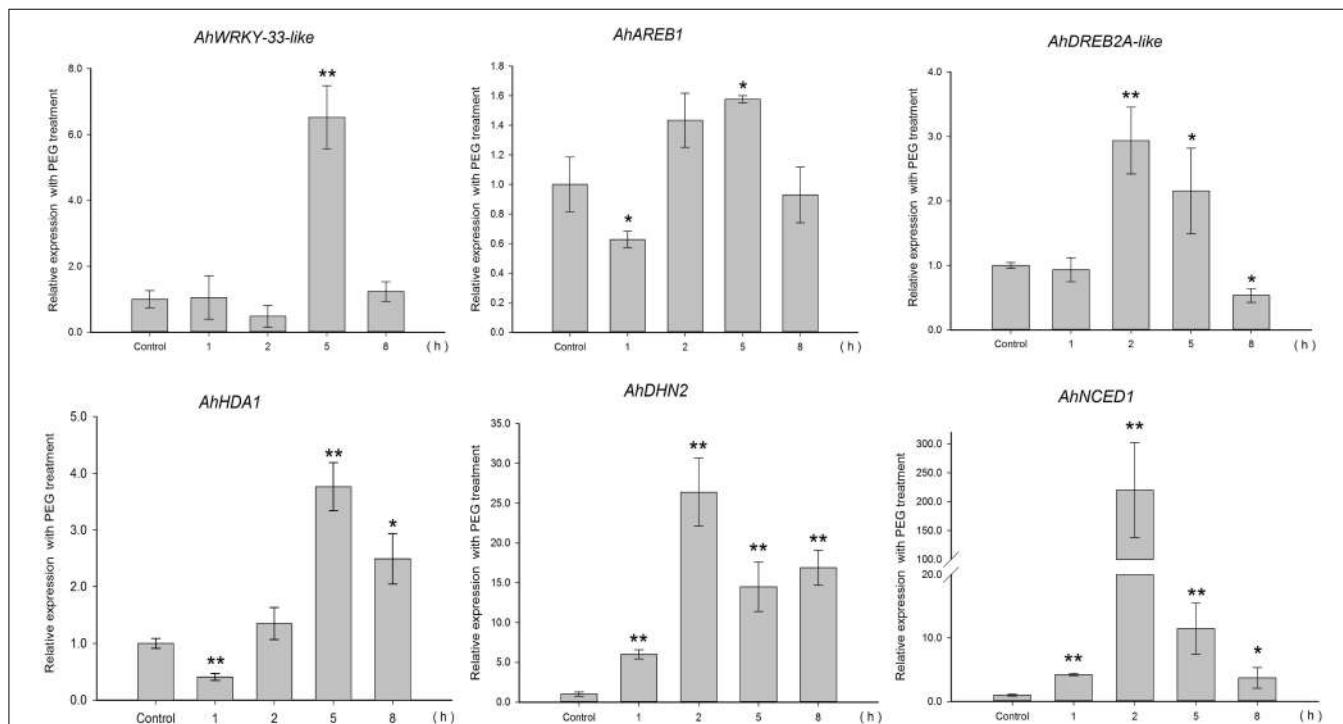


FIGURE 5 | Expression analyses of *AhHDA1* and stress resistance genes following PEG treatment by qRT-PCR. Time points of 1, 2, 5, and 8 h were sampled to observe the changing trend. The untreated group was

used as the control (no chemical treatment). Each graph shows the mean and SD of three independent experiments. **, different from control as revealed by *t*-test, $p < 0.05/p < 0.01$.

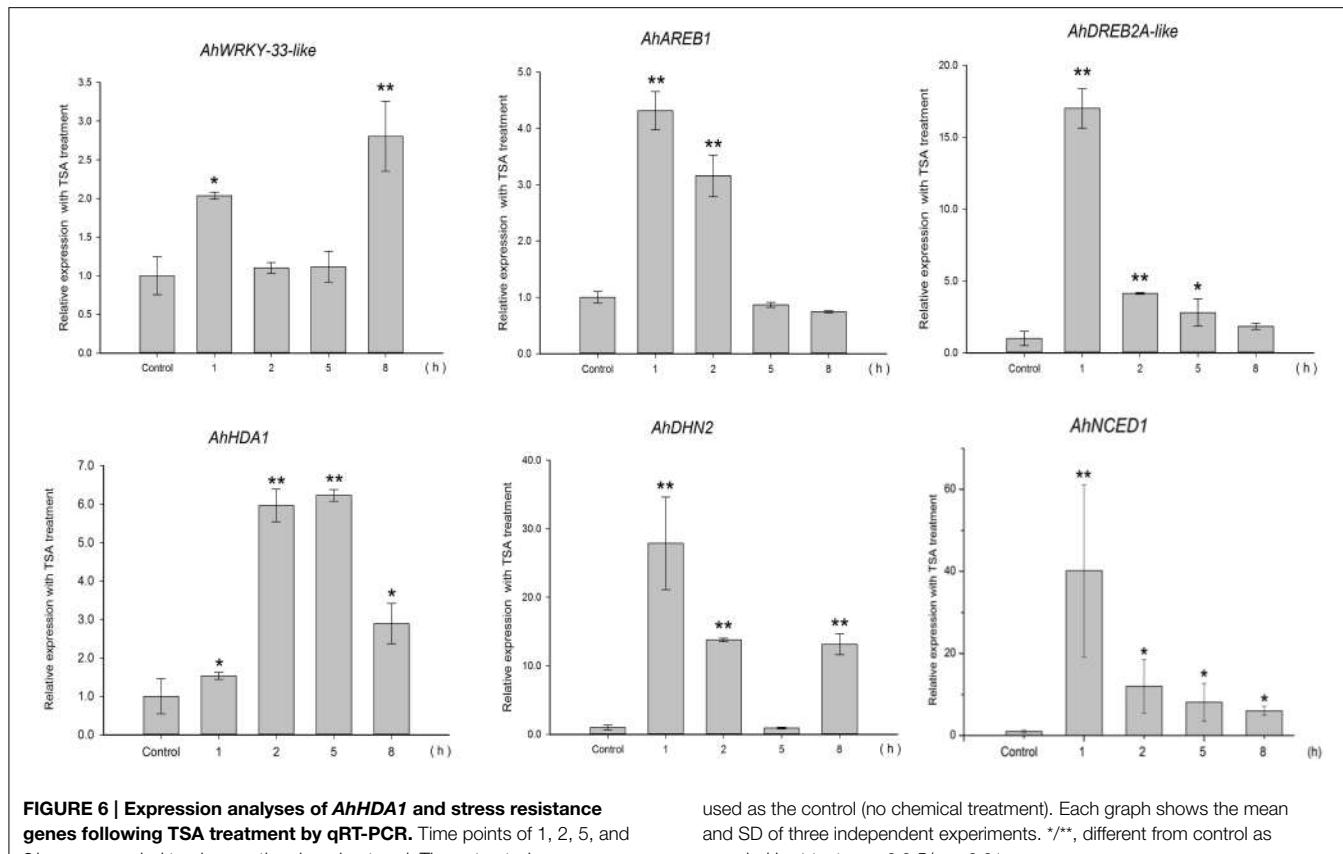


FIGURE 6 | Expression analyses of AhHDA1 and stress resistance genes following TSA treatment by qRT-PCR. Time points of 1, 2, 5, and 8 h were sampled to observe the changing trend. The untreated group was

used as the control (no chemical treatment). Each graph shows the mean and SD of three independent experiments. **, different from control as revealed by t-test, $p < 0.05/p < 0.01$.

extract from *E. coli* expressing AhHDA1 was tested for HDAC activity (Figure 7B, Figure S3). The HDAC activity of an extract from cells containing plasmid pPROEX (i.e., the expression vector control) and the HDAC activity of an extract from cells containing plasmid pPROEX-AhHDA1 without IPTG treatment were 4.6 and 5.2 U/mg, respectively. When AhHDA1 expression was induced by IPTG treatment, HDAC activity of the cell extract increased to 54.1 U/mg; purified recombinant AhHDA1 protein gave an activity of 21.0 U/mg. When the HDAC inhibitor TSA was added to either the induced cell extract or to purified recombinant AhHDA1 protein, the HDAC activity decreased to 17.0 and 7.4 U/mg, respectively. The presence of HDAC activity correlated with expression of recombinant AhHDA1 protein after induction by IPTG, suggesting that the peanut protein is functional; the results also confirm that TSA effectively inhibits AhHDA1 activity.

Discussion

The Consequences of Osmotic Stress and ABA Treatment for Histone Acetylation of H3K9 and H3K14 and Gene Expression in *Arachis Hypogaea* L.

Histone acetylation is a common modification of plant chromatin and plays a critical role in the epigenetic control of gene

expression. It is involved in the response to both drought and ABA in various plants, including Arabidopsis, rice, and maize (Hu et al., 2011; Vlachonasios et al., 2011; Fang et al., 2014). Kim et al. have proposed that enrichment of H3K9ac, but not H3K14ac, correlates with gene activation in the coding regions of drought-responsive genes in Arabidopsis (Kim et al., 2008). However, in peanut, we found that water limitation resulted in increased acetylation of both H3K9 and H3K14, albeit at different time points. Thus, H3K9ac levels were significantly enhanced by 2 h of PEG treatment, and continued to increase throughout the experiment (up to 8 h). In contrast, H3K14ac levels increased, but not until 8 h after osmotic stress was imposed (Figure 1A). A different result was obtained with ABA: increased acetylation of H3K14 was observed after treatment for 5 h (Figure 1B), but there was relatively little effect on H3K9 acetylation for the duration of the experiment. Thus, the degree of acetylation in each case indicates that osmotic stress stimulates histone acetylation mainly at the H3K9 locus, whereas ABA induces histone acetylation primarily on H3K14. An explanation for this result might be that stress-responsive gene expression is governed by two different types of TF, AREB/ABFs, and DREB2A, which operate through the ABA-dependent and ABA-independent signaling pathways, respectively (Sreenivasulu et al., 2006; Fujita et al., 2011; Yoshida et al., 2014). Thus, the SnRK2-AREB (ABA-responsive element binding)/ABF pathway governs the majority of ABA-mediated AREB-dependent gene expression

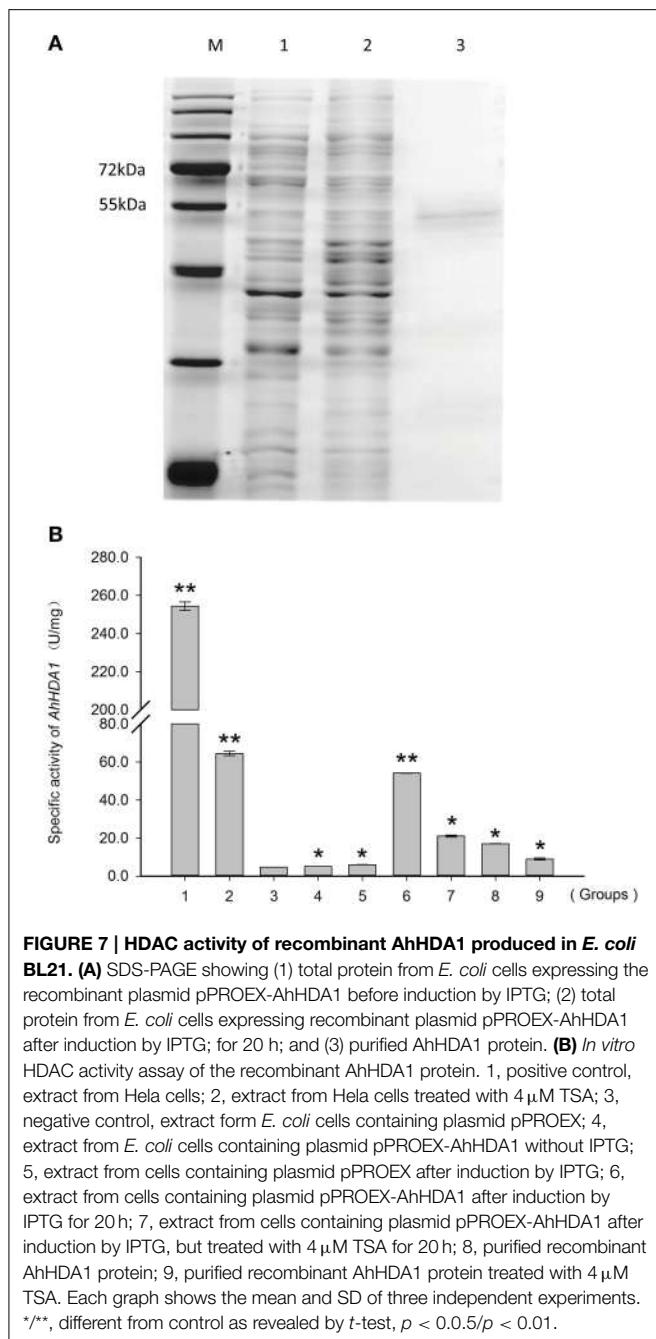


FIGURE 7 | HDAC activity of recombinant AhHDA1 produced in *E. coli* BL21. **(A)** SDS-PAGE showing (1) total protein from *E. coli* cells expressing the recombinant plasmid pPROEX-AhHDA1 before induction by IPTG; (2) total protein from *E. coli* cells expressing recombinant plasmid pPROEX-AhHDA1 after induction by IPTG; for 20 h; and (3) purified AhHDA1 protein. **(B)** *In vitro* HDAC activity assay of the recombinant AhHDA1 protein. 1, positive control, extract from HeLa cells; 2, extract from HeLa cells treated with 4 μ M TSA; 3, negative control, extract from *E. coli* cells containing plasmid pPROEX; 4, extract from *E. coli* cells containing plasmid pPROEX-AhHDA1 without IPTG; 5, extract from cells containing plasmid pPROEX after induction by IPTG; 6, extract from cells containing plasmid pPROEX-AhHDA1 after induction by IPTG for 20 h; 7, extract from cells containing plasmid pPROEX-AhHDA1 after induction by IPTG, but treated with 4 μ M TSA for 20 h; 8, purified recombinant AhHDA1 protein; 9, purified recombinant AhHDA1 protein treated with 4 μ M TSA. Each graph shows the mean and SD of three independent experiments. **, different from control as revealed by *t*-test, $p < 0.05/p < 0.01$.

in response to osmotic stress during the vegetative stage of Arabidopsis (Fujita et al., 2011), while an ABA-independent but interactive pathway acts via the dehydration-responsive element binding (DREB) 2A TF (Sreenivasulu et al., 2006).

The effect of osmotic stress and exogenous ABA function on AhHDA1 was examined and it was found to be up-regulated by both PEG and ABA early in the response to both treatments (Figures 4, 5). However, the mechanisms underlying these responses are not known, and therefore it is not clear whether ABA and osmotic stress act on AhHDA1 via a common pathway or via independent pathways. Given that AhHDA1 transcription

begins to increase significantly after 1 h of ABA treatment, but not until 2 h after PEG treatment, and that H3K14ac increases from 1 h of ABA treatment, while H3K9ac increases from 2 h of PEG treatment, it is possible that ABA-dependent stress-responsive genes are activated through modification of the H3K14 locus, and ABA-independent stress-responsive genes are activated at the H3K9 locus. The increased AhHDA1 expression induced by PEG or ABA might result from rapid changes in the HDAC and HAT “switches,” which re-balance histone and deacetylation.

AhHDA1 is a RPD3/HDA1 Histone Deacetylase Subfamily Protein and is Structurally Similar to AtHDA6

Based on our understanding of the relationship between stress and histone acetylation, together with our analysis of RNA-seq data, we may surmise that HDAC activity in peanut plays an important role in the responses to both osmotic stress and ABA treatment. In this paper, we isolated and characterized AhHDA1 from *A. hypogaea* L. cv Yueyou 7, a drought-resistant peanut variety we have reported on in previous studies (Fang et al., 2007). AhHDA1 accumulates in the stem and root in seedlings, while it predominantly accumulates in the radicle and mesocotyl in the embryo (Figure 3). Both the predicted sequence and structure of the AhHDA1 protein appear to be well-conserved as judged by multiple sequence alignment, phylogenetic analysis and comparison of AtHDA6 and AhHDA1 ribbon diagrams (Figure 2, Figure S1). The HDAC activity of AhHDA1 was demonstrated by heterologous expression of recombinant protein in bacteria and this activity was inhibited by the specific HDAC inhibitor TSA. Using the Arabidopsis *HDA6* mutant *axe1-5* and *HDA6* RNA-interfering plants, which display higher sensitivity to NaCl and ABA than wild type, Chen et al. found AtHDA6 to be involved in histone modifications that modulate seed germination and the salt stress response (Chen et al., 2010). AtHDA6 mutations also result in transcriptional gene silencing, which influences the expression of auxin-inducible genes (Murfett et al., 2001). Given its structural relatedness to AtHDA6, AhHDA1 may possess similar functions.

The Relationship between Environmental Stress, Histone Acetylation Status and Activity of Stress Resistance Genes

TSA is an HDAC inhibitor that induces transient hyperacetylation of histones H2B, H4, and H3 (Waterborg, 2011). Drought-induced *RAB18*, *RD29B*, *HSP70* and four late embryogenesis abundant protein genes (*LEA*) are up-regulated by TSA in imbibing *A. thaliana* seeds (Tai et al., 2005). In our studies, TSA promoted the expression of AhHDA1 (Figure 6) and induced acetylation of H3 (Figure S4). The expression patterns of three TF genes (*AhAREB1*, *AhDREB2A-like*, and *AhWRKY33-like*) and two functional genes (*AhDHN2* and *AhNCED1*) were also determined to study how TSA acted on stress resistance genes. TF gene expression was found to increase at an early time point after TSA treatment, then decreased as time went on. Although the expression profile of the functional

genes was similar, the transcript level of these genes remained high relative to the control groups.

Because HDACs are inhibited by TSA which induces transient hyperacetylation of histone H3 (Figure 7, Figure S4) (Finnin et al., 1999), it seems reasonable to suppose that the up-regulated expression of *AhHDA1* following TSA treatment results from a feedback mechanism to re-establish the balance of histone acetylation and diacetylation in the plant. We might speculate that the mechanism of action of environmental stress, including osmotic stress and ABA signaling, on *AhHDA1* expression is as follows: histone acetylation is enhanced in peanut leaves soon after they are exposed to osmotic stress or ABA; subsequently, upstream TFs become activated and induce the expression of functional genes. Later, TF activity is modulated to a relatively insensitive state as the products of functional genes, such as the dehydrin AhDHN2, begin to protect plant cells from environmental stress damage.

Our work on *AhHDA1* has encompassed bioinformatic analysis of the gene, *in vitro* activity analysis of the corresponding recombinant protein and analysis of the effects of osmotic stress and ABA on *AhHDA1* expression. We conclude that *AhHDA1*, which is very similar to *AtHDA6*, is up-regulated by osmotic stress, ABA, and TSA. Future studies will focus on which genes undergo specific histone acetylation in response to water

limitation and ABA treatment, and on an investigation of the critical genes in ABA-dependent and ABA-independent signaling pathways. These might help elucidate the molecular mechanisms of drought resistance, results that could be used to produce new varieties of crops for cultivation in water-limiting conditions.

Author Contributions

LCS, drafted the manuscript; BD, conducted the bioinformatics analysis; LL, LCS, BD, conceived and designed the experiments; LCS, BD, SL, LML, BH, YTZ, performed the experiments; BH, analyzed the data; LL, contributed reagents/materials/analysis tools.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00512>

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Epi-fingerprinting and epi-interventions for improved crop production and food quality

Carlos M. Rodríguez López* and Mike J. Wilkinson

Plant Research Centre, School of Agriculture, Food and Wine, Faculty of Sciences, University of Adelaide, Adelaide, SA, Australia

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Edited by:

Raúl Alvarez-Venegas,
Centro de Investigación y de Estudios
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Nacional, Mexico

Reviewed by:

Paula Casati,
Centro de Estudios Fotosintéticos y
Bioquímicos, Argentina
Igor Kovalchuk,
University of Lethbridge, Canada

*Correspondence:

Carlos M. Rodríguez López,
Plant Research Centre, School of
Agriculture, Food and Wine, Faculty of
Sciences, University of Adelaide,
Waite Campus, PMB1, Glen
Osmond, Adelaide, SA 5064, Australia
carlos.rodriguezlopez@adelaide.edu.au

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Increasing crop production at a time of rapid climate change represents the greatest challenge facing contemporary agricultural research. Our understanding of the genetic control of yield derives from controlled field experiments designed to minimize environmental variance. In spite of these efforts there is substantial residual variability among plants attributable to Genotype × Environment interactions. Recent advances in the field of epigenetics have revealed a plethora of gene control mechanisms that could account for much of this unassigned variation. These systems act as a regulatory interface between the perception of the environment and associated alterations in gene expression. Direct intervention of epigenetic control systems hold the enticing promise of creating new sources of variability that could enhance crop performance. Equally, understanding the relationship between various epigenetic states and responses of the crop to specific aspects of the growing environment (epigenetic fingerprinting) could allow for a more tailored approach to plant agronomy. In this review, we explore the many ways in which epigenetic interventions and epigenetic fingerprinting can be deployed for the improvement of crop production and quality.

Keywords: Fingerprinting, epigenetics, crop biotechnology, crop plants, crop quality, crop protection, crop improvement, priming

Context

The sustained growth in food production over the 50 years since the start of the green revolution can be at least partly explained by the introduction of molecular approaches to crop breeding (Evenson and Gollin, 2000). Systematic marker-assisted introgression has now become a mainstay of genetic improvement programs (Collard and Mackill, 2008) and yet some of the most successful varieties of several crops have arisen spontaneously, and have been identified by simple phenotypic selection. These so-called ‘sports’ are far more common in crops that are propagated vegetatively, and can often form a substantial proportion of the varieties grown. The source of the observed phenotypic divergence in sports is often assumed to have a genetic rather than epigenetic origin (Schmitz et al., 2013). In either case, the genetic divergence between sports and their progenitor lines is inevitably minimal, and so are notoriously difficult to differentiate using conventional molecular markers (Breto et al., 2001). The reality is that for the vast majority of instances we do not fully understand how phenotypic variability can be explained at the molecular level (Ball, 2013). This uncertainty is often exacerbated by poor trait definition and a lack of genomic resolution (King et al., 2010) but may sometimes arise from a mistaken presumption of genetic rather than epigenetic causality (Breto et al., 2001; Rois et al., 2013). Ever since Waddington (1942) first

proposed the term epigenotype to describe the interface between genotype and phenotype, the science of epigenetics has been progressively adding more layers of complexity to our knowledge of how information is stored and utilized within the living cell. Recent years has seen a dramatic increase in the depth of understanding of how epigenetic control mechanisms operate. There is now growing desire to better understand the stability and role of epigenetic regulatory systems in controlling development, shaping the phenotype, and determining the physiological resilience of higher organisms surviving in fluctuating environments (Geyer et al., 2011; Bräutigam et al., 2013).

Epigenetic processes can affect a phenotype without altering the genetic code (Bird, 2007) and can operate in a number of ways to alter the availability or efficacy of DNA sequences for transcription; determine transcript identity or amend the longevity of mRNA transcripts in the cell (for review, see Chahwan et al., 2011) or by changing the stability or activity of protein products. The many epigenetic mechanisms that mediate these effects include modifications of histone tags, ATP-dependent chromatin remodeling, polycomb/trithorax protein complexes, chemical modification on DNA bases and regulatory processes directing mRNA degradation and alterations to DNA chemistry driven by small RNA molecules, with circular RNA as the latest addition (Wilusz and Sharp, 2013) to the many small RNAs that fulfill this role (i.e., lncRNA, siRNA, microRNA). This array of processes is clearly interconnected and almost certainly acts in a complex, interactive and redundant fashion (Grant-Downton and Dickinson, 2005; Berger, 2007). Describing all the methods developed to study all the mentioned epigenetic layers is outside the scope of this review and we will instead focus on the potential role of the best-studied epigenetic mechanism, DNA methylation, as a route to elicit new advances in crop improvement.

Epigenetic Interventions and Crop Improvement

Applied epigenetics is an area of science that is evolving rapidly and spawning new opportunities for the enhancement of crop production. DNA methylation involves the addition of a methyl group to carbon 5 of cytosine bases (forming 5-methylcytosine, 5mC). In plants, DNA methylation can occur in three contexts (i.e., CG, CHG, or CHH, H = a nucleotide other than G). DNA methylation occurring within promoters or coding regions typically act to repress gene transcription. RNA-directed DNA Methylation (RdDM) is an important mechanism by which plants can achieve targeted DNA methylation to reduce expression of a particular gene (Wassenegger et al., 1994). This form of gene silencing is directed by small interfering RNAs (siRNAs) and is often associated with the silencing of transposable elements (TEs). However, the system can also repress the expression of endogenous genes, especially those positioned close to TEs. RdDM relies on the activity of DICER-like 3 (DCL3), Argonaute 4 (AGO4) and the DNA-dependent RNA polymerases Pol IV, and Pol V and the RNA-dependent polymerase RDR2. Collectively,

the products of these genes direct the DOMAIN REARRANGED METHYLTRANSFERASE 2 (DRM2) protein to add methyl groups to Cytosines within the targeted region and so repress expression (Naumann et al., 2011). In this way the expression of genes that regulate development or cell metabolism can be altered (Becker and Weigel, 2012). The first and most direct means of exploiting this relationship is through the deliberate perturbation of global methylation patterns via exogenous interventions. This can be achieved in several ways. Most simply, chemical inhibitors of DNA methyltransferases such as 5-azacytidine or decitabine can be used to cause partial, genome-wide DNA demethylation (Stresemann and Lyko, 2008) and so generate new ‘epigenetic’ variants that hopefully include epi-alleles that confer desirable changes to crop phenotype. Amoah et al. (2012) used this strategy when they applied 5-azacytidine to seedlings of rapeseed (*Brassica napus*) and generated novel lines that exhibited increased seed protein content. This blind tactic for the release of new variation is perhaps most analogous to mutation breeding and relies on the screening of similarly large numbers of individuals to yield positive results. It nevertheless offers the tangible benefit of not requiring a deep understanding of the mechanisms involved.

A more directed approach to epigenetic intervention is made possible by reference to the relationship between changes in the growing environment and associated changes in methylation-driven gene expression. One system by which plants can increase their resilience to challenge by biotic or abiotic threats is by intensifying the responsiveness of their immune system after recognition of specific signals from their environment. This so-called ‘priming’ provides potentially long-lasting protection and is based on eliciting a faster and/or stronger reaction upon subsequent challenge by the same or related stressor (Conrath, 2011). The primed response is made possible by increased sensitivity of previously exposed plants to signal molecules such as b-aminobutyric acid (BABA), volatile organic compounds associated with herbivore damage or to strain-specific pathogen effectors (Pastor et al., 2013). Several studies indicate that the primed response of plants to pathogen attack is mediated through early and strong activation of immune response systems such as the Salicylic Acid (SA) pathway (Kohler et al., 2002; Jung et al., 2009) and the Jasmonic Acid pathway (Turlings and Ton, 2006; Heil and Ton, 2008). It is now becoming clear that RdDM-associated DNA methylation is sometimes implicated in the improved responsiveness of primed plants. For instance, Agorio and Vera (2007) showed that AGO4 is required for full resistance in *Arabidopsis* against *Pseudomonas syringae* and by implication RdDM-mediated methylation. Yu et al. (2013) showed that some TEs become demethylated in *Arabidopsis* following exposure to *P. syringae* and that this change is associated with restricted multiplication and vascular propagation of the pathogen. The authors inferred that the widespread demethylation of the TEs may have caused prime transcriptional activation of some defense genes. Other studies have similarly shown that manipulation of the growing environment can also evoke DNA methylation-mediated changes to the expression of genes that can influence yield, such as stomatal development (Tricker et al., 2012) or aspects of product quality such as vitamin E levels (Quadrana et al., 2014). Whatever the mechanism of operation leading

to these effects, the ability to enhance the defensive capability of crop plants through the prior exposure to signal molecules or to disabled or denign pathogens has innate appeal. This prospect is most immediately tangible for clonal crops, where the effect of the conditioning treatment on methylation-mediated changes to phenotype need not pass through a filial generation. For most seed crops, however, there is the need that the induced changes to methylation status remains stable across generations for methylation-based priming to have practical utility. There is now growing evidence to suggest that at least some environmentally induced methylation marks can remain stable between generations, implying that intergenerational plant priming may also be possible.

Molinier et al. (2006) provided the first compelling evidence that environmentally induced epigenetic change can be retained over subsequent generations that were naïve to the eliciting factor. In this case, exposure to UV and flagellin (an elicitor of plant defenses) was seen to cause *Arabidopsis* to respond by increasing homologous recombination as detected by restoration of transgene function. Whilst the authors were unable to assign the effect to a particular epigenetic mechanism, they were able to demonstrate that the effect did not require presence of the transgene, was dominant, could be inherited from either parent and persisted for at least four filial generations. Boyko et al. (2007) subsequently found that progeny of tobacco mosaic virus (TMV)-infected plants show reduced methylation levels of R-gene-like genes, and enhanced resistance to different pathogens (Kathiria et al., 2010). Likewise, Slaughter et al. (2012) demonstrated that *Arabidopsis* exposed to localized infection by an avirulent strain of *P. syringae* or priming-inducing treatments with BABA produce descendants that are more resistant to *Hyaloperonospora arabidopsis*. These and many other examples of transgenerational priming of resistance (for review, see Pastor et al., 2013) imply that it may be possible to supply the grower communities with seed lots as well as clonal cuttings that are primed to enhance tolerance to biotic or abiotic stresses. Delivery of such a service will depend on stability of the effect, ability to assure that the expected change to DNA methylation has occurred, and most importantly, that there are no yield penalties associated with the priming event itself. Certainly, Luna et al. (2012) demonstrated that whilst the asymmetric DNA methyltransferase (drm1drm2cmt3) triple mutant of *Arabidopsis* (blocked for RdDM-dependent DNA methylation function) is more resistant to biotrophic pathogens such as *H. arabidopsis* and *P. syringae*, it is also more susceptible to the necrotrophic fungus *Alternaria brassicicola*. Thus, it is entirely plausible that some beneficial changes that are induced by priming may come at the expense of some associated detrimental features. The nature of such interactions will no doubt emerge with time and effort.

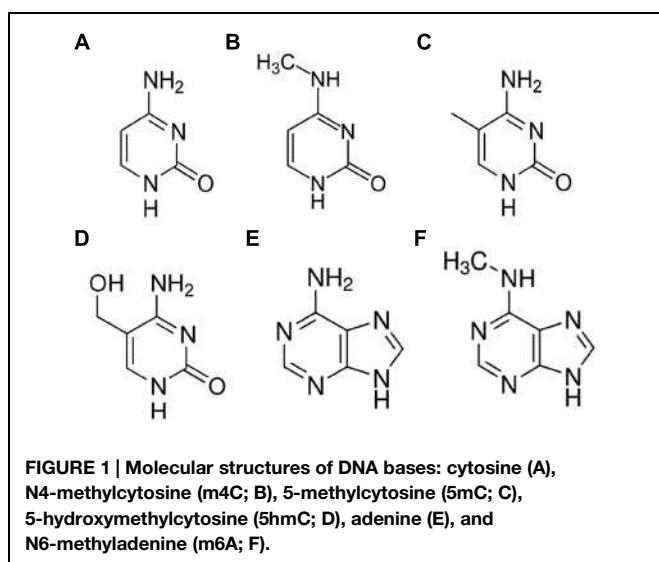
There are also more direct ways in which transgenerational stability of epi-alleles could ultimately be integrated into crop breeding efforts. In a landmark paper, Hauben et al. (2009) demonstrated that it is possible to obtain stable epigenotypes exhibiting improved energy use efficiency (an important yield determinant) through recurrent phenotypic selection of isogenic *B. napus* lines. Furthermore, crosses between these genetically identical but epigenetically divergent lines generated hybrids

with a 5% yield increase on top of heterosis. Tricker et al. (2013a) showed that environmentally induced epi-alleles associated with drought and low relative humidity tolerance can become fixed and remain stable over several generations. These observations raise the scope of targeted management of the growing environment during breeding to deliberately elicit and fix epigenetic changes responsible for control of a particular trait or developmental process. The high likelihood that genotypes will vary in their capacity to become primed or to remain stably fixed in a desired state (Daymond et al., 2011) provides scope for simultaneous genetic and epigenetic selection for (or against) aspects of plant plasticity and resilience. To our knowledge, this type of profiling has yet to be formally incorporated into commercial breeding efforts. In the following sections we therefore explore a range of specific approaches that hold promise to enhance contemporary crop improvement efforts.

At a more fundamental level, Cortijo et al. (2014) have provided an elegant illustration of how the transgenerational stability of some induced methylation marks can be usefully exploited for forward genetics efforts when they were able to construct linkage maps to describe the epigenetic basis of complex traits, so-called epiQTL analysis (Long et al., 2011; Cortijo et al., 2014). This strategy has the significant potential advantage over conventional QTL analysis by circumventing the need for functional mutational differences between parental genotypes of mapping populations used for forward genetics.

Use of DNA Methylation as a Biomarker

DNA methylation-dependent gene regulation plays an important role in orchestrating cellular differentiation and development (Rogers and Rogers, 1995; Manning et al., 2006; Henderson and Jacobsen, 2007; Feng et al., 2010; Ito et al., 2010; Yaish et al., 2011) and also provides the basis for genome–environment interactions that confer agility and plasticity of gene expression, and mediates molecular response to fluctuations in the living environment (Amoah et al., 2012). The genomes of almost all phyla include at least one alternate form of chemically modified base (Hattman, 2005), including N6-methyladenine (m6A), N4-methylcytosine (m4C), 5-methylcytosine (5mC), and 5-hydroxymethylcytosine (5hmC) (Figure 1). Of these, 5mC is by far the best studied and was originally thought to be the only functional base modification found in higher organisms (Kriaucionis and Heintz, 2009). Environmentally induced changes in 5mC have also been shown to be at least partially stable between filial generations (Tricker et al., 2013a; Cortijo et al., 2014). We are just starting to understand the mechanisms that either prevent or permit the inheritance of such epigenetic changes (Iwasaki and Paszkowski, 2014). The value of a particular 5mC as a biomarker for a particular physiological or developmental state relies partly on the consistency its association with each particular state but also on its stability. There is considerable variation in the extent to which a locus shows both consistency and stability. For example, in tomato, a spontaneous epi-allele (*cnr*) is responsible for the inhibition of fruit ripening in some epi-mutant lines (Manning



et al., 2006). The methylation status of sites within this locus are highly predictive of the observed phenotype and reversions (demethylation and associated phenotypic change) occur at a frequency of roughly one in 1000. In comparison, mutability of the epigenetic silencing of the DWARF1 gene in rice occurs in around 1 in 10 plants (Miura et al., 2009). Overall, it appears that DNA methylation patterns do not fluctuate randomly between generations or in response to the environment but neither are they completely stable (Becker and Weigel, 2012). It will therefore be desirable to identify specific sites or loci that are both stable and predictable for a particular state to maximize the capacity to apply epifingerprinting techniques across a wide range of germplasm and also between laboratories.

It is now emerging that other modified bases are also present in at least some eukaryotic organisms. These most notably include m6A and 5hmC, although relatively little is currently known about the distribution or function of these bases in plants (Ashapkin et al., 2002). The methylated modification of adenine, m6A, was first discovered in *Escherichia coli* and has since been found in a wide range of prokaryotes and simple eukaryotes (e.g., prokaryotes Dunn and Smith, 1955; ciliates, Hattman, 2005). In prokaryotes, it appears that m6A induces DNA conformational changes that alter protein-DNA interactions (Sternberg, 1985). There is indirect evidence that m6A may also be present in mammals (Polaczek et al., 1998; Ashapkin et al., 2002) although this has yet to be demonstrated unequivocally. There is stronger evidence for the presence of m6A in plants (Ashapkin et al., 2002), including the identification of a putative adenine DNA methyltransferase gene in the genome of *Arabidopsis thaliana* (Sternberg, 1985). While it is unclear whether m6A is essential for the regulation of eukaryotic genes, the detection of m6A residues in the DNA methylation maintenance gene DRM2 (Ashapkin et al., 2002) implies that this possibility is at least plausible, and that the presence and location of this modified base could be used for diagnostic purposes.

The alternate modification of cytosine, 5hmC, is present both in the nuclear (Kriaucionis and Heintz, 2009) and mitochondrial

(Shock et al., 2011) genomes of mammals. This form of the base is far less abundant than 5mC and is typically more highly tissue-specific (Muenzel et al., 2011), perhaps implying a role in tissue differentiation and development. In plants, 5hmC has only been reported in the genome of chloroplasts (Morovicá et al., 2013) although more recent publications demonstrate that it is either absent or present at undetectably low levels in plants (Erdmann et al., 2015). This form of cytosine has been proposed as an intermediate in either the active or passive demethylation of 5mC (Huang et al., 2010). However, recent evidence leads some to suggest that it may have an important functional role in its own right, at least in animals (Robertson et al., 2011a; Wu et al., 2011). Moreover, under high resolution melting (HRM) conditions 5mC has been shown to elicit a stabilizing effect to the double stranded DNA structure (Rodríguez López et al., 2010a); a feature that accords with its reported effect on the fine structure of DNA (Heinemann and Hahn, 1992). In contrast, spectroscopic (Thalhammer et al., 2011), calorimetric (Wanunu et al., 2011), and HRM (Rodríguez López et al., 2012a) analyses have all suggested that presence of the alternate base modifications (5hmC and m6A) in the DNA could reverse the stabilizing effect of 5mC. Whether or not the changes to DNA thermostability induced by 5hmC have functional impact on gene expression is still a matter of conjecture. Certainly, some authors have reported that 5mC hydroxylation is associated with the activation of gene transcription (Ito et al., 2010; Thalhammer et al., 2011; Wanunu et al., 2011) while others argue that any contribution to transcriptional activation or repression is highly context-dependent (Wu et al., 2011). Whatever role (if any) that these alternate base modifications play in gene regulation, it is already clear that they are far less abundant, if present at all, in the plant genome than 5mC and so probably hold only limited value as diagnostic marks for epifingerprinting purposes. It is therefore the distribution of 5mC in the genome that has formed the focus of attempts to link epifingerprints to the physiological, developmental, or stress status of higher organisms, including crop plants.

An array of methods has been developed to describe the global pattern of 5mC across the genome (for extensive reviews on the subject, see Tost and Gut, 2009; Chaudhry, 2010; Plongthongkum et al., 2014). All methods carry their own limitations (Rodríguez López et al., 2010a) but can be broadly grouped into three functional types that: (1) indicate the methylation status of a specific sequence; (2) reveal the degree and patterning of DNA methylation across partly characterized genomes; (3) facilitate the discovery and sequencing of new epialleles (Fraga and Esteller, 2002; Dahl and Guldberg, 2003).

The Potential Value of Epi-Fingerprinting for Agriculture

Epi-Fingerprinting of *In Vitro* Cultured Plant Material

The ability to propagate elite or desirable clones is an essential part of the seed production industry. The advent of reliable *in vitro* systems for the replication and regeneration

of plant materials has led to their widespread deployment for propagation (Bertrand et al., 2011; Etienne et al., 2012), germplasm conservation (Fang et al., 2009), and breeding purposes (Henry, 1998), as well as for more fundamental research on model species (Berdasco et al., 2008; De-la-Peña et al., 2012; Moricová et al., 2013). For micropropagation and genetic transformation systems to be efficient, it is necessary that the plants recovered from them are genetically and epigenetically faithful to the original stock material. Trueness-to-type is of particular importance when propagating elite genotypes of high value crops such as grapevine: especially in traditional vine areas where high clone quality is a prerequisite (Schellenbaum et al., 2008). In comparison to genetic somaclonal variation, divergence between DNA methylation patterns is generally wider among regenerated plants and can be directly associated with 'plastic' phenotypic variation (Miguel and Marum, 2011). The loss of epigenetic fidelity during micropropagation has been a major source of economic damage in several crops. For instance, in oil palm, mantled inflorescence syndrome was found to be associated with global changes to C-methylation status during micropropagation, and caused catastrophic reductions in yield among all affected plants and incurred huge costs to the industry (Matthes et al., 2001). Many studies have reported global changes to the distribution of cytosine methylation can be induced by *in vitro* culture spanning an impressive array of species in a wide taxonomic spread. Examples include: tobacco (*Nicotiana tabacum*, Schmitt et al., 1997); rice (*Oryza sativa*, Xiong et al., 1999); strawberry (*Fragaria Xananassa*, Hao et al., 2002); potato (*Solanum tuberosum*, Joyce and Cassells, 2002; *A. thaliana*, Bardini et al., 2003); oil palm (*Elaeis guineensis*, Jaligot et al., 2004); and cocoa (*Theobroma cacao*, Rodríguez López et al., 2010b). On the other hand, some forms of such 'somaclonal variation' may offer a source of valuable new variation that has potential applications in plant breeding (Henry, 1998). Furthermore, different studies have shown that epigenetic regulation plays an important role during plant development *in vitro* (Rodríguez López et al., 2010b; Nic-Can et al., 2013). Regardless of whether the change in methylation status evokes a desirable or unwanted outcome, there is clearly great value in the ability to detect these changes or at least to predict the scale of any phenotypic or physiological divergence. Advances in our understanding of the links between gene expression and phenotype mean that the ambition may now turn from simply viewing these plants as a new source of variation for breeding and toward a more targeted approach that deliberately manipulates the process for use in crop improvement efforts.

Epi-Fingerprinting for Breeding and Varietal Selection

The majority of agricultural land is cultivated with commodity crops that are either highly inbred or clonal. These genetically invariant populations nevertheless exhibit measurable morphological or developmental plasticity, even when grown under controlled conditions, which may be at least partly explained by stable epigenomic states (Hauben et al., 2009). It has

recently been argued that these epigenetic sources of variation may even be greater than those attributable to genetic causes (Hirsch et al., 2013; Schmitz et al., 2013). Several authors have linked genotype-specific changes to DNA methylation to yield components or to other agronomically desirable traits (e.g., Gourcilleau et al., 2010; Alonso et al., 2014; **Table 1**). The first classic example of a single epiallelic gene variant was attributed to hypermethylation of the CYCLOIDEA gene of *Linaria vulgaris*; a state which causes radial symmetry of previously bilaterally symmetric flowers (Cubas et al., 1999). Other epigenetic variants have subsequently emerged with features that have economic potential. For instance, the hypomethylation of the rice gene FIE1 induces its ectopic expression and results in a dwarf and flower-aberrant phenotype (Zhang et al., 2012). Goettel and Messing (2013) reported that cytosine methylation of a gene (P1-rr) encoding for a Myb-like transcription factor that mediates pigmentation in floral organs and grains, is negatively correlated with transcription and pigment levels. These mutations are thought to have arisen spontaneously by somatic epimutation and later became fixed after repeated passage through meiosis.

Systematic selection for fixed epi-loci is not the only possible source of new varietal material with potential to improve crop production or quality. Environmentally induced epi-alleles also offer an important potential source of exploitable variation. For many inbreeding and clonal crops, environmentally induced epigenetic variation can sometimes outweigh genetic variation, with such changes being induced by exposure to various aspects of the living environment (Raj et al., 2011; Tricker et al., 2012; Hirsch et al., 2013). These properties can lead to an epigenetic convergence of populations when grown under similar conditions (Schulz et al., 2014) but can also lead to spontaneous divergence of fixed epigenetic states (Becker et al., 2011). Tricker et al. (2013b) proposed an approach in which the deliberate manipulation of the specific aspects of the growing environment could be used to induce desirable changes in tolerance to low humidity and periodic drought. Nevertheless, the disentanglement of this kind of epigenetic variation from the genetic background that underpins the capacity to produce new variability continues to pose major technical difficulties (Cortijo et al., 2014) and is probably still some way from commercial reality.

For vegetatively propagated perennial crops such as grapevine (Zufferey et al., 2000) or *Pinus radiata* (Fraga et al., 2001) the need to fix between generations is circumvented. For these crops there is a long association between productivity and quality characteristics and plant age. The possibility that this relationship has an epigenetic basis and so is amiable for manipulation is especially appealing. Certainly, it is known that DNA methylation changes progressively during maturation and aging, for both plants and animals species (Theiss and Follmann, 1980; Quemada et al., 1987; Fraga et al., 2005). There is also evidence that these changes are associated with altered expression of genes that are implicated in morphological changes in plants (Galaud et al., 1993) and animals (Zhang et al., 2002). More specifically, the extent of genomic DNA methylation in pine is a strong indicator of aging and can provide

TABLE 1 | Examples of plant genes involved in agronomic traits affected by DNA methylation.

Epiallele type	Locus	Epigenetic regulation	Trait	Reference
Stress response epialles	LFR	Regulated by DNA methylation	Disease resistance	Yu et al. (2013)
	Plastooyanin-like domain	Differentially methylated	Low pH and aluminium stress in sorghum	Kimata et al. (2011)
	CalB domain family protein	Possible regulation by DNA methylation	Abiotic stress signaling	Dubin et al. (2014)
	BAL (BAL)		Pathogen resistance	Stokes et al. (2002)
	CIPK		Abiotic stress response in plants	Shan et al. (2013)
	CBS domain-containing protein	Differentially methylated under cold stress	Abiotic stress response in plants	Shan et al. (2013)
	Phosphoribulokinase/Uridine kinase family		Photosynthesis and energy metabolism	Pan et al. (2011)
	SPEECHLESS	Differentially methylated under low relative humidity	Stomata development control	Tricker et al. (2012)
	FAMA	Differentially methylated under low relative humidity	Stomata development control	Tricker et al. (2012)
	NTGPDL	Differentially methylated under abiotic stress	Abiotic stress response in plants	Choi and Sano (2007)
Developmentally regulated epialles	CRK8	Methylated in rice/Differentially methylated in maize under cold stress	Transposon	Shan et al. (2013)
	CWF19	Methylated	Cell cycle control protein	Jeon et al. (2015)
	EMB71 (MAPKKK4, YDA)	Possible regulation by DNA methylation	Embryo and in stoma development programmes	Tricker personal communication
	ARF2	Target for srRNA	Repressor of cell division and organ growth	Zhang et al. (2014)
	GT-2 related proteins	Regulated by DNA methylation	Organ morphogenesis	Song et al. (2012)
	MEG1 Cys-rich protein	Maternal parent-of-origin expression	Regulates seed development in maize	Gutierrez-Marcos et al. (2004)
	MEA Polycomb protein		Regulates seed development in maize	Grossniklaus et al. (1998)
	FIS2 Transcription factor		Regulates seed development in maize	Luo et al. (1999)
	FIE Polycomb protein		Regulates seed development in maize	Ohad et al. (1996)
	PHERES1 MADS TF		Regulates seed development in maize	Kohler and Makarevich (2006)
Maternally imprinted	MPC Poly(A) binding protein FWA	Maternally imprinted	Positive regulator of flowering	Tiwari et al. (2008)
	SUPERMAN	Hypermethylation induce mutant floral morphologies	Regulation of floral whorls development	Kinoshita et al. (2004)
	AGAMOUS	Hypermethylation induce mutant floral morphologies	Regulation of floral whorls development	Jacobsen and Meyerowitz (1997)
	RIN	Differentially methylated during fruit development	Regulator of shelf life and quality	Ito (2012)
	NOR	Differentially methylated during fruit development	Regulator of shelf life and quality	Zhong et al. (2013)
	PG2A	Differentially methylated during fruit development	Regulator of shelf life and quality	Zhong et al. (2013)
	PSY	Differentially methylated during fruit development	Regulator fruit color	Zhong et al. (2013)
	PDS	Differentially methylated during fruit development	Regulator fruit color	Zhong et al. (2013)

(Continued)

TABLE 1 | Continued

Epiallele type	Locus	Epigenetic regulation	Trait	Reference
pectinesterase-1		Differentially methylated under cold stress in maize	ABA signalling	Shan et al. (2013)
Hexokinase		Differentially methylated under cold stress	Meiosis	Shan et al. (2013)
MADS-box protein		Differentially methylated under cold stress in maize	Cell wall formation	Shan et al. (2013)
Kinesin-like protein		Differentially methylated under cold stress in maize	Flowering time	Shan et al. (2013)
NAM		Hypomethylation-dependent up-regulation in pluripotent protoplasts	Determining the pluripotent state of the cells	Avivi et al. (2004)
ATAF1		Hypomethylation-dependent up-regulation in pluripotent protoplasts	Determining the pluripotent state of the cells	Avivi et al. (2004)
CUC2		Hypomethylation-dependent up-regulation in pluripotent protoplasts	Determining the pluripotent state of the cells	Avivi et al. (2004)
MAPK12		Hypermethylated in callus and cell suspensions		Berdasco et al. (2008)
GSTU10		Hypermethylated in callus and cell suspensions		Berdasco et al. (2008)
<i>In vitro</i> culture induced epialleles				
BXL1		Hypermethylated in callus and cell suspensions		Berdasco et al. (2008)
TTG1		Hypermethylated in cell suspensions		Berdasco et al. (2008)
GSTF5		Hypermethylated in cell suspensions		Berdasco et al. (2008)
SUVH8		Hypermethylated in cell suspensions	Regulates ripening	Berdasco et al. (2008)
CNR		Spontaneous epiallele in tomato		Manning et al. (2006)
SP11		Regulated by Methylation		Shiba et al. (2006)
CYCLOIDEA				
P1		Hypermethylated mutant	Contro floral symmetry <i>Lirnia vulgaris</i>	Cubas et al. (1999)
D1		Hypermethylated in metastable Epi-d1	Grain colour in maize	Das and Messing (1994)
GUN4		Regulated by DNA methylation in rice	Plant height in rice	Mitura et al. (2009)
XTH1		Regulated by DNA methylation in potato	Chlorophyll biosynthesis	Li et al. (2014)
				Da et al. (2012)

molecular evidence of reinvigoration (Fraga et al., 2001). Thus, there is scope to manipulate the methylation status of crop genomes either chemically using methyltransferase inhibitors, by exposure to signaling molecules or by manipulation of the growing environment. Individuals exhibiting stable, rejuvenated methylation profiles, and associated phenotypes could then be selected and used for commercial planting.

Epi-Fingerprinting as an Indicator of Plant Health

In addition to the generation of new variation there is also considerable scope for deploying epigenetic fingerprinting approaches to improve the efficacy of agronomic or prophylactic interventions. Plants are sessile organisms and so unable to avoid abiotic or biotic stresses. They must instead rely on rapid and effective stress response systems to withstand harmful changes to the living environment to enhance their chances of survival. Plants have amassed an array of mechanisms for detecting and then responding to stresses in ways that can include substantial amendments to key metabolic pathways (Madlung and Comai, 2004). Such responses can be activated in a number of ways including the adjustment of the transcriptional control of genes through differential cytosine methylation (Aceituno et al., 2008).

Several authors have noted that large numbers of biotic and abiotic stresses induce global changes to the methylation patterns of plants (Stokes et al., 2002; Boyko and Kovalchuk, 2008, 2011; Chinnusamy and Zhu, 2009). This feature means there is often a clear relationship between the detection of a particular stress by a plant and overall C-methylation profile. This property means that there is scope for the use of C-methylation fingerprinting approaches as a tool to diagnose the early onset or asymptomatic exposure of a crop to a range of stresses. Several workers have demonstrated that diagnostic changes in methylation fingerprints are associated with exposure to a wide range of abiotic stresses including drought (Raj et al., 2011; Tricker et al., 2013b), low relative humidity, (Tricker et al., 2012), low temperatures (Pan et al., 2011), salt and heavy metals (Choi and Sano, 2007; Verhoeven et al., 2010), and low nutrient levels (Verhoeven et al., 2010). The same is seemingly also true for exposure to biotic stresses, with changed DNA methylation profiles also being reported following plant-herbivore (Verhoeven et al., 2010; Herrera and Bazaga, 2013) and plant-pathogen interactions (Mason et al., 2008; Boyko and Kovalchuk, 2011). These observations have yet to be used as a basis to develop a robust set of methylation markers to routinely diagnose exposure of crops to these stresses but this aspiration appears both attractive and tractable within a relatively short time period.

There is also opportunity to use C-methylation profiling to gain better understanding of the relationship between the stress and the physiological response of the plant to that stress. Herrera and Bazaga (2013) reported that phenotypic changes adopted by the plant in response to stress (such as prickly leaves induced by herbivory) positively correlated to global changes in DNA methylation. Resistance to *Rhizoctonia solani* in maize is similarly linked to global shifts in DNA methylation (Li et al., 2011).

Sequence characterisation of these differentially methylated loci may ultimately provide a useful route through which to discover candidate genes that are implicated in these responses. This approach has been adopted in other cases. For instance in rice, where resistance to bacterial blight is linked to plant age, it has been shown that acquired resistance is regulated by the hypo/hypermethylation of several loci. Such methylation changes correlate with the expression levels of several genes including a putative Gag-Pol polyprotein, a putative RNA helicase of the Sk12 subfamily and a putative receptor-like protein kinase (Sha et al., 2005). There has also been interest in tracking changes in DNA methylation associated with virus silencing in plants (English et al., 1996).

The apparent stability of some C-methylation sites following induction allows for stress detection long after initial exposure and means that carefully selected epimarkers potentially provide a more robust source of *a posteriori* stress diagnosis than more ephemeral changes within the cell such as the abundance of mRNA (transcriptomics), proteins (proteomics), or metabolites (metabolomics). Furthermore, this ‘memory of stress’ is not limited to cells and cell lineages but as described above can also persist through filial generations. Boyko and Kovalchuk (2011) showed that changes to the DNA methylation patterns of plants associated with continuous interactions with pathogens were successfully transmitted and fixed in their progeny seemingly also potentially allowing for the diagnosis of parental stress exposure.

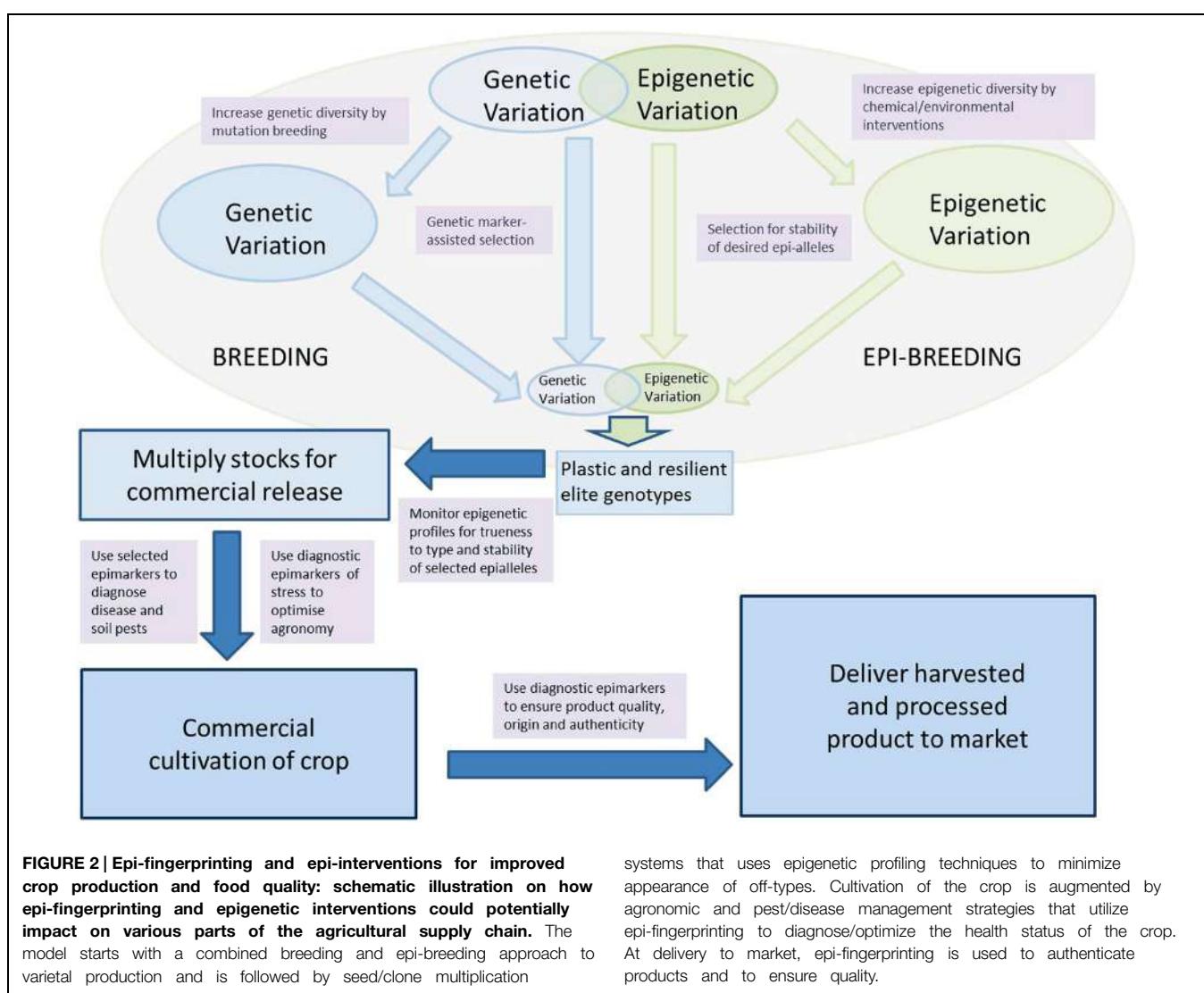
Looking ahead, it seems inevitable that in the relatively near future there will be methylation markers developed for many crops able to track developmental progression and also the exposure and response of the plants to the stresses they are experiencing. The long-term possibility of using these markers as sentinels of health and developmental state leads to the enticing prospect that they may ultimately be integrated into models to predict yield. If applied onto a broader scale, it is even possible that epigenetic fingerprinting of airborne pollen samples for signatures of stress could eventually augment existing monitoring of the landscape for the effects of climate change or to track new epidemiological events, and so facilitate more timely and targeted interventions.

Epi-Fingerprinting and Product Quality

The high market value of ‘top end’ agricultural products used for nutritional or medicinal properties frequently attracts fraudulent labeling of lesser products with lower quality or commercial value (Mader et al., 2011). Certifying the authenticity and origin of such products is a legal requirement in many jurisdictions to avoid unfair competition and assure consumers protection against fraudulent practices (Reid et al., 2006). Although there is an increasing demand by consumers for high quality food products (Luykx and van Ruth, 2008), the majority of authentication techniques for food products have focussed on species or varietal identification or on the chemical composition of processed foods (Sentandreu and Sentandreu, 2011). However, quality traits of plant products are not only influenced by the choice of species or cultivars. In some agricultural products, quality can be primarily determined by the harvested components of the crop used to generate a product (Srancikova et al., 2013) or

else by climate, location, crop age, management systems used to cultivate the crop (e.g., industrialized versus organic farming, manure versus *N*-fertilizer; Posner et al., 2008). Equally, soil conditions, as well as the interactions of different environmental conditions or “terroir” can be viewed as important quality determinants of products such as wine (van Leeuwen et al., 2004). These conditions affect plant composition variables such as dry matter content and furthermore starch, crude protein, amino acids, nitrate, sugars, and citric acid (Müller and Hippe, 1987). The measurement of such components has often necessitated development of a series of independent tests to detect fraudulent labeling. The use of methylation profiles as a diagnostic tool relating to several different aspects of crop quality is therefore appealing because it provides a ‘plant’s perspective of the growing environment.’ This area of methylation profiling is still untested but would be especially alluring if evidence can be provided to distinguish between agronomic practices (such as those used for organic farming) that are currently primarily verified only by certification.

New evidence is now emerging to suggest that this may be possible. For example, Boyko et al. (2010) showed that exposure of *A. thaliana* to a range of mild abiotic modifications (salt around the roots, UVC, cold, heat, and flood) could be detected by reproducible changes in DNA methylation patterns. Similarly, in clonally propagated poplar grown under different conditions of water availability, differences in genome-wide DNA methylation paralleled differences in transcriptome, suggesting an epigenomic basis for the clone history-dependent divergence (Raj et al., 2011) and illustrating the plausibility of epigenetic profiling to characterize watering regime. Indeed, cultivation conditions of a wide variety of plants have now been shown to induce differences at methylome level (i.e., Dandelions, Verhoeven et al., 2010; mangrove, Lira-Medeiros et al., 2010; alligator weed, Li et al., 2013). These findings open the door for deploying epigenetic profiling approaches to diagnose growth conditions and geographical region of origin of otherwise identical crops and their processed products.



It is therefore tempting to speculate that quality traits associated with crop management may also be detectable using the same C-methylation markers. There is equally scope also to differentiate between products generated from parts of the plant with different market value. Certainly, it is now well established that different cell types or tissues within an organism can have markedly different methylation profiles (Baron et al., 2006; Feng et al., 2010; Rodríguez López et al., 2010b, 2012b) and that the use of epigenetic markers has been proven to be an effective means of generating organ-specific epigenetic markers as a tool for identifying the tissue of origin in plant (Rodríguez López et al., 2010b) and animal (Rodríguez López et al., 2012b) products. This gives rise to the prospect of simplifying global methylation patterns to generate smaller numbers of highly diagnostic epimarkers for use in food quality assessment. Such markers could not only have potential value in identifying the cultivating system and product composition, but also to other factors affecting quality such as storage, transport and processing conditions.

Conclusion

Epigenetic control mechanisms provide the crop plant with an ability to respond to the many and varied challenges posed to them by an ever-changing growing environment during growth and development. Of all these mechanisms, histone tail modifications and DNA methylation are by far the better studied. Of the two, DNA methylation way of action is the better understood, the easier to analyze and the one with most associated epialleles in the literature.

We have shown that the deliberate manipulation of this relationship through direct (chemical) and indirect (environmental) interventions holds the potential to generate new and useful variability to the crop. In some cases the induced changes can alter the genome regulatory system

of the crop in such a way as to allow it to better cope with particular, anticipated stress types. The capability to fix at least some of these states across generations offers the tantalizing possibility of a targeted system of epigenetic breeding to augment existing breeding efforts, and has particular appeal for long-lived clonal crops. We have also shown that gaining a better understanding of the relationship between the stress elicitor and the changed epigenetic state offers new opportunities for the identification of candidate genes that are important in conferring resilience against important stresses. Such stable epigenetic markers, especially if associated to commercially interesting traits, can be of interest to plant breeders. Apart from variations in the gene sequence, epigenetic variation may contribute to commercially interesting traits.

However, it is perhaps as a diagnostic tool of stress that there is the greatest source of unexplored opportunity for short-term step improvements to crop management and production. A plethora of works have shown that there is a clear and strong relationship between a vast array of stresses and the C-methylation status of crop plants. Conversion of these global differences into specific diagnostic epimarks of stress detection and stress-induced physiological response by the crop plants offers a range of opportunities for the improvement in varietal selection, crop management, for the control of pests and disease, and to control and regulate the quality of agricultural products. Moreover, the methylome epifingerprinting can be considered as a measure of the phenotype of the crop's genome. Such an 'epphenotype' not only provides a new diagnostic tool to study stress responses and developmental progression but also provides a useful bridge that allows direct functional relationships to be inferred between the growing environment and associated genome regulation. In the medium term we expect the collective impact of these developments to enable substantive advances in crop production and protection; an epigenetic revolution (**Figure 2**).

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Crop epigenetics and the molecular hardware of genotype × environment interactions

Graham J. King^{1,2,3*}

¹ Southern Cross Plant Science, Southern Cross University, Lismore, NSW, Australia, ² National Key Laboratory for Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China, ³ Crops for the Future, Biotechnology and Breeding Systems, Semenyih, Malaysia

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Raúl Alvarez-Venegas,
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Switzerland
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and Industrial Research Organisation,
Australia

***Correspondence:**

Graham J. King
graham.king@scu.edu.au

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Crop plants encounter thermal environments which fluctuate on a diurnal and seasonal basis. Future climate resilient cultivars will need to respond to thermal profiles reflecting more variable conditions, and harness plasticity that involves regulation of epigenetic processes and complex genomic regulatory networks. Compartmentalization within plant cells insulates the genomic central processing unit within the interphase nucleus. This review addresses the properties of the chromatin hardware in which the genome is embedded, focusing on the biophysical and thermodynamic properties of DNA, histones and nucleosomes. It explores the consequences of thermal and ionic variation on the biophysical behavior of epigenetic marks such as DNA cytosine methylation (5mC), and histone variants such as H2A.Z, and how these contribute to maintenance of chromatin integrity in the nucleus, while enabling specific subsets of genes to be regulated. Information is drawn from theoretical molecular *in vitro* studies as well as model and crop plants and incorporates recent insights into the role epigenetic processes play in mediating between environmental signals and genomic regulation. A preliminary speculative framework is outlined, based on the evidence of what appears to be a cohesive set of interactions at molecular, biophysical and electrostatic level between the various components contributing to chromatin conformation and dynamics. It proposes that within plant nuclei, general and localized ionic homeostasis plays an important role in maintaining chromatin conformation, whilst maintaining complex genomic regulation that involves specific patterns of epigenetic marks. More generally, reversible changes in DNA methylation appear to be consistent with the ability of nuclear chromatin to manage variation in external ionic and temperature environment. Whilst tentative, this framework provides scope to develop experimental approaches to understand in greater detail the internal environment of plant nuclei. It is hoped that this will generate a deeper understanding of the molecular mechanisms underlying genotype × environment interactions that may be beneficial for long-term improvement of crop performance in less predictable climates.

Keywords: crop epigenetics, chromatin dynamics, thermal homeostasis, ionic homeostasis, DNA methylation, phenotypic plasticity, G × E interactions

INTRODUCTION

Crop plants are sessile autotrophs, represented by relatively few monocotyledon and dicotyledon angiosperm species which lack the internal thermoregulation of hot blooded animals. Modern breeding programs have contributed to increases in yield, with major advances made during a period of relative climate stability. However, the planet has entered a period of climate variability, in which higher global temperatures also increase amplitude and temporal variance of climate parameters, and temperature accounts for over 30% of global crop yield variability (Porter and Semenov, 2005; Ray et al., 2015). These effects are compounded by the progressive salinization of many available arable soils (Pimental et al., 2004).

Such issues require a deeper understanding of the molecular mechanisms underlying plant responses to the environment (Baulcombe and Dean, 2014). Crop performance, yield and quality are sensitive to interactions between genotype and environment ($G \times E$), with built-in phenotypic plasticity required for crop cultivars to cope with variable environments (Bloomfield et al., 2014). This is particularly critical where management of the internal thermal and ionic environment affects growth rates and developmental phase transitions. The internal ionic status of a plant is strongly dependent on external nutrient availability, with mineral fertilizers a major cost for crop production (Timilsena et al., 2014). In particular, the major macronutrient potassium plays a key role in metabolic adjustment during plant development, affecting yield and responses to salinity, drought and cold.

The detection of temperature by plants is required for appropriate responses on multiplexed timescales covering periods from seconds to years (Blanchard and Runkle, 2011; Way and Pearcy, 2012), with supply of mineral ions varying on an intermediary timescale (Le Bot et al., 1998). Crop yields are sensitive to the pattern of diurnal variation in air and soil temperature that affects the rate of growth and development (Schlenker and Roberts, 2009; Lobell and Gourdji, 2012; Gourdji et al., 2013). Productivity is dependent both on the ability to perceive minor fluctuations in ambient temperature, as low as $\pm 1^{\circ}\text{C}$ (Porter and Semenov, 2005; Hüve et al., 2006; Blanchard and Runkle, 2011), and plastic responses that involve keeping tally of accumulated thermal history during specific developmental phases (Muchow et al., 1990; McMaster and Wilhelm, 1997; Tan et al., 2000). Thus fluctuations in thermal environment that perturb the ontogenetic timeline have potential for a significant impact on crop yield and quality (Craufurd and Wheeler, 2009; Bloomfield et al., 2014; Ray et al., 2015).

Although many crop traits and developmental phase changes are dependent upon thermal and ionic signals, conventional genetic models have not provided a complete understanding of the relevant signal transduction pathways and behavior (Hammond et al., 2011; McClung and Davis, 2010). More recently it has become apparent that epigenetic marks play a significant role and are able to provide a mechanistic framework in the context of chromatin dynamics.

Environment is detected in a number of ways, including via effector proteins, small RNAs (Mirouze and Paszkowski, 2011)

and directly by chromatin (Kumar and Wigge, 2010). As we will see, sophistication and ruggedness in crop plasticity depends to a great extent upon epigenetic feedback loops that contribute to genomic regulation, with crosstalk between physiological and sub-cellular systems (Bloomfield et al., 2014; Kissoudis et al., 2014; Kulcheski et al., 2015). Whilst many of these mechanisms involve specific gene networks and epigenetic marks, at the molecular level within the plant nucleus the relationship between temperature and electrostatic interactions mediated by ion concentration merits investigation.

Plant growth and development progress within biophysical and thermodynamic constraints imposed by the molecular composition of cells (Lintilhac, 2014; Wolfe, 2015). In eukaryotes, sub-cellular compartmentalization reduces the impact of environment on key sub-systems (Millar et al., 2009), and helps preserve the integrity of enzymes and other informational macromolecules. In the nucleus, the dynamic composition and status of chromatin plays a central role in genomic regulation, and is sensitive to local ionic and thermal environment (Gan and Schlick, 2010; Woodcock and Ghosh, 2010; Allahverdi et al., 2015). Management of temperature and ionic homeostasis represents a major energetic and organizational overhead (Jones and Rotenberg, 2001; Alekseeva et al., 2007; Watling et al., 2008), and involves complex signal transduction systems that are fine-tuned to generate appropriate physiological and developmental responses (Wilson, 2013). These systems include epigenetic processes that provide an environmental memory heritable through mitosis, and in some situations through meiosis (Heard and Martienssen, 2014). Thus it has become clear that RNA-mediated epigenetic mechanisms, along with DNA methylation and histone protein epigenetic marks, significantly extend the adaptive responses of plants (Bräutigam et al., 2013; Pikaard and Scheid, 2014). More complex crop plant genomes, with a high load of repetitive sequences and associated pool of epigenetic marks, may offer greater opportunities for regulation of phenotypic plasticity (Bloomfield et al., 2014).

The performance potential of crop plants relies on maximizing harvest index, the ratio of harvestable to total biomass (Unkovich et al., 2010). This is dependent upon the timely transition between distinct phases of development, where genomic and phenotypic plasticity enables this to be orchestrated in the context of variation in the cultivation environment and crop management practices (Bloomfield et al., 2014). It is recognized that coping with the more extreme environmental fluctuations during a crop cycle is likely to rely on secondary systems (Cramer et al., 2011), as well as gene neo-functionalization, with novel genetic loci conferring distinct regulation or function in order to maintain plant homeostasis (Mickelbart et al., 2015).

This review describes the macromolecular components of the chromatin hardware in which plant genomes are embedded. It explores their biophysical behavior in the context of the ionic and thermal environment of the nucleus, and how this is affected by the local distribution of DNA and histone epigenetic marks. Evidence from *in vitro* and molecular modeling studies is placed where possible in the context of *in vivo* observations for model species and crop plants. The contribution of ions to mediating electrostatic interactions of chromatin and epigenetic marks is

placed in the context of ionic variation at whole plant level. Recent advances in understanding how specific epigenetic marks mediate plant thermosensory signaling and other responses to abiotic environment are placed in the context of chromatin dynamics and biophysics.

A preliminary speculative framework is outlined, based on the evidence of what appears a cohesive set of interactions at molecular, biophysical and electrostatic level between the various components contributing to chromatin conformation and dynamics (**Figure 1**). It proposes that within plant nuclei, general and localized ionic homeostasis plays an important role in maintaining chromatin conformation, whilst maintaining complex genomic regulation that involve specific patterns of epigenetic marks. More generally, reversible changes in DNA methylation appear to be consistent with the ability of nuclear chromatin to manage variation in external ionic and temperature environment. Whilst tentative, this framework provides scope to develop experimental approaches to understand in greater detail the internal environment of plant nuclei. It is hoped that this will generate a deeper understanding of the molecular mechanisms underlying genotype × environment interactions that may be beneficial for long-term improvement of crop performance in less predictable climates.

THE GENOME AT HOME IN THE NUCLEUS

Crop plants have derived from taxa that represent different levels of genome complexity (King, 2002). Some are well adapted to the relatively uniform annual environments of the tropics and subtropics (Gepts, 2008), while others must manage variability in length of temperate seasons and severity of cold winter periods (Rosenzweig and Liverman, 1992; Craufurd and Wheeler, 2009). Compared with the condensed genome of *Arabidopsis* (125 Mbp), crop genome sizes vary over 60-fold, from around 265 Mbp (peach, *Prunus persica*) to the larger cereal genomes (barley, 5.1 Gbp; wheat: 17 Gbp; Michael and Jackson, 2013). In common with all eukaryotes, this variation is directly proportional to nuclear volume (Cavalier-Smith, 1985), which suggests strong selective pressure to maintain an optimal dense nuclear environment, where crowding due to proteins, DNA and RNA leads to a macromolecular concentration calculated at over 100 mg ml⁻¹ (Hancock and Hadj-Sahraoui, 2009; Hancock, 2012a), considerably higher than those typically used for many *in vitro* experiments (Hancock, 2012a). This greatly reduced effective solvent volume also means that the equivalent molar concentrations of mono- and divalent ions may be considerably different from those regarded as cytoplasmic or physiological. At present few reliable estimates of nuclear water content exist and, as already noted, it appears that a significant proportion of ions are bound to chromatin and other macromolecules (Garner, 2002; Hancock and Hadj-Sahraoui, 2009).

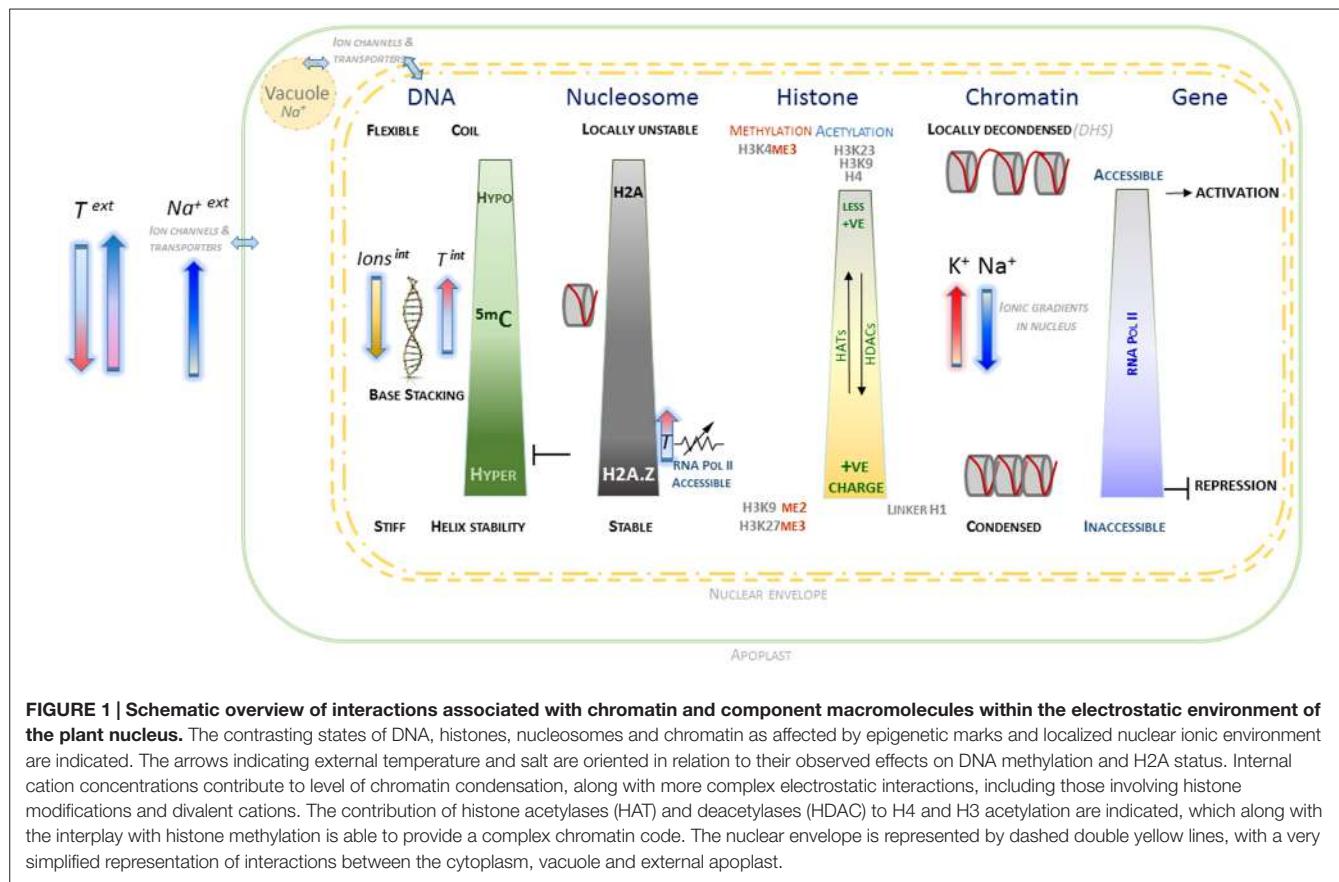
Compared with the cytoplasm, the rate of molecular mobility, expressed as diffusion constants, is about five times lower in the nucleus, and 10-fold lower in the nucleolus (Bancaud et al., 2009). Despite this, regulatory factors appear still able to locate genomic targets rapidly, due to the presence of electrochemical

gradients generated by ion distributions in the remaining space, which have been modeled as channels between chromatin fibers represented by percolation clusters (Wedemeier et al., 2007; Fritsch and Langowski, 2010; Bancaud et al., 2012). Such an environment may also encourage diffusiophoresis (Hancock, 2012a), a process that involves dispersed particles moving spontaneously in a fluid induced by a diffusion or concentration gradient. Indeed, *in vivo* imaging of HeLa nuclei indicates a “mesh spacing” of chromatin fibers on the order of 63 nm, significantly larger than the typical size of diffusing protein complexes (Weidemann et al., 2003). From this and other *in vivo* studies, these authors concluded that all nuclear locations are accessible for diffusing protein complexes (Weidemann et al., 2003). Thus signal transduction via diffusion of regulatory factors to the genome does not appear to be a time-limiting factor for regulatory systems involving rapid transcription, or epigenetic response to external environment. At 7–19 mg ml⁻¹, the 30 nM chromatin fiber represents about 10% of the nuclear volume (Strickfaden et al., 2012) and so the time for a non-interacting protein to explore the whole nucleus is of the order of a few seconds, and four times faster than in water (Strickfaden et al., 2012). For example, GFP (27 kDa) may move 12 μM sec⁻¹, a similar rate as between the cytoplasm and nucleus (Pack et al., 2006). The extent of this molecular crowding within the nucleus has led to the suggestion that entropic forces, such as those associated with polymer elasticity, may be more significant for chromatin structure and dynamics than some of the electrostatic forces observed under typical *in vitro* conditions (Hancock, 2012b).

CHROMATIN ARCHITECTURE AND DYNAMICS

Eukaryote nuclear chromosomes represent the complex macromolecular structures formed of chromatin, in which genomic DNA is embedded along with highly charged proteins and varying amounts of RNA. Nucleosomes represent the building blocks of chromatin, with ~147 bp of DNA wrapped around core nucleosome particles, each of which consists of a H3-H3-H4-H4 histone tetramer and two H2A-H2B dimers. Within this octamer, the H2A-H2B dimers occupy the peripheral 2 × ~30 bp DNA, and the H3-H4 tetramer the inner region, with tails that interact both within and between nucleosomes and contribute to accessibility for transcriptional apparatus (Iwasaki et al., 2013). Post-translational changes to histones, including acetylation and methylation, are able to generate a combinatorial code of epigenetic marks (Lothrop et al., 2013), along with variants such as H2A.Z.

Based on *in vitro* and modeling studies, electrostatic interactions appear to be the dominant factor affecting overall nucleosome stability (Fenley et al., 2010), with higher ionic concentration a major source for destabilization and disruption (Gansen et al., 2009). The wrapping and unwrapping of DNA around the histone core is sensitive to the charge state of the globular core (Fenley et al., 2010), with *in vivo* post-translational acetylation of a single lysine residue in H3 or H4 able to decrease the charge and decrease the strength of binding to



DNA. This reduces the tendency of chromatin to fold into highly compact structures, making it more accessible to transcription factors (TFs) and other interacting proteins, as well as having greater sensitivity to DNase I (Garcia-Ramirez et al., 1995; Tse et al., 1998). This epigenetic switch is mediated by histone acetyltransferases (HATs) that neutralize the positive charge on lysine, and histone deacetylases (HDACs) which play an inverse role, by providing a more positive net charge (Eberharter and Becker, 2002).

Nucleosome Positioning and Epigenetic Marks Define Chromatin State

Nucleosome positioning with respect to genomic sequence is sensitive to many intrinsic and external factors (Tsankov et al., 2011; West et al., 2014), especially in euchromatin, where positioning is dynamic and responsive to cellular identity and internal nuclear environment (Hughes et al., 2012). Gene activation is facilitated by DNA and nucleosome thermodynamics, the nucleosomal surface and chromatin higher order structure (Dechassa and Luger, 2012). Thus initiation and progress of transcription is dependent upon RNA polymerase II (RNA Pol II) gaining access to DNA wrapped around nucleosomes, by harnessing fluctuations that locally unwrap DNA, rather than unwrapping nucleosomes (Mack et al., 2012). Targeted protein and snRNA regulatory factors are able to effect rapid and reversible changes in transcription

as a result of this highly dynamic behavior (Ivashuta et al., 2011; Chen and Rajewsky, 2014; Franco-Zorrilla et al., 2014), which is mediated by the epigenetic marks of histone and DNA modifications (Pikaard and Scheid, 2014). The detection of an association between nucleosome phasing, introns, and RNA splicing (Schwartz et al., 2009a; Tilgner et al., 2009; Chodavarapu et al., 2010) also highlights the central role nucleosomes play in managing RNA Pol II transcription in complex eukaryote genomes.

The distribution of epigenetic marks is an important contributor to the organization of nucleosomes in promoter regions, with histone modifications able to mediate very specific access to DNA to enable gene activation (Bannister and Kouzarides, 2011). This can result in distinct and dynamic histone landscapes associated with specific plant processes, such as de-etiolation in *Arabidopsis* (Charron et al., 2009). Experimental data and statistical mechanics thermodynamic predictions both indicate that nucleosomes are able to block the binding of many TFs by competing with common binding sites, as well as contributing to cooperative binding between TFs (Raveh-Sadka et al., 2009). In general, core histones and variant forms tend to be stably bound to DNA on a timescale of hours (Rippe, 2012), whilst the half-life turnover of histone acetylation is on the order of minutes (Nightingale et al., 2007). This confers a combination of high thermodynamic stability whilst being sensitive to factors that allow rapid access to DNA when required.

Chromatin Accessibility

Decondensed or open chromatin is detectable by the presence of DNase I hypersensitive sites (DHSs), which provide an accurate experimental indication of where the DNA is exposed and accessible (Wu et al., 2014). For example, in *Arabidopsis* ~90% of the binding sites of the MADS-box TFs APETALA1 and SEPALLATA3 are covered with DHSs (Zhang et al., 2012), indicating that these form a barrier for nucleosome formation. However, in general only a subset of nucleosomes are reproducibly positioned, with phasing associated with flanking of transcription start sites (TSS) of active genes in *Arabidopsis* (Zhang et al., 2012). In rice, DHSs are associated with regions flanked by strongly phased nucleosome arrays (Wu et al., 2014). This is consistent with a barrier model, where intergenic and other regions in which regulatory proteins may be bound to the genomic DNA to provide a barrier that facilitates phased nucleosome arrays to organize either side (Mobius and Gerland, 2010). Rice DHSs may also span a single phased nucleosome (Wu et al., 2014). For promoters of constitutively transcribed genes a DHS detectable barrier may be permanent (Wu et al., 2014), while for binding sites of TFs associated with tissue-specific, organ-specific or environmentally induced gene expression such barriers may be transient, thus allowing nucleosome rearrangement to facilitate transcription (Zhang et al., 2012).

EPIGENETIC MODIFICATIONS MODIFY CHROMATIN ARCHITECTURE

Although many components of chromatin and epigenetic marks are conserved between plants and animals, it is important to be aware of a number significant plant-specific features. These include 5'-methylcytosine (5mC) occurring in all context in plants compared with solely CG in animals, the presence of hydroxymethylated cytosine (5 hmC) in animals, the distinct plant methyltransferases CMT3, DRM1/2, and MET (Jang et al., 2014) and defined distribution of methylation with respect to *cis*-regulatory and gene body sequences (Cokus et al., 2008). In addition, plants display a characteristic pattern of nucleosome distribution (Chodavarapu et al., 2010), and tighter distribution of intron length (Wu et al., 2013) compared with animals. Plants and animals also appear to have evolved distinct DNA demethylation systems, with the DEMETER (DME) family DNA glycosylases able to remove 5mC efficiently in plants, resulting in DNA demethylation and transcriptional activation of target genes (Jang et al., 2014).

Management of DNA Methylation

Various experimental approaches have shown that DNA cytosine methylation contributes to regulating higher order chromatin structure in plants (Tariq and Paszkowski, 2004), primarily through interactions with histones, and ultimately affecting nucleosome positioning. There is extensive evidence that gene silencing and repression of active euchromatin is associated with hypermethylation of DNA in plants (Vaillant and Paszkowski, 2007). Although there is less evidence for direct involvement of 5mC in condensation of heterochromatin (Gilbert et al., 2007), at the primary chromatin level DNA methylation has been shown to

have a strong interaction with nucleosome formation (Pennings et al., 2005), and particularly in plants (Chodavarapu et al., 2010). These phenomenological observations appear to be consistent with what is known of the underlying properties of the component molecules and their interactions.

5mC has been shown to shift the preferred rotational position of nucleosomes *in vitro* by 3 bp (Buttinelli et al., 1998). In mammals, the stabilizing effect of 5mC on DNA duplexes is able to be reversed by hydroxymethylation to 5 hmC (Rodriguez Lopez et al., 2010; Thalhammer et al., 2011). However, the 5 hmC modification appears absent in plant chromatin (Erdmann et al., 2014), and at present it is unclear whether functional analogs for regulating chromatin dynamics exist.

In plants, the involvement of DNA cytosine methylation (5mC) in regulation of gene expression makes a significant contribution to definition of cellular identity and coordination of ontogeny (Vaillant and Paszkowski, 2007; Heard and Martienssen, 2014). The specific molecular attributes of 5mC compared with unmethylated C, and the dynamic nature of DNA methylation, are critical in providing a “toggle switch” mechanism. Thus 5mC provides a versatile heritable epigenetic mark able to define tissue specific expression patterns, and mediate responses to the environment (Zhang et al., 2011; Bloomfield et al., 2014; Widman et al., 2014). The higher density of 5mCG observed within plant genes compared with promoters (Cokus et al., 2008) appears to have a greater effect on transcription due to inhibition of elongation (Chodavarapu et al., 2010; Gelfman et al., 2013). Within the rice genome, the methylation map indicates single peaks close to start codons (Li et al., 2008). In *Arabidopsis* transcriptional units, 5mC appears to be enriched over the first nucleosome in a transcription unit, with strong periodicity of ~180 bp in methylation over subsequent nucleosomes (Chodavarapu et al., 2010), and a strong signal associated with positioning in exons.

The processes underlying hypomethylation that are associated with reprogramming, particularly in establishing pluripotency and imprinting effects in plant and animal systems, are now starting to be uncovered (Feng et al., 2010). Dynamic control of DNA methylation involves a cyclic enzyme cascade that consists of cytosine methylation, iterative oxidation of the methyl group by TET (ten eleven translocation) dioxygenases which act as 5mC oxidase, and replacement with unmodified cytosine (Zhao and Chen, 2014). In plants, this latter step of active DNA demethylation is primarily carried out by a small group of bifunctional DNA glycosylases that include ROS1, DME, DML2, and DML3 (Gong and Zhu, 2011). These remove the methylated cytosine base and create an abasic site, with the gap refilled by an unmethylated cytosine through a base-excision-repair pathway (Gong and Zhu, 2011).

RNA Directed DNA Methylation

The RNA-directed DNA methylation (RdDM) epigenetic pathway is the primary mechanism by which plants mediate responses involving small RNAs, and is dependent upon the RNA polymerases Pol IV and Pol V, which are specific to plants, along with various accessory proteins currently being characterized (Matzke and Mosher, 2014). There is increasing evidence for

involvement of RdDM in a wide range of developmental and physiological processes that include stress responses, pathogen defense as well as reproductive development (Boyko et al., 2010; Gutierrez-Marcos and Dickinson, 2012; Matzke and Mosher, 2014). This is in addition to the major role played in repression of subsets of transposons as well as protein coding genes, and the interplay between these in complex crop genomes has yet to be fully explored.

Within the nucleus snRNAs operate to repress epigenetic modifications such as 5mC and histone methylation directly at specific target sites, resulting in transcriptional gene silencing (TGS; Simon and Meyers, 2011; Matzke and Mosher, 2014). This involves processing of Pol IV transcripts within the nucleus and cytoplasm, and re-introduction into the nucleus, where siRNAs are able to facilitate targeting of Pol V nascent transcripts (Simon and Meyers, 2011). Recruitment of methyltransferase leads to *de novo* methylation of cytosines in each of the CG, CHG, CHH contexts, and although Pol V-mediated RdDM operates over many genomic regions, there appears to be a preference toward euchromatin, more recently acquired intergenic TEs, and genes containing TEs or other repeat sequences in their promoters and introns (Matzke and Mosher, 2014). A large proportion of RdDM targets are also modified by modified histone H3K9me, which can provide a feedback loop with DNA methylation to reinforce TGS.

Biophysical Properties of 5mC

The methylation of cytosine (5mC) affects a wide range of DNA biophysical properties, with variation in the localized patterns of steric and conformational energy, as well as hydrophobic modifications to polarity (Hausheer et al., 1989; Wanunu et al., 2011). Together with electrostatic alterations that affect internal base pair dynamics, and variation in base stacking energy, these lead to variation in DNA flexibility (increased flexibility or bending propensity = decreased stiffness) and duplex stability. In particular, stacking energies between neighboring dinucleotides in DNA are represented by elastic force constants that contribute both to DNA flexibility and helical opening (Severin et al., 2011). In 5mC, these effects appear to be specifically associated with molecular polarizability of the pyrimidine, which increases the base stacking energy and reduced flexibility (Norberg and Vihinen, 2001; Acosta-Silva et al., 2010). This is due in part to the protrusion of the hydrophobic methyl group into the major groove, which alters the steric arrangement and local charge environment (Song et al., 2013). The base stacking interactions can generate local distortions in DNA (Acosta-Silva et al., 2010; Yusufaly et al., 2013) and inhibit CG:CG step overtwisting, which in turn decreases flexibility (Yusufaly et al., 2013).

In addition to DNA flexibility, stacking energies also contribute to the cooperative melting associated with the DNA helical-coil transition that is observed both in naked form (Anselmi et al., 2000) as well as within protein complexes (Perez et al., 2004), with helical stability also proportional to the local cation environment (Yakovchuk et al., 2006). Atomic force experiments and molecular dynamics simulations suggest that the contribution of 5mC to increased cooperative DNA helical stability may also depend on methylation level and sequence context, with perhaps more significant effects on mechanical stability and relative

stiffness (Severin et al., 2011). Independent experimental evidence based on high resolution melting has also shown that 5mC confers increased helical stability compared with unmodified C (Rodriguez Lopez et al., 2010; Wanunu et al., 2011).

These contributions of 5mC to increased DNA duplex stability and reduced flexibility also appear to affect some aspects of nucleosome positioning, as well as the ability of nucleotide sequences to wrap around the histone complex (Dantas Machado et al., 2015). A picture that is emerging from recent plant whole genome methylome and nucleosome positioning studies (Pennings et al., 2005; Chodavarapu et al., 2010; Gelfman et al., 2013) suggests a discontinuous variation of 5mC in nucleosomal regions.

H3.3 Distribution

Histone H3 is a substrate that provides considerable molecular complexity in terms of epigenetic marks for most eukaryotes, having two major variants, H3.1 and H3.3, as well as accommodating a range of post-translational modifications in the N-terminal amino acid residues. In *Arabidopsis*, H3.1 is enriched in silent areas of the genome, including those with the H3K27me3 and H3K9me3 modifications that contribute to transcriptional repression, as well as with DNA methylation (Stroud et al., 2012). In contrast, H3.3 has been shown to play a role in maintaining accessible chromatin (Jin and Felsenfeld, 2007), and is enriched in actively transcribed regions, particularly in the 3' of *Arabidopsis* genes, where it is correlated with H3K4me3 and H3B ubiquitylation (Stroud et al., 2012).

Histone H2A.Z Distribution

The histone variant H2A.Z is evolutionarily conserved, and often ~60% identical to canonical H2A within a species, while being ~80% conserved between species (Zlatanova and Thakar, 2008). The variant plays an important role in marking the epigenetic state of nucleosomes (To and Kim, 2013), and is preferentially localized toward the 5' of genes in *Arabidopsis*, where it has been shown to be excluded from sites of heavily methylated DNA within actively transcribed genes (Zilberman et al., 2008). This inverse relationship between the H2A.Z and 5mC has been interpreted as providing a mechanism whereby H2A.Z protects DNA from cytosine methylation in euchromatic regions (Meneghini et al., 2003; Zilberman et al., 2008).

Increasing the wrapping of DNA around the core of H2A.Z containing nucleosomes can reduce the intrinsic fluctuations in DNA accessibility which facilitate transcription (Bowman and Poirier, 2015). Thus H2A.Z marked nucleosomes are often found in regions flanking TSS, and these provide a “molecular rheostat” for initiation of RNA Pol II transcription (Weber et al., 2014; Subramanian et al., 2015).

H1 Linker

H1 histones interact with the linker DNA between adjacent nucleosomes, and cooperatively contribute to formation of the stable and compact 30 nm fiber (McBryant et al., 2010). Although the linker histones ensure compaction and stabilization of higher order chromatin, the variant forms also mediate variation in

conformation and accessibility (Wong et al., 2007). It should be noted that linker H1 facilitates self-association of chromatin fibers at salt concentrations considerably lower than for nucleosomal arrays lacking H1 (McBryant et al., 2010). The stoichiometrical relationship between H1 and core nucleosomes has been shown to range from 0.5 to 1 in different tissues (Woodcock et al., 2006), with linker length conventionally described as a diagnostic feature of chromatin from different taxa and/or tissues (Woodcock et al., 2006).

Transient binding of H1 determines the trajectory of DNA entering and exiting the nucleosome (Bednar et al., 1998) by asymmetric binding of an entry or exit linker with the dyad axis (Brown et al., 2006), and constrains an additional 19–20 bp beyond the nucleosome core (Noll and Kornberg, 1977; Simpson, 1978). This is achieved primarily by neutralizing the negative charge of linker DNA, with the binding of the H1 C-terminal domain contributing to chromatin condensation (McBryant et al., 2010). More recently, additional roles for H1 histones have been uncovered, with the C terminal ends associated with molecular “hubs” that recruit proteins involved in accessing and modifying the chromatin fiber (McBryant et al., 2010).

Plants appear to have a wider range of H1 variants than animals (Over and Michaels, 2014), with many monocot and dicot species having at least one shorter variant that may be induced under drought stress (Jezmanowski et al., 2000). For example, in *Arabidopsis*, H1.3 is drought inducible and has greater binding to chromatin (Ascenzi and Gant, 1999). However, it should be noted that not all “drought inducible” H1 variants are associated with drought, and may contribute other functions during development (Over and Michaels, 2014). Depletion of the variants H1.1 and H1.2, along with removal of H2A.Z, is consistent with the global pattern of chromatin decondensation observed in *Arabidopsis* female megasporangium mother cells (She et al., 2013).

Various lines of evidence have suggested a close interaction between linker H1 and ordered DNA methylation in plants (Wierzbicki and Jezmanowski, 2005). For example, knockdown of H1 in *ddm1* mutants of *Arabidopsis* can lead to restoration of DNA methylation by RdDM (Zemach et al., 2013), suggesting that DDM1 is able to remove H1 to facilitate access to the DNA methylation machinery (Over and Michaels, 2014). Additional evidence has come from analysis of parent-of-origin imprinted loci in the MEDEA (MEA): DEMETER (DME) system, where DME acts as a 5mC demethylase and physically interacts with H1.2 (Rea et al., 2012). The same study has shown that *H1* mutants increase DNA methylation in maternal copies of *MEA* and *FWA* promoters in *Arabidopsis* endosperm. More recently, analysis of *h1.3* mutants has indicated that the absence of H1.3 can lead to significantly reduced stress-related DNA methylation, with this being most evident in the CHH context (Rutowicz et al., 2015). The requirement of H1.3 for a significant proportion of the DNA methylation associated with environmental stress suggests that this linker histone variation may facilitate chromatin accessibility in direct competition with the primary variants H1.1 and H1.2 (Rutowicz et al., 2015). More generally H1 depletion and DNA hypomethylation, along with H3K27me3 demethylation, appear to be key contributors to pluripotency that is facilitated by chromatin decondensation (Alatzas et al., 2008; He et al., 2012).

Biophysical Properties of Histone Modifications

The complimentary roles of 5mC and H2A.Z associated with nucleosome stability may also be based on their respective biophysical and thermodynamic properties. H2A.Z has been shown to contribute to increased nucleosome stability compared with the canonical H2A, with structural and thermodynamic evidence for a more stable interface via the extended acidic path of the H2A.Z dimer and the charged tails of the (H3–H4)₂ tetramer (Dechassa and Luger, 2012). These differences in electrostatic potential and size affect the interface with neighboring nucleosomes and other nuclear proteins (Chakravarthy et al., 2005), and can also contribute to compaction of the 30 nM fiber (Fan et al., 2002).

Sequence analysis of human H2A.Z and H2A-containing nucleosomes has also indicated a prominent association with DNA flexibility at nucleosome boundaries (Gervais and Gaudreau, 2009), with H2A.Z being slightly more rigid than corresponding H2A sequences. Moreover, a DNA flexibility model is able to predict the presence of H2A.Z bordering TSS (Gervais and Gaudreau, 2009). Biophysical studies have also indicated a decreased sensitivity of H2A.Z to ionic strength, with reduced organization of only ~118 bp of core nucleosomal DNA compared with the canonical 147 bp (Doyen et al., 2006).

By adding a negative charge, phosphorylation of H1 generally has the effect of weakening the electrostatic interaction between H1 and DNA, thus increasing H1 mobility (Roque et al., 2008; Over and Michaels, 2014). Although the precise arrangement of H1 in relation to linker and nucleosome is still unclear (Woodcock et al., 2006), recent models suggest increased bending of DNA at the ends of the nucleosome core (Cui and Zhurkin, 2009).

NUCLEAR IONIC STATUS AND CHROMATIN DYNAMICS

The Ionic Environment of the Nucleus

The nuclear envelope provides a boundary within which the genome resides and benefits from a distinct ionic environment buffered from the cytoplasm (Mekhail and Moazed, 2010; Van de Vosse et al., 2011). The two membranes of the nuclear envelope provide an interface, with discrete functions serving the nucleus and cytoplasm. In plants, the vacuole provides the primary store for inorganic ions (Seidel et al., 2013). However, it has become apparent that in all eukaryotes the perinuclear space between inner and outer nuclear envelope provides a store for calcium and other inorganic ions (Matzke et al., 2010), which may contribute to intracellular signaling (Draguhn et al., 1997), including rapid responses for maintaining selective homeostasis of ions such as K⁺ to sustain nuclear function (Wyn Jones and Lunt, 1967).

Although nuclear pores are not freely permeable to Na⁺ and K⁺ the outer membrane of the nuclear envelope contains distinct ion channel classes (Franco-Obregon et al., 2000), including K⁺ channels, which contribute to the Na⁺/K⁺ gradients between the perinuclear lumen, the nucleus and cytoplasm both in animals (Garner, 2002) and plants (Charpentier et al., 2008). Early X-ray microanalysis of oocytes demonstrated that only a portion of the

K^+ in interphase nuclei is in free ionic state, with the remainder being absorbed to the nuclear macromolecules, including DNA and histones (Cameron, 1985). As we shall see, the conformation of chromatin can be modulated by the electrostatic interaction mediated by ions such as K^+ . More generally, it is recognized that monovalent cations that are actively transported through nuclear channels are likely to play an important role in modulation of chromatin structure and gene expression (Garner, 2002).

Chromatin Sensitivity to Cation Environment

The dynamic state of chromatin is subject to variations in the immediate thermal and ionic environment (Spadafora et al., 1979; Caño et al., 2006; Arya and Schlick, 2009). As we have seen, the genome exists in a crowded nuclear environment, embedded in chromatin and serviced by an array of RNA and protein molecules, with access to the read-only transcriptional capability being affected by thermodynamic and biophysical properties of the constituent macromolecules. The ionic environment of chromatin has significant effects on higher level chromatin conformation, with salt-dependent chain folding indicated by *in vitro* (Bertin et al., 2007) and electron microscope (Thoma et al., 1979) studies. As well as being guided by genomic sequence and distribution of epigenetic marks, global aspects of nucleosome assembly and disassembly appears to be dependent upon salt concentration, with the internal $(H3-H4)_2$ tetramer of the nucleosome binding DNA more often at higher ionic strengths than the H2A-H2B dimer (Dechassa and Luger, 2012).

Evidence from molecular combing experiments, which generate a uniformly stretched array of DNA, has suggested that both Na^+ and K^+ inhibit binding of histone to DNA, whilst divalent cations significantly enhance binding, with Mn^{++} inducing condensation and aggregation of histone-DNA complexes *in vitro* (Liu et al., 2005). Thus increasing ionic strength is able to condense the 10 nm nucleosome fiber to form the 30 nm chromatin fiber as part of a reversible process arising from electrostatic repulsion overcoming nucleosome stacking interactions (Poirer et al., 2002). While an increase in monovalent cations above normal range may result in destabilization of interphase chromatin, low concentrations (10 mM) of divalent cations are able to condense chromatin (Visvanathan et al., 2013), possibly as a result of Mg^{++} mediating attraction between single negative charges along chromatin (Poirer et al., 2002).

Large scale sensitivity to the ionic environment is also apparent from the fact that attractive electrostatic interactions in chromatin can be screened by a high ion density with salt concentrations >100 mM (Poirer et al., 2002). In terms of visible phenotype, this has been found to lead to unfolding and expansion of chromosomes in *Notophthalmus viridescens* (eastern newt; Poirer et al., 2002). This may also account for the early observation that increasing the external supply of K^+ up to 0.3 M in *Lolium temulentum* (ryegrass) had the effect of increasing meiotic chiasmata frequency at 30°C, although with little effect at 20°C (Law, 1963), and with little effect from Ca^{+} .

Of more general significance, different lines of evidence from molecular modeling and *in vitro* studies now suggest that K^+ and Na^+ ions have distinct roles in condensation of DNA and

chromatin, with recent *in vitro* evidence indicating that Na^+ promotes the folding into 30 nm fibers in the presence of Mg^{++} , whereas K^+ limits this effect (Allahverdi et al., 2015). This appears to be due to the different binding behaviors of each ion to DNA, with K^+ binding to the electronegative sites of DNA bases in the major and minor grooves, and Na^+ interacting preferentially with the phosphate groups (Cheng et al., 2006). Moreover, there appears to be greater variation in the mobility of both water and ions in the K-DNA system than the Na-DNA system (Allahverdi et al., 2015). The consequences of this phenomenon for transcription, as well as the maintenance of Na:K ratios within the plant nucleus, have yet to be fully explored, but may have extensive ramifications for our understanding of how plant genomes harness and respond to the complex electrostatic environment within the nucleus.

Ionic Variation in Plants

Cellular organisms expend a substantial proportion of their energy ensuring that the biochemical and other components within the cell are able to operate within boundaries of a relatively consistent ionic environment (Alekseeva et al., 2007). The internal concentration of specific mono- and divalent cations in plants appears to be under strong selection, with considerable variation observed across plant taxa based on data derived from tissue level assessment (Thompson et al., 1997; Broadley et al., 2004; Harada and Leigh, 2006). Whilst internal K, N, and P concentrations have been found to vary sixfold to ninefold between species of related angiosperm taxa (Thompson et al., 1997), concentrations of the divalent cations Mg^{++} and Ca^{++} appear much more variable, with a 49-fold variation in Ca^{++} and a 24-fold variation in Mg^{++} . Most of this variance is allocated between monocot and dicot species (Thompson et al., 1997), and compares with P, where most of the variance is found at or below the species level.

Potassium (K^+) is the most abundant inorganic cation in plants, representing up to 10% of dry weight (Watanabe et al., 2007), which is significantly more than required for optimal growth. Since the greater proportion is sequestered into the vacuole, most research attention has focused on its role as an osmoticant in the vacuole and cytosol, as well as an enzyme activator (Leigh and Wyn Jones, 1984), rather than its role in the nucleus. Much of the intra-specific variation appears to be under genetic control, with a greater than twofold variation in shoot K observed in *Brassica oleracea*, and quantitative trait loci (QTL) analysis suggesting a significant role for variation in K^+ transporters (White et al., 2009). In the same species, levels of shoot Ca and Mg vary two and twofold to threefold respectively (Broadley et al., 2008), with a range of genes contributing to the uptake and homeostasis in leaf tissue (Hammond et al., 2011; Graham et al., 2014).

In general, plant species are able to exclude most of the salt (NaCl) present in soil solution, allowing about 2% to be transported in the xylem to shoots (Munns et al., 2006). Na^+ severely inhibits most enzymes at levels >100 mM, and since more than 50 enzymes require K^+ as a co-factor these are sensitive to Na^+ and high Na^+/K^+ ratios (Tester and Davenport, 2003; Munns et al., 2006). Thus, while halophytic plants continue to grow at >250 mM, a number of crops, including rice, are

compromised and die if soil salinity rises to 100 mM NaCl (Munns et al., 2006). Na^+ toxicity is strongly associated with a plant's ability to maintain uptake of K^+ , as well as the within plant distribution (Kader and Lindberg, 2005). Rice appears to have evolved in a low salt environment with plentiful supply of fresh water (Zong et al., 2007), and so yields start to decline at 30 mM Na^+ , compared with wheat at between 60 and 80 mM (Munns et al., 2006).

External Ionic Conditioning and Epigenetic Variation

An early study using *Medicago* callus cultures indicated that while short term exposure to NaCl had no effect on histone variant composition, it did lead to major increases in acetylation of H3.1, H3.3, and H4 (Waterborg et al., 1989). This was interpreted as an altered intra-nuclear ionic environment in the presence of salt, and possibly also representing an adaptive response in chromatin structure to permit chromatin function as Na^+ increases (Waterborg et al., 1989). Since this study, a number of studies have detected an interaction between salt stress and histone modifications including both acetylation and methylation (Kim et al., 2015).

Although global DNA methylation is not significantly different between *Arabidopsis* shoots and roots, those regions of the genome that are differentially methylated tend to be preferentially ($1.85\times$) hypermethylated in shoots (Widman et al., 2014), consistent with findings in rice (Karan et al., 2012). Within *Arabidopsis*, these sites in genes are primarily in the CG context, with a higher proportion at transcription initiation and termination boundaries. This also corresponds to a higher nucleosome density in these regions for the differentially transcribed genes, with the corresponding gene body being less nucleated. Overall it appears that roots tend to have a higher nucleosome density over genic regions and a more marked periodicity of DNA methylation (Widman et al., 2014). Moreover, genes with $>10\times$ higher level of transcription in root tissue are more nucleosome-rich in the boundary regions compared with shoot tissue. The relationship between such observations and the prevailing intracellular thermal and ionic environments has yet to be explored. However, based on the findings in rice it has been suggested that the relative DNA hypomethylation observed in roots provides greater plasticity or preparedness for salt response genes (Karan et al., 2012).

DNA methylation has been shown to provide levels of environmental responsiveness in plant phenotypes, while providing some evolutionary flexibility in terms of heritability (Heard and Martienssen, 2014). Previous reviews have addressed a broad range of genetic and some epigenetic responses to extreme temperature or salt stress events (Madlung and Comai, 2004; Atkinson and Urwin, 2012; Mickelbart et al., 2015). Initial evidence that epigenetic mechanisms are more extensively involved in a range of plant responses to abiotic stress has come from reactivation of transgenes silenced by DNA methylation (Ito et al., 2011; Lang-Mladek et al., 2010). Thus, in tobacco the elevation of cold, salt, and metal ions all lead to hypomethylation of coding regions (Choi and Sano, 2007). The wider contribution of 5mC to management of stress responses has been revealed by

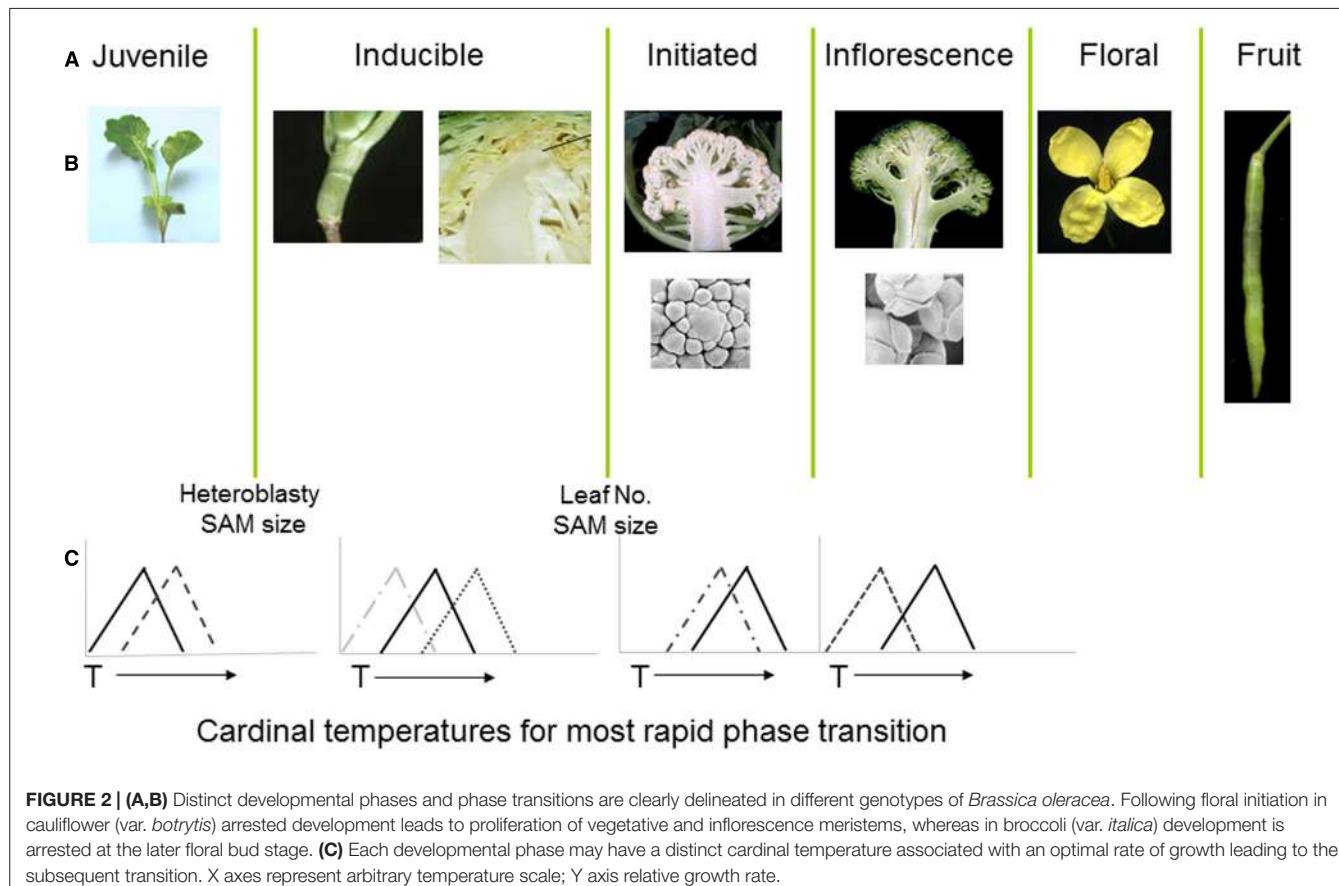
the identification of large numbers of differentially methylated genomic regions, many with associated transcriptional changes, as a result of induction by stresses including bacteria and abiotic factors (Dowen et al., 2012). Although transposons may occur in these differentially methylated regions, such responses appear to be accompanied by up-regulation of 21-nt siRNAs, with many coupled to changes in transcription of the transposon itself and/or nearby genes (Dowen et al., 2012).

In soybean, salt stress has also been shown to induce hypomethylation, along with transcriptional activation of salt stress-induced TFs (Song et al., 2012). From evidence in rice, it appears that remodeling of DNA methylation may play a more general role in conditioning salt tolerance. For example, two salt tolerant genotypes have been found to have a significant level of hypermethylation compared with hypomethylation in two salt-sensitive genotypes (Feng et al., 2012). Although an independent study (Karan et al., 2012) did not find any specific methylation pattern associated with salt tolerant or susceptible genotypes under salt stress, there was a significant association with level of methylation and salt treatment in the shoot of four genotypes and in the root of two others. The authors concluded that many methylation changes associated with salinity were not directed (Karan et al., 2012), which may suggest a more generalized effect on the genome, particularly in the context of 5mCG gene-body methylation. This would be consistent with chromatin being in a more repressed (condensed) state, as well as the observation that stress leads to hypermethylation in satellite DNA at (non-genic) CHG sites within the halophyte *Mesembryanthemum crystallinum* (Dyachenko et al., 2006). It also appears consistent with the observation that mutations in components of the HDAC complexes reduce the ability of *Arabidopsis* to cope with salt and cold (Zhu et al., 2008; Chen et al., 2010), where more condensed chromatin may provide some protection against these stresses.

THERMAL PHYSIOLOGY AND CONTRIBUTIONS TO CHROMATIN CONFORMATION

Thermal Physiology of Crop Plants

A wide range of crop phenotypic traits are affected by thermal environment over multiplexed time-scales, from transitory responses through diurnal, circadian, and annual cycles (Bita and Gerats, 2013). Progress through the sequential developmental phase transitions associated with the detection, initiation, onset and progression of inflorescence development is co-ordinated by integration of environmental signals (Baurle and Dean, 2006; Huijser and Schmid, 2011; **Figure 2**). Detection of temperature is particularly important for the regulation and integration of signals contributing to onset of flowering, including the vernalization and photoperiod pathways (Huijser and Schmid, 2011). Many annual and perennial plants have evolved to fine-tune the sensing and integration of thermal signals (Patel and Franklin, 2009), with the ability to integrate periods of prolonged exposure below critical temperatures, together with thermal responsiveness, directly affecting crop performance (Luo, 2011; Robertson et al., 2013).



The cauliflower crop (*B. oleracea* var. *botrytis*) provides a useful demonstration of the consequences of temperature variation at different phases of development, with each phase transition typically sensitive to a genotype-specific optimal temperature range (Figure 2). Following seedling emergence, the plant remains in a vegetative juvenile phase during which it is unable to detect signals to initiate floral development (Wurr et al., 1994; Guo et al., 2004). The duration of this phase is sensitive to ambient temperatures (Wurr et al., 1995) with considerable variation dependent upon genotype (Fellows et al., 1999; Wurr et al., 2004). In most cultivars, including non-winter types, the plant undergoes a vernalization phase during which the vegetative apical meristem is responsive to accumulated thermal units in a range around a “cardinal” temperature optimum, with considerable genotypic variation also associated with this cardinal temperature (Wurr et al., 2004).

The developmental program in cauliflower leads to a proliferation of vegetative and inflorescence meristems to form the harvested curd (Smith and King, 2000), which is also sensitive to temperature variation (Wurr et al., 1990; Rahman et al., 2007). Radiation of the cauliflower and related broccoli crops from their centre of diversity in Italy (Massie et al., 1999) has resulted in cultivars containing alleles able to provide distinct cardinal temperature optima for length of juvenile period, vernalization, floral initiation, curd, and inflorescence initiation (Irwin et al., 2012). For some genotypes,

high temperatures at the curd arrest stage may lead to the development of bracts, with a reduction in marketable quality (Kop et al., 2003), whilst others grown at temperatures below an optimal range may develop an undesirable “ricey” phenotype (Fujime and Okuda, 1996), where the arrested floral meristems progress to a later stage in development. This diversity of responses and phenotypic consequences are indicative of sophisticated gene regulatory mechanisms that are able to manage complex thermal signals in the context of distinct phases of development.

Chromatin Responses to Thermal Variation

A range of properties associated with the chromatin macromolecular complex are affected by thermal variation. Thermal fluctuations are able to induce partial unwrapping of DNA from nucleosomes *in vitro*, and introduce twist or loop defects into the DNA wrapped round the core particle, resulting in repositioning in relation to the DNA (Blossey and Schiessel, 2011). Increased affinity (free energy, or enthalpy) due to association of histone proteins with DNA may also contribute to nucleosome translational or rotational positioning (Lowary and Widom, 1997), while relatively small quantities of free energy appear sufficient to precipitate association/disassociation with histone H1 (Rippe, 2012). In the absence of ATP-dependent DNA remodeling complexes, translational sliding of nucleosomes along DNA is temperature

dependent, with repositioning taking place relatively slowly, at a rate of a few hours per 200 bp (Blossey and Schiessel, 2011). More generally, a range of histone modifications have been found to be associated with the heat stress response (Kim et al., 2015).

H2A.Z occupancy, especially at the TSS+1 nucleosomes of temperature-induced genes, has been shown in *Arabidopsis* to decrease with temperature, independent of transcription (Kumar and Wigge, 2010), so that when H2A.Z deposition is prevented, plants have a constitutive warm temperature response. Thus the canonical H2A nucleosomes do not contribute to unwrapping of DNA from the nucleosome in response to temperature, while H2A.Z nucleosomes become increasingly accessible to RNA Pol II as temperature rises. Where gene transcription decreases with temperature this may be due to H2A.Z providing greater access to binding of repressors at these loci, or by facilitating *de novo* DNA methylation (Kumar and Wigge, 2010).

Involvement of H2A.Z in Thermosensory Flowering

The autonomous flowering pathway was conventionally regarded as being independent of environmental signals such as photoperiod. However, mutant analysis has demonstrated that genes of this pathway are also directly involved in mediating the effects of ambient temperature (Blázquez et al., 2003), with consequent effects on the expression of *FLOWERING LOCUS T* (*FT*), the mobile integrator gene of the floral pathway.

The mechanism by which the *FT* locus mediates the thermosensory flowering pathway (Halliday et al., 2003; Balasubramanian et al., 2006) is now being unraveled. It appears that while H2A.Z is enriched in the promoter region of *FT*, it is depleted at higher temperature, providing an explanation for acceleration of flowering in *Arabidopsis arp6* mutants deficient in H2A.Z (Kumar and Wigge, 2010). The chromatin modification that results from the heat-induced removal of H2A.Z from nucleosomes provides access to the *FT* promoter by the bHLH TF PIF4 (Kumar et al., 2012). In terms of control logic, H2A.Z is able to provide a genome-wide mechanism that is directly and rapidly coupled to temperature, and thus facilitate fine-tuning of phenotypic plasticity in response to environment.

This generic mechanism appears conserved in *Brachypodium*, a close relative of the major Pooideae grain crops, where H2A.Z nucleosomes appear responsible for the observed increase in thermal sensitivity of endosperm compared with vegetative tissue in the major monocot grain crops (Boden et al., 2013). Notably, H2A.Z nucleosome occupancy was more responsive to increases in ambient temperature in grain reproductive tissues, and correlated with the sensitivity to increased ambient temperature during early maturity. Thus the genomic organization of H2A.Z in *Brachypodium* results in limited impact of temperature on the phase transition from vegetative to reproductive stage, whilst retaining sensitivity at grain filling—a major contributor to yield in temperate grain crops. Perturbing the deposition of H2A.Z was found to be sufficient to mimic the effects of a warm temperature environment on grain development (Boden et al., 2013).

Temperature Effects on RdDM

Temperature also appears to play a role in mediating RNA silencing (Szittya et al., 2003; Romon et al., 2013; Zhong et al., 2013; Liu et al., 2015). Post transcriptional gene silencing (PTGS) is characterized by accumulation of snRNAs, targeted degradation of mRNAs and DNA methylation of target genes. This can be inhibited in *Arabidopsis* by increasing growth temperature from 22 to 30°C (Zhong et al., 2013), and inherited through meiosis, affecting DNA methylation status as a result of exposure to higher temperatures in the previous generation. The release of PTGS appears to be due to a reduction in the formation of dsRNA required for production of siRNAs in the RNA silencing pathway, where the temperature increase reduces abundance of SUPPRESSOR OF GENE SILENCING 3 (SGS3). When over-expressed, SGS3 can reverse the warmth-induced inhibition of siRNA biogenesis and so reduce the transgenerational epigenetic memory (Zhong et al., 2013). Moreover, temperature induced release of sense transgene-mediated PTGS is dose dependent and stochastic between 24 and 28°C, but becomes deterministic at 30°C, with associated variation in warmth-induced DNA methylation within the target transgenes.

Basal heat tolerance in *Arabidopsis* also involves the RdDM pathway (Popova et al., 2013), with consequences for transcription and epigenetic regulation of transposons. *Arabidopsis* plants defective in either NRPD2, a subunit of RNA Pol IV and V, or in HDA6, an Rpd3-type histone acetylase, are hypersensitive to heat exposure, and these genes have independent roles in transcriptional reprogramming in response to temperature stress (Popova et al., 2013).

Thermal Memory

Detection of accumulated thermal units or growing day degrees above or below a threshold has been resolved to major QTLs (Sadok et al., 2007; Bogard et al., 2014; Sánchez-Pérez et al., 2014), even in complex crop genomes such as *Brassica napus* (canola, rapeseed), where candidate genes at such loci appear to account for differences between over-wintering and summer crop types (Wang et al., 2012; Nelson et al., 2014). Natural flowering responses in *Arabidopsis* have also been localized to the *cis*-regulatory regions of the *FT* locus (Schwartz et al., 2009b). In *B. napus*, six copies of *FT* appear to have contributed to more complex mechanisms of floral regulation and niche adaptation compared to *Arabidopsis* (Wang et al., 2012) with promoter analysis (Wang et al., 2012) indicating that one copy (*FT*-C2) has been repressed by transposon insertion, with high levels of 5mC in both *B. napus* and the ancestral *B. oleracea*. Meanwhile the *FT*-A7/C6 homologs are specifically silenced in winter type *B. napus*, but abundantly expressed in spring type cultivars under vernalization-free conditions.

In *Arabidopsis*, dissection of the molecular mechanisms underlying vernalization has uncovered the role of epigenetic marks, particularly the polycomb-mediated additive effect of histone modifications, which under cold conditions regulate silencing of the flowering repressor *FLOWERING LOCUS C* (*FLC*, Sheldon et al., 2009; Romera-Branchat et al., 2014). Subsequently, it has been found that long non-coding RNAs

(lncRNAs) COOLAIR (Swiezewski et al., 2009) and COLDAIR (Heo and Sung, 2011) are embedded within the *FLC* locus, and also induced during vernalization in *Arabidopsis* by periods of cold. COLDAIR has been proposed to recruit the H3K27me3 mark to the *FLC* gene, thus contributing to *FLC* repression (Heo and Sung, 2011), whereas some alternatively spliced isoforms of COOLAIR may contribute to activation of *FLC* (Csorba et al., 2014; Romera-Branchat et al., 2014).

Temperature Conditioning and Epigenetic Variation

An early study by Burn et al. (1993) found that cold temperatures lead to hypomethylation in *Arabidopsis* and a *Nicotiana* cell line. Subsequently Finnegan et al. (1998) studied the effect of imbibing *Arabidopsis* C24 seed for 4 weeks at 8°C, and detected a large (86%) albeit transitory effect of hypomethylation in mature leaves compared with untreated controls at the same stage of development. These phenomena appeared to be reversible, as after 7 days growth at 22°C DNA methylation in seedlings developed from the vernalized controls was comparable to those of control seedlings. Cold stress has since been found to lead to genome-wide demethylation in maize seedlings (Steward et al., 2002), while growth of *Antirrhinum majus* in low-temperature conditions results in hypomethylation of the transposon *Tam3* (Hashida et al., 2006). A related phenomenon is also observed with heat-stress, where inducible alterations in endogenous loci generally lead to hypomethylation of retro-elements, with depression of transcription along with transient changes in nucleosome density (Lang-Mladek et al., 2010; Pecinka et al., 2010; Ito et al., 2011).

Histone methylation at H3K27me3 has also been shown to decrease gradually during cold exposure in two *Arabidopsis* cold-responsive genes *COR15A* and *ATGOLS3* (Kwon et al., 2009). It appears that in this case gene activation leads to removal of H3K27me3 and that this mark is able to be inherited quantitatively, providing a memory of recent transcriptional activity.

A SPECULATIVE FRAMEWORK: ELECTROSTATIC AND EPIGENETIC INTERACTIONS WITHIN THE PLANT NUCLEUS

Our current understanding of the direct involvement of ionic and temperature variation on chromatin structure and transcription is fragmentary. However, it is clear that some key biophysical properties of chromatin components and epigenetic marks are affected by electrostatic and thermal interactions, and these more fundamental observations are starting to align with observations at cellular and whole organism level. In order to unravel these relationships, experimental approaches need to distinguish between direct effects and those mediated by signal transduction pathways that sense variation in external ionic or temperature environment and are then apparent at the level of epigenetic modifications.

Few studies have systematically evaluated the ensemble of interactions that place the ionic and thermal environment, the

biophysical attributes of 5mC, H2A.Z and other epigenetic marks in the context of chromatin dynamics and genomic regulation (McClung and Davis, 2010). Thus, at present there is no cohesive model that takes into account the contribution made by each of the distinct epigenetic marks to chromatin conformation, and the ability of plants to maintain complex genomic regulation under fluctuating external environmental conditions. However, from the information presented in this review it is clear that temperature and ionic conditions both play an important role in determining the biophysical behavior of histone and DNA macromolecules, their interaction in forming nucleosomes, and in higher order chromatin conformation.

Taken together, the various lines of evidence outlined here appear to be internally consistent in describing contributions to accessible versus inaccessible chromatin. The framework that emerges is based on what appears to be a cohesive set of interactions at molecular, biophysical and electrostatic level between the various components that affect chromatin conformation and dynamics. This is represented in a simple schematic (**Figure 1**) that outlines the behavior of the key components of DNA, histones, and nucleosomes in the context of epigenetic marks and ionic environment within the plant nucleus. From this set of interactions, it is possible to speculate that within plant nuclei, general and localized ionic homeostasis plays a significant important role in maintaining chromatin conformation, whilst maintaining complex genomic regulation involving specific patterns of epigenetic marks.

The contributions of 5mC to local DNA stability and reduced flexibility appear to be consistent with the association of 5mC with stable, more ordered nucleosomes and localized transcription, with denser methylation leading to tighter chromatin and gene repression. The complementary contributions of 5mC and H2A.Z to less accessible chromatin is consistent with their observed relative mutual exclusivity in chromatin (Zilberman et al., 2008), and H2A.Z providing the transcriptionally responsive mark in response to external temperature (Kumar and Wigge, 2010).

Complex electrostatic interactions within the nucleus contribute to the condensation state of chromatin, with the localized net charge state of the interaction between DNA and histone affecting position and stability of nucleosomes. Post-translational histone modifications play a major role, although it is currently unclear how these may be affected by the proposed differential roles played by Na⁺ and K⁺ in chromatin condensation (Allahverdi et al., 2015). The latter findings have yet to be incorporated into our understanding of ionic variation in plants, and the dearth of knowledge about the ionic environment of plant nuclei, although Na⁺ and K⁺ gradients are observed within some eukaryote nuclei (Garner, 2002). Given the huge resources devoted to K fertilizer use of around 30 mt per annum (Timilsena et al., 2014) and concerns about salinization of cultivated land, it would seem timely to explore these phenomena in more detail at a molecular level to understand mineral ion availability in the nuclei of crop plants.

Whilst tentative, this framework provides scope to develop experimental approaches to understanding in greater detail the internal environment of plant nuclei. It is hoped that this will generate a deeper understanding of the molecular mechanisms

underlying genotype × environment interactions that may be beneficial for long-term improvement of crop performance in less predictable climates.

CONCLUDING REMARKS

A high proportion of crop traits exhibit quantitative inheritance, many with relatively low penetrance. For example, in rapeseed hundreds of significant environment-specific QTL have been identified for yield and other traits in a “fixed” segregating population of homozygous lines grown in 10 environments, with relatively few coinciding in multiple environments (Shi et al., 2009). In many cases the extensive genotype × environment interactions associated with crop yield traits has limited the ability to identify underlying genes. However, it is now apparent that QTL may also be accounted for by changes in DNA methylation status, with, e.g., 60–90% of heritability for the complex traits of flowering time and primary root length being detected in epiRILs of *Arabidopsis* (Cortijo et al., 2014). It is worth noting that in *Arabidopsis*, spontaneous transgenerational epiallelic variation can occur at a rate 10^3 times higher than the genetic mutation rate (Becker et al., 2011), with hypermethylated alleles associated with siRNA production and TGS (Schmitz et al., 2011). This stochastic generation of epialleles has the potential to alter transcriptional behavior and generate novel phenotypic variation subject to selection. Thus formation of random epialleles mediated by RdDM may be of more significance than genetic variation (Matzke and Mosher, 2014). For crop breeding, there are clear indications that such variation needs to be under active selection, and attention given to maintenance of germplasm to ensure that epigenetic plasticity is hard-wired into new cultivars.

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- At present we have only a partial understanding of how the various epigenetic components confer dynamic functional information content in the context of the ionic and thermal environment of the nucleus. It is becoming clear that most regions of complex eukaryotic genomes play some role in gene regulation (Haudry et al., 2013). However, there is a need for systematic analysis of the relationships between plant genome complexity, the known taxonomic variation in ionic composition, the distribution of epigenetic marks and measures of genome ruggedness or plasticity. For example, does the relatively compact genome of rice, with less scope for redundancy in epigenetic regulation, also contribute to its inability to manage more extreme and complex abiotic stresses?
- The ability of plants to accommodate fluctuations in thermal and ionic environment is an essential fitness attribute and a key determinant for crop performance, and requires a deeper understanding of the interactions at intra-cellular and intra-nuclear level, including those with epigenetic marks and processes. The availability of comprehensive tissue-specific epigenome, nucleosome and snRNA datasets will contribute to more comprehensive models of interactions between genome organization, chromatin dynamics, and epigenetic signaling systems. These can help provide new tools and approaches for breeding selection and agronomic management of crops able to perform in changeable environments.

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Fibrillarin methylates H2A in RNA polymerase I trans-active promoters in *Brassica oleracea*

Lloyd Loza-Muller¹, Ulises Rodríguez-Corona¹, Margarita Sobol², Luis C. Rodríguez-Zapata³, Pavel Hozak² and Enrique Castano^{1*}

¹ Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, Mexico

² Department of Biology of the Cell Nucleus, Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic, ³ Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Mérida, Mexico

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Institute of Cancer Research, UK

*Correspondence:

Enrique Castano
enriquec@cicy.mx

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Fibrillarin is a well conserved methyltransferase involved in several if not all of the more than 100 methylations sites in rRNA which are essential for proper ribosome function. It is mainly localized in the nucleoli and Cajal bodies inside the cell nucleus where it exerts most of its functions. In plants, fibrillarin binds directly the guide RNA together with Nop56, Nop58, and 15.5ka proteins to form a snoRNP complex that selects the sites to be methylated in pre-processing of ribosomal RNA. Recently, the yeast counterpart NOP1 was found to methylate histone H2A in the nucleolar regions. Here we show that plant fibrillarin can also methylate histone H2A. In *Brassica* floral meristem cells the methylated histone H2A is mainly localized in the nucleolus but unlike yeast or human cells it also localizes in the periphery of the nucleus. In specialized transport cells the pattern is altered and it exhibits a more diffuse staining in the nucleus for methylated histone H2A as well as for fibrillarin. Here we also show that plant fibrillarin is capable of interacting with H2A and carry out its methylation in the rDNA promoter.

Keywords: histones, methylation, RNA polymerase I, *Brassica*, phosphoinositide

INTRODUCTION

The nucleolus is the largest structure inside the cell nucleus. The main function of this structure is ribosome biogenesis. This process involves transcription of rDNA, processing of rRNA and assembly of ribosomal proteins (Kressler et al., 1999). Ribosomal genes (rDNA) in eukaryotes are in a tandem arrayed of 100–1000s (depends on the species) copies at chromosomal loci,

Abbreviations: aFib, archaea fibrillarin; AtFib1, *Arabidopsis thaliana* fibrillarin 1; AtFib2, *Arabidopsis thaliana* fibrillarin 2; BoFib, *Brassica oleracea* fibrillarin; DABCO, 1,4-Diazabicyclo(2.2.2)octane; DAG, diacylglycerol; DAPI, 4',6-diamidino-2-phenylindole; DFC, dense fibrillar component; DTT, dithiothreitol; FAA, formalin – acetic acid – alcohol; FACT, facilitator of chromatin transcription; FC, fibrillar center; GAR, arginine glycine rich domain; GC, granular component; GMSA, gel mobility shift assays; HRP: horseradish peroxidase; HsFib, homo sapiens fibrillarin; IPTG, Isopropylthiogalactoside; IRES, internal ribosome entry site; NCBI, national center for biotechnology information; NE, nuclear extract; Nop1, nucleolar protein 1; Nop56: nucleolar protein 56; Nop58, nucleolar protein 58; PBS, phosphate-buffered saline; PBST, Phosphate-buffered saline tween; PI4,5P2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C; PVDF, polyvinylidene difluoride membrane; rDNA, ribosomal DNA; rRNA, ribosomal RNA; SAM, S-adenosyl methionine; SMN, survival of motor neuron; snoRNA, small nucleolar RNA; snoRNP, small nucleolar ribonucleoprotein; TBS, Tris-buffered saline; TBST, Tris-buffered saline tween; U2OS, human osteosarcoma cell line; UBF, upstream binding factor.

known as nucleolus organizer regions. Each rRNA gene is transcribed within the nucleolus by RNA polymerase I to produce a primary transcript that is processed to form the 18S, 5.8S, and 25–28S rRNAs (Nemeth and Langst, 2011). However, the nucleolus is also involved in several other processes like genetic silencing, cell cycle progression, senescence and biogenesis of snRNA and tRNAs (Jacobson and Pederson, 1998; Cockell and Gasser, 1999; Garcia and Pillus, 1999). In plants the nucleolus consists of four components: FCs, DFC, GC and the nucleolar vacuole (NV). Fibrillarin is a methyltransferase involved in the processing of the primary ribosomal transcript and is mainly located in the FC and DFC region of the nucleoli where it is directly involved in several steps of ribosome biogenesis (Rodriguez-Corona et al., 2015). Fibrillarin is known to be part of the snoRNP that methylate rRNA (Tollervey et al., 1993). Biochemical evidence for the process with eukaryotic fibrillarin is lacking but it has been demonstrated using aFib in order to recapitulate the methylation process on rRNA (Tran et al., 2003). High resolution crystal structure data from this complex has been obtained by several laboratories (Aittaleb et al., 2003; Oruganti et al., 2007; Ye et al., 2009) and have shown a well conserved overall structure (Rodriguez-Corona et al., 2015). The snoRNA acts like a guide to help direct aFib together with Nop56/58 and L7Ae that interact with the rRNA in order to methylate at specific sites. In eukaryotes fibrillarin has been shown to form a complex with Nop56, Nop58, protein 15.5Ka and different guide RNAs like U3, U6, etc. The guide RNA recognizes specific regions to be methylated on rRNA. Fibrillarin is also involved in the earliest steps of ribosomal transcription initiation and this step requires the interaction with PI4,5P2 (Sobol et al., 2013; Yildirim et al., 2013) linking the rRNA processing with rRNA transcription initiation where PLC can inhibit transcription initiation (Yildirim et al., 2013). Overproduction of fibrillarin in mammalian cells can lead to alteration in ribosomal methylation and as a result there is an alteration in the process of translation. Highly methylated ribosomes surpass IRES leading to misread translation that results in some types of cancers (Marcel et al., 2013). In plants, fibrillarin has been shown to be part of the mediator of RNA polymerase II transcription (subunit 36a) (Backstrom et al., 2007). Two different RNA binding sites have been determined in fibrillarin from *Arabidopsis thaliana* (Rakitina et al., 2011). Plant fibrillarin has also been a link between both rRNA gene binding and pre-rRNA processing by analyzing the fractions containing the snoRNP complex in both promoter complex and rRNA cleavage sites (Saez-Vasquez et al., 2004). Moreover, plant umbravirus life cycle suggest the requirement of fibrillarin. Fibrillarin is redistributed upon infection to the cytoplasm and participates in the formation of viral ribonucleoproteins able to move through the plant phloem resulting in complete infection of the plant (Kim et al., 2007). Recently, fibrillarin has been shown to be involved in epigenetic nucleolar mechanism. Fibrillarin methylates histone H2A in yeast and human cells at position Q105 and this methylation is unique to the nucleolus (Tessarz et al., 2014). The FACT (facilitates chromatin transcription) is a protein complex known to facilitate transcription elongation of RNA pol II derived transcription where it has a preferential interaction

to histone H2A/H2B dimers. In RNA pol I transcription FACT interacts preferentially with the methylated H2A to reorganized nucleosomes in the active promoters for rRNA (Tessarz et al., 2014). Nevertheless, the ribosomal promoter has been shown to differ significantly between mammalian and plants (Perry, 2005; Knight et al., 2014). We show that plant fibrillarin is also capable to methylate histone H2A while bound to the rDNA. Our results also showed that *in vivo* methylated histone H2A in *B. oleracea* can also be found at other locations besides the nucleolar regions, this modification in plants may have additional epigenetic roles than what is found in animal cells.

MATERIALS AND METHODS

Maintenance and Propagation of Cell Culture

U2OS osteosarcoma cells were kept in DMEM with 10% fetal calf serum in 5% CO₂/air, 37°C, humidified atmosphere.

Antibodies

Rabbit polyclonal anti-H2A (Q105Met) was a kind gift from Tessarz et al. (2014). Rabbit Fibrillarin Antibody (H-140): Santa cruz sc-25397; Anti-Histone H2A antibody ChIP Grade (ab15653) Abcam. Anti-Histone H3 (mono methyl K4) antibody – ChIP Grade (ab8895). Goat Anti-Rabbit IgG H&L (Alexa Fluor 647) (ab150079) Abcam. (Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate (Invitrogen) (A-11008).

Nucleotide Sequence Data base

Fibrillarin nucleotide sequence from *B. oleracea* (BoFib) was obtained from the database for *B. oleracea* (<http://www.ncbi.nlm.nih.gov/bolbase/>) with the accession number: Bol39546. All other nucleotide sequence were obtained from NCBI: *Saccharomyces cerevisiae* fibrillarin (Nop1: CAA98572.1), *Homo sapiens* fibrillarin (HsFib: CAA39935.1) and *Arabidopsis thaliana* fibrillarin 1 and 2 (AtFib1: NP_568772.3, AtFib2: NP_567724.1; respectively).

Plasmids

pET15b::Fibrillarin contain the sequence from *A. thaliana* fibrillarin 2 (NP_567724.1). The *pHis::PLC* that expresses recombinant PLC were received from Dr. Hitoshi Yagisawa. All expression vectors were in frame with the histidine tag from the plasmid. pLLMP1 plasmid was constructed by cloning rDNA promoter (-265 to +163) from a PCR of the genomic DNA of *B. oleracea* into pGEM. The oligos used for the PCR of rDNA (fwd 5'-TCGGTAC CGAGTTAGGATGTCAAGT-3' rev TAGGATCCGGAAAAGTCGCC GGAAAAG-3') (Chen and Pikaard, 1997). pUC18 was from Thermo Fisher Scientific.

Recombinant Protein Expression and Purification

Expression vectors were transformed in *Escherichia coli* BL21 (DE3) pLysE from Invitrogen and allowed to grow to an OD of

0.5 at 600 nm. 1 mM IPTG was added after and incubated at 25°C for 3 h. Followed by 10 min centrifugation at 4000 × g, suspension was carried out in a denaturing buffer (20 mM Tris HCl, pH 7.9, 8M Urea, 0.1 M NaH₂PO₄, 0.5 M KCl, 20 mM imidazol) and sonicated three times. The re-suspended lysate was centrifuged at 4000 × g for 10 min to remove cell debris and the supernatant was allowed to binding 0.1 ml of Ni²⁺- nitrilotriacetic acid resin for 1 h. The column was wash with 5 ml of the denaturing buffer. Finally 0.3 ml of elution where recovered in a denaturing buffer containing 250 mM Imidazole.

Nuclear Extract and Histone Purification

Brassica oleracea nuclear extraction was carried out as described by Gustavsson et al. (1991). Briefly we used 60 g of fresh weight for the maceration in liquid nitrogen and suspended at 4°C with an extraction buffer 50 mM Tris-Cl pH 8.0, 3 mM EDTA, 2 mM EGTA and 0.2% NP 40. Debris was removed and the extract collected. Centrifugation of the extract was carried out and the nuclei were responded in a hypotonic buffer for 30 min at 4°C followed by addition of an extraction buffer 10 mM Tris-Cl pH 8.0, 1.5 M NaCl, 0.05% NP40 to obtain the NE after centrifugation at 6500 g for 10 min. The extraction of histones from *B. oleracea* was carried out from the left over nuclear pellet and high salt extraction buffer 10 mM Tris-Cl pH 8.0, 2.5 M NaCl, 0.05% NP40 was added for 30 min under rotation at 4°C. Centrifugation at 16000 g for 10 min. was carried out and the remaining extract contain a large amount of histones.

Western Blot Analysis

Proteins were separated on a 15% SDS-PAGE and transferred to nitrocellulose membrane (Pall Corporation, USA). After 1 h of blocking with 5% non-fat milk in TBST (TBS, 0.1% Tween-20), the membrane was incubated with either anti-H2AQ105 or anti fibrillarin as mention in the legends in TBST with 5% milk over night at 4°C then washed with TBST. Immunoreactive bands were detected with anti-rabbit antibodies conjugated with HRP followed by AlkPhos direct labeling reagents (Amersham).

Immunofluorescence

The plant tissue was fixed in tubes containing FAA with aspiration for 24 h. They were dehydrated through an ethyl alcohol series and embedded in paraffin (melting point 54–56°C) with a graded series of tertiary butyl alcohol. The paraffin blocks were sectioned serially at 5 µm thickness using a microtome. The deparaffinization was carried out with four washes with Histology grade Xylene for 2 min and by removal of xylene with absolute ethanol. Seventy percent ethanol followed by water for 1 min each. *B. oleracea* inflorescence and surrounding tissue were permeabilized with 0.1% Triton X-100 in PBS for 15 min, respectively. After washes with PBST they were either incubated with anti-H2AQ105me or anti-fibrillarin. Secondary antibodies donkey anti-rabbit IgG conjugated with Alexa 488 (Invitrogen), goat anti-rabbit IgG conjugated with Alexa 647 (Invitrogen). After being washed for 30 min with PBST cells were mounted with moviol (DAPI-DABCO). Images were taken in confocal microscope (Leica TCS SP5 AOBS TANDEM) and a laser-scanning microscope FV100 Olympus with 60X (NA 1.4)

oil immersion objective lens. U2OS were treated as published previously (Sobol et al., 2013).

Gel Mobility Shift Assays

Gel mobility shift assays were carried as previously published (Castano et al., 1997), with minor modifications. End-labeled rDNA promoter was incubated for 30 min with 10 ng of purified protein at room temperature using the binding reaction contained 5 ng of probe (5000 c.p.m./ng), 25 mM HEPES (pH 7.4), 80 mM NaCl, 10% glycerol, 0.5 mM PMSF and 1 mM leupeptin in a final volume of 20 µl. The mixture was separated in a native 6% PAGE at 4°C followed by autoradiography.

Methylation

For assays on purified histones, 0.2 µg of Atfib2 was assayed on 1 µg of purified histones in the presence of 100 µM SAM (H³) in 1/2 TBS and 1 mM DTT for 30 min at 30°C. Half of the reaction was loaded on SDS-polyacrylamide gel electrophoresis for Coomassie staining and 20% of the reaction for western blotting or for scintillation counting.

Farwestern

Purified histones were separated on an 15% SDS PAGE and transfer to a PVDF membrane. Membrane was blocked with PBST with 5% of non-fat milk (PBS, 0.1% tween-20) for 1 h at room temperature, then washed three times with PBST. After blocking the membrane was incubated with AtFib2 (0.5 µg) as bait in protein binding buffer (20 mM Tris pH 7.6, 100 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.1% tween-20, 2% non-fat milk, 1 mM DTT) at 25°C for 4 h, then washed three times with PBST and incubated with anti-Fibrillarin (for 12 h at 4°C). Immunoreactive bands were detected with anti-rabbit antibodies conjugated with HRP followed by AlkPhos direct labeling reagents (Amersham). Striping of the membrane was incubated at 50°C for 45 min under agitation in a buffer (50 mM Tris HCl pH 6.8, 2% SDS and β-mercaptoethanol(8ml/l)) followed by rinsing the membrane with water.

Transcription Pull-down In Vitro

Methodology published in Castano et al. (2000). Brief explained a reaction mixture containing either NEs or purified transcription factors were mix with 100 ng of rRNA promoter in the presence of 0.5 mM NTP, 5 mM MgCl, 5 mM DTT, in 20 mM HEPES KOH pH 8.4, in 20 ul reaction volume. In order to assay if H2A methylation was bound during the transcription, rDNA promoter region was bound to magnetic beads (Dynabeads MyOne Streptavidin C1, 650.01, Invitrogen). The promoter was obtained by PCR from the plasmid containing the RNA pol I promoter sequence from *Brassica oleracea*. The oligos used were a 5'-biotin labeled oligo TCGGTACCGAGTTT AGGATGTCAAGT-3' (promoter region from -265 to -248) and a reverse oligo 5'-TAGGATCCGGAAAAGTCGCCGGAAAAG-3' from +142 to +163 (published by Chen and Pikaard, 1997). Control oligos 5'-biotin labeled pUC18 CCC AGTCACGACGTTGTAA and a reverse CGCAACGCAATTATGTGAG were purchase from

Sigma-Aldrich. Before adding the NEs the bound sequences were blocked with 5% BSA for 1 h at 4°C. The beads were then incubated with NE in a transcription buffer without nucleotides for 1 h, after incubation the beads were washed six times with a buffer containing 20 mM Tris pH 7.9, 100 mM KCl, 0.1 mg/ml BSA, 10% Glycerol, 0.2 mM EDTA pH 8.0. The full amounts of beads were loaded into a PAGE for western blot analysis. PVDF membranes were soaked in Ponceau S stain [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] to verify protein transfer.

RESULTS

Fibrillarin sequence can be divided into four regions: The GAR domain, Space region, the Central domain with the RNA binding region and the Alpha helix rich domain. The GAR domain is typically the least conserved and contains a non-structural motif that is methylated in human cells. **Figure 1** shows the sequence alignment and domain position for fibrillarins. The comparison between human, yeast, *A. thaliana* and *B. oleracea* reveal that the GAR domain contains the lowest degree of conservation with 31.82% of similarity between AtFib1 and Nop1 as the lowest and with 49.32% of similarity between AtFib2 and BoFib as the highest. The RNA binding domain is well conserved in all species with 72.04% of similarity between AtFib1 and Nop1 as the lowest and with 98.92% of similarity between AtFib1 and AtFib2 as the highest. The alpha helix rich domain differs by 63.44% of similarity between HsFib and Nop1 as the lowest and with 90.72% of similarity between AtFib1 and AtFib2 as the highest, and is known to interact with other proteins in mammalian cells like SMN (Pellizzoni et al., 2001). The red marked amino acids indicate the sites for mutations that allowed Nop1 to be a temperature sensitive mutant. These are key amino acids in Nop1 and are essential for yeast viability at 37°C. We find that for the most part are well conserved, with two alterations between *B. oleracea* and Nop1 located at the N terminus. The two green slash boxes highlight the sequences defined by Rakitina et al. (2011) to be responsible for RNA binding in *Arabidopsis*, while the green letters define the human RNA binding domain. The bold blue label arginine amino acids in the sequence are known to be methylated in human cells. The yellow boxed serine is known to be phosphorylated and the black boxed lysine to be acetylated in human fibrillarin. Although the exact function of all the modifications has still to be defined in any species, and may reflect the high versatility of this protein in different complexes that may occur in the cells (Rodriguez-Corona et al., 2015).

In order to assay the effect of phospholipids in specific histone binding to the rDNA *in vitro* we tested the NE and purified histones from *B. oleracea* on rDNA promoter binding. We used magnetic beads with the rDNA promoter region as bait. **Figure 2A** shows a typical pull-down experiment, the asterisks indicate the proteins that were specifically bound to the promoter. The amount of these bound proteins increased with the pre-incubation of PLC to the NE. PLC acts on PI4,5P2 which is a known phosphoinositide that binds several nuclear

proteins including fibrillarin and histones (Yu et al., 1998; McLaughlin et al., 2002; Yildirim et al., 2013). Since several of the proteins bound to the promoter had a similar profile to that of histones, we further tested that PLC treatment would affect the interaction of histones to the promoter. We carried out a GMSA (**Figure 2B**) with purified histones from *B. Oleracea* and used them to bind the rDNA promoter. Histones bound readily to the promoter and PI4,5P2 degradation by PLC showed an increased histone binding to the promoter. However, the NE reduced significantly the binding of histones to the promoter. This indicates a competition for the interaction between the histones and the ribosomal promoter with NE components that prevent this interaction. PLC treatment under these conditions did not show a significant increase in binding.

Histone H2A found in active rDNA has been recently shown to be methylated in the nucleolus by fibrillarin in yeast and human cells (Tessarz et al., 2014), therefore we decided to test if plant fibrillarins can also methylate H2A at the rDNA promoter. Purified histones from *B. olearace* were used (**Figure 3A**) and tested for both protein-protein interactions and methylation using AtFib2 (**Figure 3B**) which is 88% identical to BoFib.

To test for protein-protein interactions we used a far-western approach where the transferred histones were used as bait for AtFib2. Western blot of AtFib2 shows the amount used in the assay (**Figure 3B**). The binding of AtFib2 to histone H2A is shown by farwestern (**Figure 3C**). Histone H2A was verified by western blot after stripping (**Figure 3D**). We expected fibrillarin to tightly bind their substrates until the enzymatic reaction could be accomplished plus previously this possible interaction was obtained from a two hybrid system in the interactome data published (Krogan et al., 2006). Krogan et al. (2006) showed H2A among several other proteins that can bind human fibrillarin. After farwestern blot analysis we proceeded to carry out a methylation assay to verify if fibrillarin methylate histones *in vitro* (**Figure 3E**). This was done by mixing tritium radiolabeled SAM, AtFib2 and histones from *B. oleracea*. After the reaction, the histones were separated in a 15% SDS PAGE and stain histones were measured on a scintillation counter showing specific addition of the radiolabeled SAM by the addition of AtFib2.

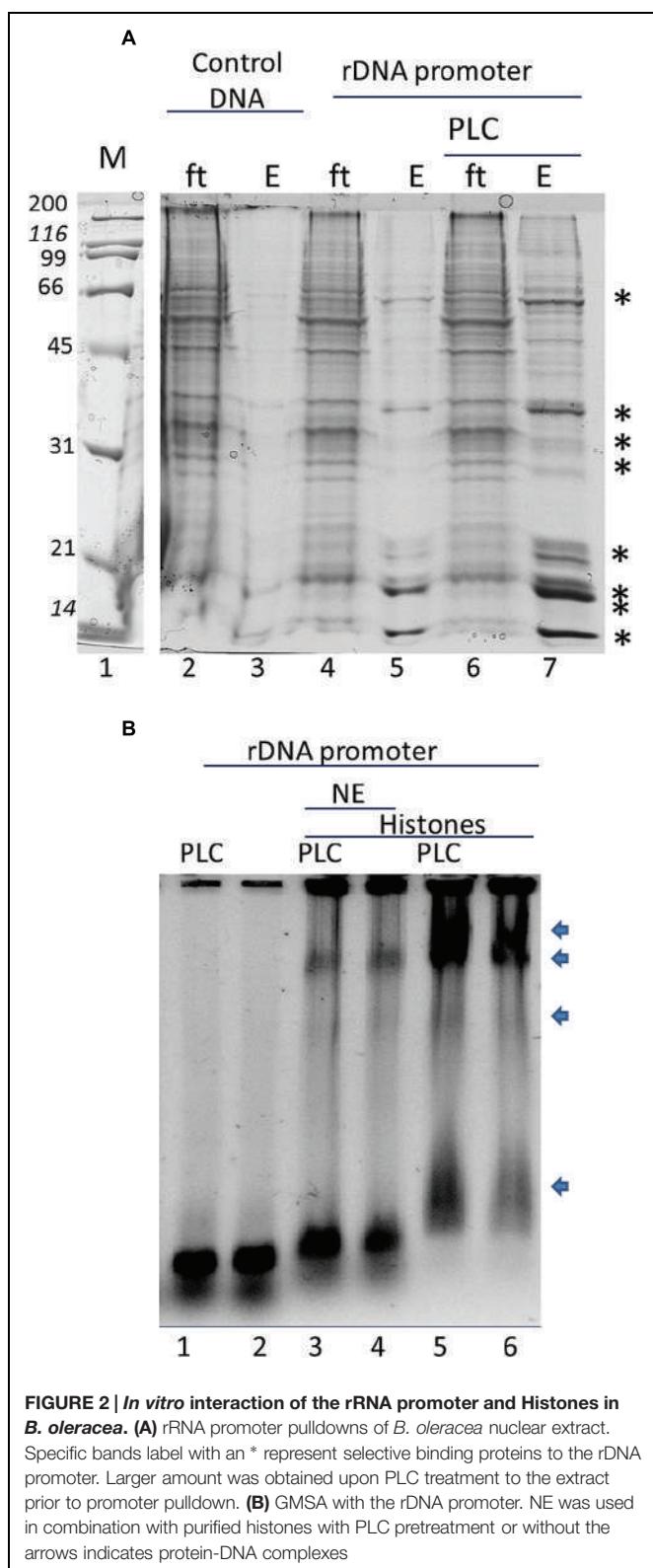
H2A methylation was further checked by western blot (**Figure 3F**). The aid of anti-H2AQ105me previously used to check H2A methylation by yeast fibrillarin Tessarz et al. (2014) showed successfully that AtFib2 methylate histones H2A from *B. oleracea*. Moreover, AtFib2 methylated H2A while bound to the rDNA promoter. We tested this by allowing the histones from a methylation reaction bind to the rDNA promoter attached to magnetic beads for 1 h. The rDNA promoter bound proteins were resolved on a 15% SDS PAGE and western blot was carried out with anti-H2AQ105me detecting large amounts of methylated H2A as compared with a pUC18 sequence bound to magnetic beads used as control (**Figure 3F**). Since control and rDNA promoter beads were incubated in a buffer containing BSA, the loaded amount was verified by staining the membrane with ponceau and checking that BSA amounts should be equal.

GAR domain		
A. thaliana 1	--MRPPVTG-----GRGGGGFRGG-RDGGGRGFGGGRSFGGGRSG-DRGRS--GPRGR	47
A. thaliana 2	--MRPPLTGSGGGFSGGRGRGGYSGG-RGDG--GFSGGRG-GGGRRGGRGFSDRGGRGR	53
B. oleracea	--MRPPLTG-----GRGGGGFSGG-RGGG--GFSGGRSGGRGRAG-GRGFGDRGGGRS	47
H. sapiens	--MKPGFSPRGFG-GRGGFGDRGG-RGGRG-GFGGGRGGFRGRGRGGGGGGGGGG	55
Yeast (Nop1)	MSFRPGSRG--GSRGGSRGFGGRGGSRGGARGGSRGFGGRGGSRGGARGGSRGFGGR	58
Space region		
A. thaliana 1	GRGAPRG----RGG--PP-RGGMKG---GSKVIVEPHRHAGVFIAKGKEDALVTKNLVPG	97
A. thaliana 2	GRGPPRGG--ARGGRGPAGRGGMKG---GSKVIVEPHRHAGVFIAKGKEDALVTKNLVPG	108
B. oleracea	GRGMRGRGERGRNGRGPGRGGMKG---GSKVIVEPHRHPGVFIAKGKEDALVTKNLVPG	104
H. sapiens	GGGRGGGG-FHSGGNRGRGGKRGNQSGKVNVMVEPHRHEGVFICRGKEDALVTKNLVPG	114
Yeast (Nop1)	GGSRGGARGGSRGGR-GGAAGGARG---GAKVVIIEPHRHAGVIAARGKEDLLVTKNMAPG	114
central domain		
A. thaliana 1	EAVYNEKRISVQN---ED GTKVEYRVWNPFRSKLAAAIILGGVDNIWIKPGAKVLYLG	151
A. thaliana 2	EAVYNEKRISVQN---ED GTKTEYRVWNPFRSKLAAAIILGGVDNIWIKPGAKVLYLG	162
B. oleracea	EAVYNEKRISVQN---ED GTKTEYRVWNPFRSKLAAAIILGGVDNIWIKPGAKVLYLG	158
H. sapiens	ESVYGEKRV[S]S---EG DDKIEYRAWNPFRSKLAAAIILGGVDQIHICKPGAKVLYLG	167
Yeast (Nop1)	ESVYGEKRISEEPSKEDGVPP[KVEYRVWNPFRSKLAAGIMGLDEFIAPGKKVLYLG	174
RNA binding region		
A. thaliana 1	AASGTTVSHVSDIVGPE[GCVYAVEF]SHRSGRDLVNMAKKRTNVIPIIEDARHPAKYRMLV	211
A. thaliana 2	AASGTTVSHVSDIVGPE[GCVYAVEF]SHRSGRDLVNMAKKRTNVIPIIEDARHPAKYRMLV	222
B. oleracea	AASGTTVSHVSDIVGPEGCVYAVEFSHRSGRDLVNMAKKRTNIIPIIEDARHPAKYRMLV	218
H. sapiens	AASGTTVSHVSDIVGPGLVYAVEFSHRSGRDLINLAKKRTNIIPIIEDARHPHYRMLI	227
Yeast (Nop1)	AASGTSVSHVSDVVGPE[GVVYAVEF]SHRPGRRELISMAKKRPNIIPIIEDARHPQKYRMLI	234
Alpha helix rich domain		
A. thaliana 1	GMVDFVIFSDVAQPDQARI[LALNASFF]LKTGGHFVSIKANCIDSTVAAEAVFQSEVKLQ	271
A. thaliana 2	GMVDFVIFSDVAQPDQARI[LALNASFYFLKSGGHFVSIKANCIDSTVPAEAVFQTEVKLQ	282
B. oleracea	GMVDFVFA[DVAQPDQARI]VADNSSFFLKTGGHFVSIKANCIDSTVPAEAVFQSEVKLQ	278
H. sapiens	AMVDVIFADVAQPDQTRIVALNAHTFLRNGGHFVISIKANCIDSTASAEEAVFASEVKMQ	287
Yeast (Nop1)	GMVDCVFADVAQPDQARI[LALNSHMF]LKDQGGVVISIKANCIDSTVDAETVFAREVQKLR	294
A. thaliana 1	QEQQPKPAEQVTLEPFERDHACVGGYRMPKKQKTPAS-	308
A. thaliana 2	QEQQPKPAEQVTLEPFERDHACVGGYRMPKKPKAATAA	320
B. oleracea	QEQQPKPAEQVTLEPFERDHACVGGTYRAPKKTVA--	314
H. sapiens	QENMKPQEQQLTLEPYERDHAVVVGVYRPPP[PKVN]---	321
Yeast (Nop1)	EERIKPLEQLTLEPYERDHICIVVGRYMRSGLKK----	327

FIGURE 1 | Fibrillarin sequence comparison relationships of taxa. The analysis included the sequences from both *Arabidopsis thaliana* fibrillarin (AtFib NP_568772.3 and AtFib2 NP_567724.1), fibrillarin sequence from *Brassica oleracea* (BoFib BoI039546), fibrillarin sequence from *Homo sapiens* (HsFib CAA39935.1) and the yeast fibrillarin Nop1 CAA98572.1. All the domains are label in different colors Gar domain in blue, space region in gray, central domain in purple and the alpha rich domain in orange. Arginines known to be methylated are marked in a red circle. Key amino acids that were mutated in Nop1 are marked in red. The phosphorylated serine is marked in a yellow square and the acetylated lysine in a black square. The dotted underline sequence marks the methyl transferase domain. The slash boxes in green indicate the RNA binding domains in *Arabidopsis thaliana* fibrillarins. Green label amino acids indicate the define RNA binding region.

We proceeded with the *in vivo* localization of methylated H2A by immunolocalization in cells of *B. oleracea*. The immunolocalization pattern of anti-H2AQ105me and fibrillarin was compared between human U2OS cells and *B. oleracea* cells. Both plant and human cell lines showed a primary stain of

fibrillarin and methylated histone H2A in the nucleoli. Human U2OS cells were used as a control since the immunolocalized pattern for H2A Q105me had already been published (Tessarz et al., 2014). Here we show a higher magnification the staining of anti-H2AQ105me in human cells. As can be



seen the stain at the nucleolus is not homogenous and there is a weak diffuse nuclear stain (**Figure 4A**). Floral meristem *B. oleracea* cells showed fibrillarin stain located in the nucleolus

(**Figure 4B**). The secondary antibody did not stain the cells and was used to set the intensities of the signals (**Figure 4C**). The staining with anti-H2AQ105me shows an additional stain on the periphery of the nucleus and additional stain outside the nucleus (**Figure 4D**). This pattern of stain was reproducible in three independent experiments and in all the fresh cauliflower floral meristem buds that have a round nucleus. The staining was specific to anti-H2AQ105me as addition of just secondary antibody did not stain the cells (**Figure 4E**). The additional stain of the anti H2AQ105me outside the nucleus also shows exactly in the same position a weak DAPI stain at the extra nuclear regions, we were surprised by this extra nuclear DNA, but it is been consistent in three independent experiments with different reagents. This extranuclear DNA could be either an aggregation of organelles like mitochondria from the meristematic cells as previously shown by Kuroiwa et al. (1992). We also checked the pattern in specialized cells. The vascular inflorescence cells showed a different stain as seen in **Figure 5**. Due to the type of tissue, these cells are elongated in order for them to carry out their function. The nucleus is also elongated and thinner than in meristem cells. Here the fibrillarin stain was not only localized to the nucleolus but showed a diffuse stain in most of the nucleus (**Figure 5A**). This is a typical localization of fibrillarin in cells that are under stress (Mironova et al., 2014). As well as in cells that overexpress fibrillarin. Specialized transport cells in plants may reflect this pattern for unknown functional roles at this time. None of these specialized cells showed additional extrachromosomal staining as compared with all of the meristem cells that had a weak extranuclear DAPI stain. The Anti-H2A (Q105M) staining showed a similar pattern to that of fibrillarin. However, these cells had no perinuclear staining or additional extra nuclear stain (**Figure 5B**). These are the first results that show nucleolar methylated histone H2A in plants and may involve a conserved epigenetic rDNA transcriptional mechanism for all eukaryotic cells nucleoli. The immunolocalization of Dimethylated lysine 4 in histone 3 in these cells shows an overall nuclear pattern, with no selectivity for the nucleolus as compared with methylated H2A (**Figure 5C**). Furthermore, the methylation of histone H2A in specialized cells can be involved in other epigenetic mechanism that can be specific to plants outside the nucleoli as shown by the immunolocalization pattern of H2A.

DISCUSSION

Fibrillarin sequence in all eukaryotic cells differs from Archaea organisms by addition of the GAR sequence (Rodriguez-Corona et al., 2015); this highly methylated region is responsible for nucleolar localization and protein–protein interaction and is the less conserved sequence in all fibrillarins (Snaar et al., 2000). The lack of conservation in the GAR domain can indicate that only methylated arginine charges are involved for these activities. Although up to date, there is no biochemical data that provides clear function besides the nucleolar localization

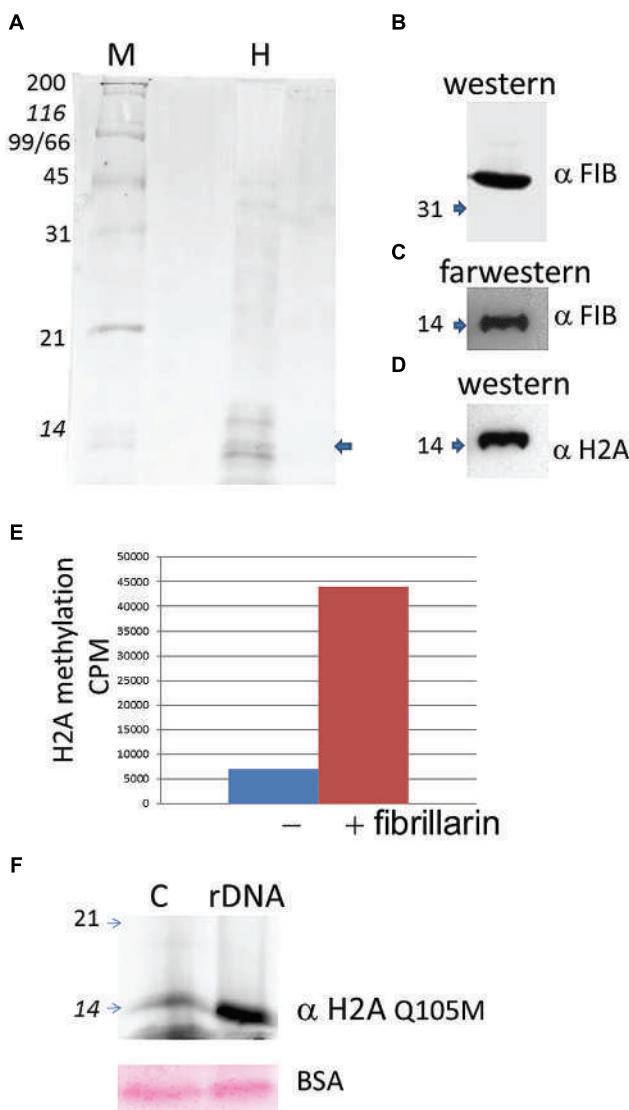


FIGURE 3 | Histone H2A methylation in *B. oleracea*. **(A)** Purified histones from *Brassica* as seen in a coomassie stain. M indicate protein weight marker and H the purified histones. The arrow shows the band that is labeled by farwestern in the position of H2A. **(B)** Western blot of the purified Atfib2. **(C)** Farwestern of the purified histone fraction. Purified Atfib2 was used to screen the histones and find specific interacting partners. **(D)** Western blot of H2A to mark the position of this histone after striping the membrane. **(E)** *In vitro* methylation assay with or without AtFib2 with the *B. oleracea* purified histones. **(F)** Western blot with anti H2A Q105me on histone pulldowns with a control beads (C) or with beads with the rDNA promoter (rDNA). Below is shown the ponceau stain of BSA from the transfer membrane. Numbers indicate the KDa by the marker.

(Rodriguez-Corona et al., 2015). The central domain, the RNA binding region and the Alpha helix rich domain form the methyl transferase region that allow fibrillarin to methylate rRNA and histones. Tessarz et al. (2014) showed recently that yeast and human fibrillarin can methylate histone H2A and the previously thermo-sensitive yeast fibrillarin (Nop1) mutant (Tollervey et al., 1993) showed a reduction of methyl transferase activity of H2A from Nop1 at the non-permissive temperature after 3 h (Tessarz et al., 2014). Thus showing that Nop1 is responsible for this methylation and the methylation is under constant evaluation by the cell. Probably this is part of the mechanism that helps the cell define the number of

ribosomal promoter regions that to be active. Interestingly the key mutated amino acids in the alpha helix in yeast are not well conserved in plants as seen in Figure 1. This may reflect the difficulty of some fibrillarins to recapitulate fully all the functions of fibrillarin in a yeast complementary assay (Jansen et al., 1991; Pih et al., 2000). The promoter of the rDNA from *B. oleracea* was reported by Chen and Pikaard (1997) and has been used previously *in vitro* transcription assays. We used the same assay as bait for nuclear proteins in particular histones and fibrillarin. Since PI4,5P2 is known to interact with fibrillarin and histones we tested if the degradation of PI4,5P2 by the recombinant PLC added into

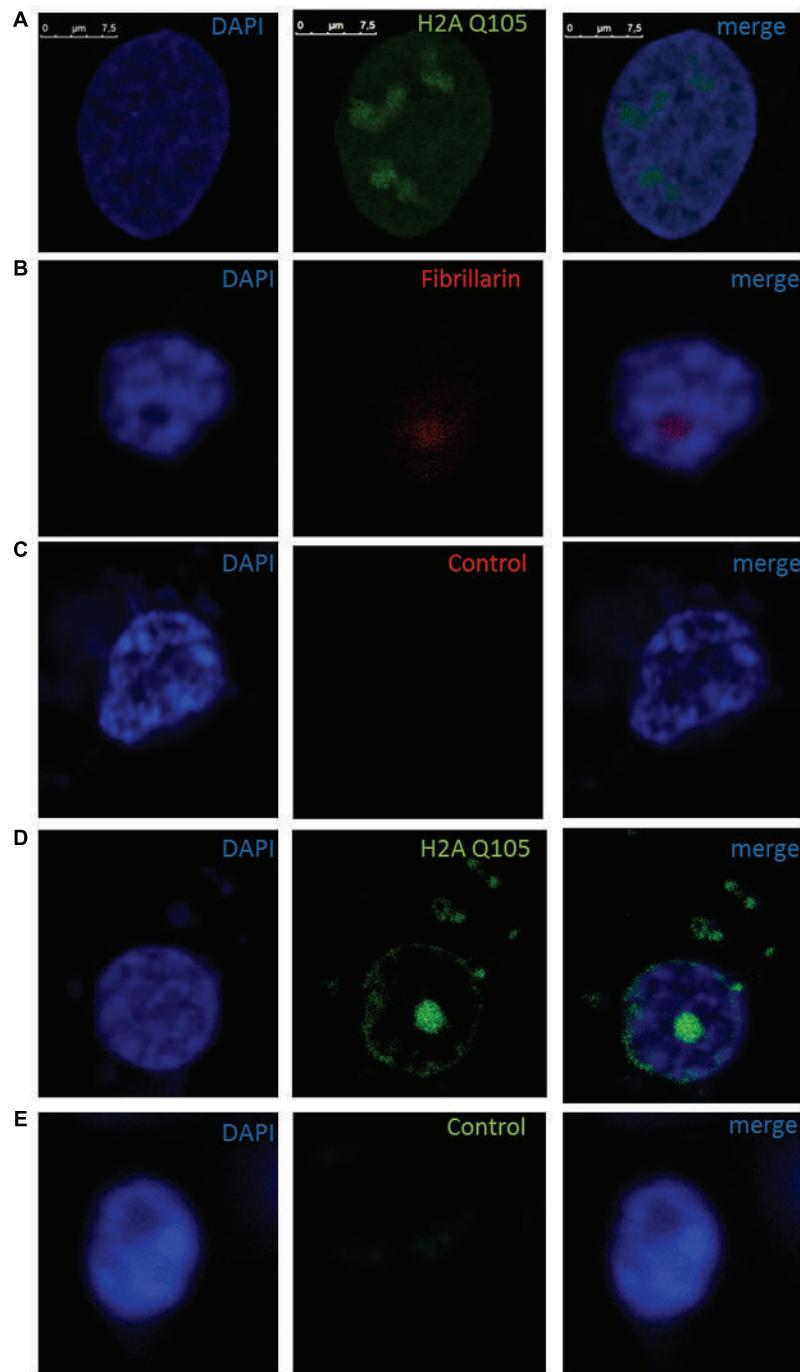


FIGURE 4 | Immunolocalization of methylated H2A in *B. oleracea*. All cells were stained with DAPI. **(A)** U2OS cells immunostained with anti H2A Q105me show a specific nucleoli stain. **(B–E)** Nucleus from *B. oleracea* fresh cauliflower floral meristem cells with round nucleus. **(B)** Immunostained with antibodies against fibrillarin. **(C)** Control secondary antibody only couple to alexa 555. **(D)** Immunolocalization of methylated H2A. **(E)** Control secondary antibody only couple to alexa 488.

the assay would alter the amount of nuclear proteins that bind the promoter. The results correlates with the studies on histone H1 and H3 interaction with PI4,5P₂ where it was suggested that this lipid may promote the formation of less accessible interaction of RNA pol II to the promoter due

to higher binding of the histones (Yu et al., 1998). PI4,5P₂ is well known phosphoinositide in the signal transduction mechanism in the cell membrane (McLaughlin et al., 2002; Lemmon, 2008; Boss and Im, 2012), where it is digested by PLC into PIP3 and DAG. However, the nuclear form of

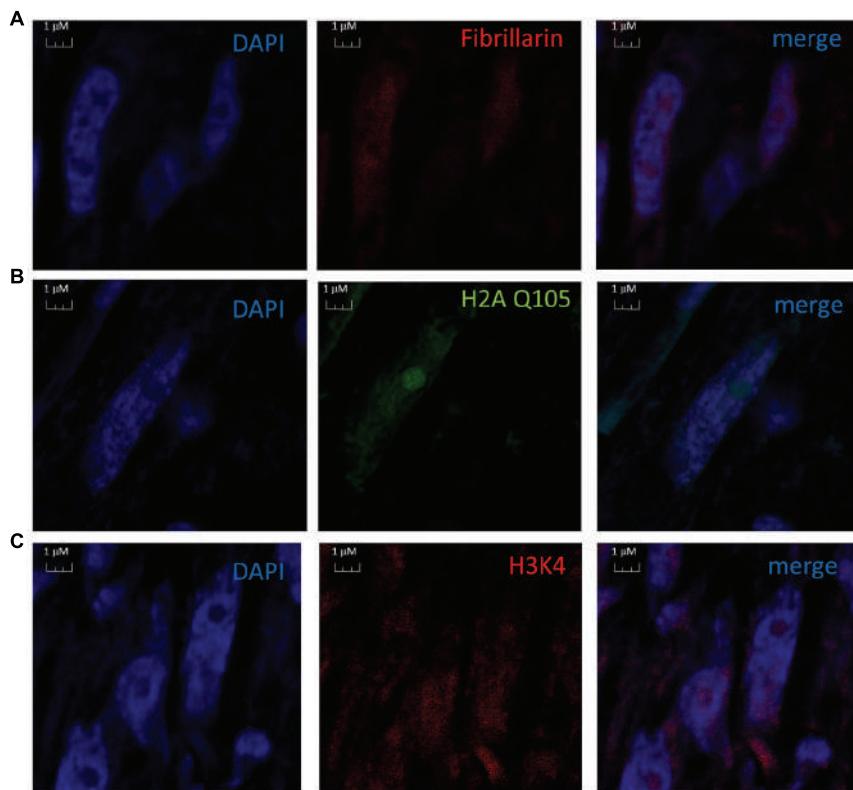


FIGURE 5 | Nuclear immunolocalization of *B. oleracea* vascular cells. All vascular cells were stained with DAPI. **(A)** Immunolocalization of Fibrillarin. **(B)** Immunolocalization of methylated H2A showing a similar pattern as fibrillarin immunostain. **(C)** Histone H3 dimethylated in lysine 4 was used as control for nuclear staining a different nuclear pattern from H2A Q105me pattern is observed.

this lipid has only come into play during the last decade (Osborne et al., 2001; Yildirim et al., 2013). PI4,5P₂ is known to bind histone H1, H3 as well as fibrillarin, StarPAP, UBF etc. (Yu et al., 1998; Jiang et al., 2006; Mellman et al., 2008; Yildirim et al., 2013) and localized in transcriptionally active ribosomal promoters in human cells. Up to date it is not clear what is the mechanism by which PI4,5P₂ is affecting transcription and it's interesting that its removal increases binding of several proteins to the rDNA promoter as seen in **Figure 2**. H1 was reported to increase its binding activity as a result of PI4,5P₂ loss (Yu et al., 1998). However, there is no studies yet done on chromatin structure alteration by phosphoinositides.

The *in vitro* methylation of *B. oleracea* histones by AtFib2 is similar to the results obtained recently by Tessarz et al. (2014) with purified Nop1. This epigenetic mechanism involves fibrillarin marking histone H2A on active ribosomal promoters. Our pulldown experiments with the rDNA promoter show a preferential binding of methylated H2A as compared to a control sequence. As previously published that fibrillarin and histone bind well to the human rDNA promoter (Yildirim et al., 2013). On a recent model (Leonhardt and Hake, 2014). Fibrillarin interacts with RNA pol I and such interaction represses FACT complex action on chromatin remodeling. This model is interesting considering that fibrillarin in plants

has been shown to be part of the mediator for RNA pol II transcription, as up to date there is a missing functional data to explain the function of fibrillarin on the mediator. It may help in the process of chromatin remodeling in other parts outside the nucleolus. It was observed on the immunolocalization of both fibrillarin and methylated histone H2A in the *B. oleracea* nucleus. There is a clear label outside the nucleolus in plant cells that is not seen in human U2OS cells. This may indicate plant fibrillarin role with RNA pol II, however more experiments are required to test this hypothesis. Fibrillarin is primarily located in the nucleoli, in particular in the DFC and FC regions. However, in these regions, several processes take place, the transcription initiation, elongation and first stages of rRNA processing take place in this region and may involve different functions of fibrillarin, which is well known to methylate rRNA for further processing. Methylation of H2A may help discriminate between active and inactive rDNA and its nucleoli organization. There is evidence that core histone H3 is also located in mitochondria in *B. oleracea*, however, this is not recognized by highly specific antibodies for the N terminal tail region of H3. One possibility is that the N terminal region of H3 is modified and is not detected with these antibodies (Iwasaki et al., 2013). A similar scenario could explain the methylated H2A signal in the extra nuclear stain in the fresh cauliflower

floral meristem buds. Since *B. oleracea* meristem cells are the most exposed cells it would follow that it may also have this additional function. Furthermore other explanation may involve ribonucleoproteins known to interact with fibrillarin that can form U bodies structures found in the cytoplasm (Liu and Gall, 2007) although it is unclear why methylation of H2A would be required for this outside the nucleus. Although it is known those histones H2A/H2B have antimicrobial action in particular cells that are closer to the surface as published (Stekhoven et al., 2004). The absence of this signal in vascular inflorescence cells can be due to a reduction in the number of mitochondria for this cell type or lack of U bodies. Plant viruses that interact with fibrillarin may take advantage of the broad distribution of this protein in this transport cell. The spread of the virus through the plant aided by fibrillarin has been published (Kim et al., 2007) and the diffusion of fibrillarin in vascular cells may help viruses tag alone for distribution through the phloem. The diffusion of the methylated H2A in transport cells correlates well with the diffusion pattern of fibrillarin. However, it is early to define the role of this epigenetic marker and its functional significance in this type of cells. Tessarz et al. (2014) had shown a particular interaction with FACT and it is known that in many cell types FACT facilitates the remodeling of RNA pol II promoter more

than RNA pol I promoters. Recently it was shown that FACT-Histone interactions identifies a role of Pob3 C-terminus in H2A-H2B binding (Hoffmann and Neumann, 2015). So it is possible that methylation of H2A in specialized cells may reflect this interaction as suggested by Hoffmann and Neumann that FACT interactions are altered by histone posttranslational modification.

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Chromatin versus pathogens: the function of epigenetics in plant immunity

Bo Ding¹ and Guo-Liang Wang^{1,2*}

¹ State Key Laboratory of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China, ² Department of Plant Pathology, The Ohio State University, Columbus, OH, USA

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***Correspondence:**

Guo-Liang Wang,
Department of Plant Pathology,
The Ohio State University,
2021 Coffey Road, Columbus,
OH 43210, USA
wang.620@osu.edu

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To defend against pathogens, plants have developed a sophisticated innate immunity that includes effector recognition, signal transduction, and rapid defense responses. Recent evidence has demonstrated that plants utilize the epigenetic control of gene expression to fine-tune their defense when challenged by pathogens. In this review, we highlight the current understanding of the molecular mechanisms of histone modifications (i.e., methylation, acetylation, and ubiquitination) and chromatin remodeling that contribute to plant immunity against pathogens. Functions of key histone-modifying and chromatin remodeling enzymes are discussed.

Keywords: Chromatin, histone modification, chromatin remodeling, plant immunity

Introduction

Throughout their life cycles, plants are exposed to abiotic stresses, including temperature fluctuation and nutrition deficiency, and biotic threats, including attack by herbivores and microbial pathogens. With respect to microbial pathogens, plants are unlike animals in that they lack an adaptive immune system that produces antibodies and also lack mobile circulatory cells that detect and prevent or reduce infection. Instead, plants mainly rely on an innate immunity system to resist microbial attack. In plants, the salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signaling pathways play pivotal roles in defending against biotrophic and necrotrophic pathogens (Pieterse et al., 2009). After detecting a pathogen, the plant activates a cascade of defense responses to establish local and systemic acquired resistance (SAR; Durrant and Dong, 2004).

Transcription of defense genes is tightly regulated by many transcription factors (TFs) that fine-tune the defense response (Thilmony et al., 2006). This requires that plants rapidly and precisely re-program gene expression. In particular, activation of an appropriate stress signaling pathway following pathogen detection is integrated in the plant cell nucleus through a set of regulatory cascades that prioritize defense over growth-related cellular functions (Moore et al., 2011). Research over the last decade has revealed that this transcriptional re-programming and regulation of defense-related genes often involves chromatin modifications and remodeling in *Arabidopsis* (Alvarez et al., 2010). In this review, we summarize and discuss the roles of chromatin modifications and remodeling in plant defense.

Plant Innate Immunity

Plant innate immunity is triggered by pattern recognition receptors (PRRs) located on the external cell surface. PRRs can recognize specific pathogen-/microbe-associated molecular patterns

(PAMPs/MAMPs), including cell wall components, short peptides, and lipopolysaccharides derived from the pathogen, leading to PAMP/MAMP-triggered immunity (PTI/MTI), which is the first layer of defense (Boller and Felix, 2009). Several early defense responses, including the generation of reactive oxygen species (ROS), calcium flux, plant cell wall modification, and the activation of a cascade of mitogen-activated protein kinases (MAPKs), are triggered during PTI. To overcome PTI, pathogens may deliver effector proteins into host cells, resulting in effector-triggered susceptibility (ETS). An additional level of resistance associated with vigorous defense induction may occur when specific intracellular receptors/sensors called resistance (R) gene products to recognize such race-specific avirulence (avr) effectors, thereby activating effector-triggered immunity (ETI; Jones and Dangl, 2006). ETI usually triggers a localized cell death at the infection site, in a process known as the hypersensitive response (HR), which along with antimicrobial effects may restrict most pathogen growth (Caplan et al., 2008). In addition to these PTI and ETI responses after initial local infection, the uninfected portions of the plant usually develop SAR, providing resistance in distal plant tissues against subsequent pathogen challenges (Durrant and Dong, 2004; Mishina and Zeier, 2007).

Chromatin Modification in Plant Innate Immunity

Chromatin Structure and Modifications

The basic, repeated unit of chromatin is the nucleosome that contains 147 base pairs (bp) of DNA wrapped around a histone octamer, which in turn consists of two copies of the following core histones: H2A, H2B, H3, and H4 (Luger et al., 1997). The linker histone, H1, associates with DNA between two nucleosomes and participates in higher order chromatin structure formation and remodeling. Extending from the globular nucleosome core, the histone tails may harbor diverse post-translational modifications (PTMs), i.e., acetylation, methylation, phosphorylation, ubiquitination, sumoylation, carbonylation, and glycosylation. PTMs can directly affect chromatin structure or can recruit specific “readers or effectors,” thereby regulating gene expression mainly by altering nucleosome stability and positioning, which affect the accessibility for regulatory proteins or protein complexes involved in transcription, DNA replication, and repair (Kouzarides, 2007). In general, histone acetylation by histone acetyltransferases (HATs) is associated with transcriptional activation, while histone deacetylation by histone deacetylases (HDACs) is associated with transcriptional suppression (Eberharter and Becker, 2002). Depending on the context of targets, histone methylation and/or ubiquitination can either be an active or repressive marker for transcription. Generally, tri-methylations of H3K4 and H3K36 (H3K4me3 and H3K36me3) and mono-ubiquitination of H2B (H2Bub) are enriched at actively expressed genes (Xu et al., 2008; Zhang et al., 2009), H3K27me3 is associated with repressed genes, while H3K9me2 and H4K20me1 are enriched at constitutive heterochromatin and silenced transposons (Zhang et al., 2007a,b;

Bernatavichute et al., 2008). In addition to histone modification, ATP-dependent chromatin-remodeling enzymes use the energy of ATP hydrolysis to remodel chromatin structure by modifying the interaction between DNA and histone to relocate or dissociate nucleosomes, move histone octamers, and catalyze the incorporation of specific histone variants. ATP-dependent chromatin-remodeling enzymes thus play crucial roles in nucleosome assembly/disassembly and allow the transcriptional machinery to access the DNA (Smith and Peterson, 2005; Clapier and Cairns, 2009).

Many studies have documented that histone modifications and ATP-dependent chromatin remodeling result in rapid, reversible, or trans-generational changes in gene expression associated with various developmental processes, such as flowering time control, cell fate determination and maintenance, and seed development. These mechanisms, however, have only recently attracted attention as potential transcriptional regulators in plant innate immunity (Table 1).

Histone Acetylation

Histone lysine acetylation is regulated by the antagonistic interactions between HATs and HDACs. Plant HDACs can be divided into four major groups or families. In addition to a plant-specific type-II HDAC (HD2) family, three other major families are designated as reduced potassium dependency 3 (RPD3), HDA1, and silence information regulator 2 (SIR2); this grouping is based on homology to yeast counterparts. Among these groups, HDA19 from *Arabidopsis* has been well-studied with regard to its roles in plant defense against pathogen attack. HDA19, which belongs to the RPD3 subfamily, was initially reported to be involved in the ET/JA signaling pathways of defense responses based on two lines of evidence. First, the expression of *HDA19* is induced by wounding, by challenge with the pathogen *Alternaria brassicicola*, and by treatment with the plant hormone JA. Second, the knock-down mutant of *HDA19* exhibits decreased transcription of several ET/JA pathway genes (*ERF1*, *CHI-B*, and *BGL*) and increased susceptibility to fungal pathogens, while overexpression results in the opposite disease phenotypes (Zhou et al., 2005). Similarly, HDA6, another *Arabidopsis* RPD3-type HDAC, is induced by treatments with JA and the ET precursor ACC, whereas the expression of other members of *Arabidopsis* RPD3-type HDACs is not inducible by these hormones (Zhou et al., 2005). In addition, HDA6 interacts with an F-box protein, coronatine insensitive 1 (COI1), which mediates JA signaling (Devoto et al., 2002). The expression of the JA-responsive genes, i.e., *PDF1.2*, *VSP2*, *JIN1*, and *ERF1*, is down-regulated in *axe1-5* (HDA6 loss-of-function mutant) and HDA6-RNAi plants (Wu et al., 2008), suggesting redundant roles of HDA6 and HDA19 in plant defense against infection by necrotrophic pathogens. In addition to its role in the JA/ET defense pathway, HDA19 positively regulates SA-mediated basal defense and the expression of pathogenesis-related gene 1 (PR1) by physically interacting with WRKY38 and WRKY62 and inhibiting their transcriptional-activator activities (Kim et al., 2008). On the other hand, the basal expression of the SA-induced *PR1* and *PR5* is upregulated in the *hda19* mutant when it is not challenged by pathogens, reflecting the negative role of HDA19 in defense responses. *PR1*

TABLE 1 | Histone-modifying enzymes and chromatin-remodelling factors involved in plant responses to pathogens.

Modification category	Sub-category	Name	Gene locus	Mutant phenotype and biological role	Reference
Histone acetylation	Histone deacetylase (HDAC)	<i>HDA19/AtHD1</i>	At4G38130	Increases sensitivity to <i>Alternaria brassicicola</i> and <i>Pst</i> DC3000; down-regulates ET/JA pathway genes (PDF1.2, VSP2, and ERF1), and enhances basal expression of SA-responsive genes (PR1, PR4, and PR5)	Zhou et al. (2005), Kim et al. (2008), Choi et al. (2012)
		<i>HDA6/Axe1</i>	At5G63110	Down-regulates expression of ET/JA pathway genes (PDF1.2, VSP2 ERF1)	Zhou et al. (2005)
		<i>AtSRT2</i>	At5G09230	Increases resistance to <i>Pst</i> DC3000; down-regulates expression of SA-biosynthesis genes (PAD4, EDS5, and SID2)	Wang et al. (2010)
		<i>HDT701</i>	Os5G51830	Increases resistance to rice blast in RNAi plants; up-regulates mitogen-activated protein kinases (MAPK6), WRKY53	Ding et al. (2012)
Histone methylation	Histone acetylase	<i>HAC1</i>	At1G79000	Mutants deficient in priming of the PTI	Singh et al. (2014a)
	Histone methyltransferase	<i>ATX1/SDG27</i>	At2G31650	Down-regulates expression of SA-pathway genes (WRKY70 and PR1); up-regulates expression of ET/JA pathway genes (PDF1.2, VSP2)	Alvarez-Venegas et al. (2007)
		<i>SDG8/ASHH2/EFS/LAZ2</i>	At1G77300	Increases sensitivity to <i>Botrytis cinerea</i> ; down-regulates expression of ET/JA pathway genes; increases sensitivity to <i>Pst</i> DC3000, down-regulates the basal expression of R genes (LAZ5 and RPM1) and SA-inducible genes (WRKY70 and PR1)	Berr et al. (2010), Palma et al. (2010), De-La-Pena et al. (2012)
		<i>ASHR1</i>	At2G17900	Increases sensitivity to <i>Pst</i> DC3000, down-regulates the expression of SA-inducible genes (WRKY70 and PR1)	De-La-Pena et al. (2012)
Histone demethylation	Histone demethylase	<i>FLD/RS1</i>	At3G10390	Decreases resistance after systemic acquired resistance (SAR) induction, down-regulates expression of SAR-inducible WARY6 and WRKY29	Singh et al. (2013, 2014b)
		<i>JMJ705</i>	Os1G67970	Increases sensitivity to Xoo, down-regulates the basal and MeJA-inducible defense genes	Li et al. (2013)
Histone ubiquitination	H2B ubiquitination-ligase	<i>HUB1</i>	At2G44950	Increases sensitivity to <i>B. cinerea</i> and <i>A. brassicicola</i> , does not alter expression of PDF1.2; decreases resistance to <i>Pst</i> DC3000 in <i>snc1</i> and <i>bon1</i> background, down-regulates the expression of the R gene SNC1	Dhawan et al. (2009), Zou et al. (2014)
Chromatin remodeling factors	SWI2-like group	<i>DDM1</i>	At5G66750	Increases resistance to <i>Pst</i> DC3000 in <i>mos1/snc1</i> background, up-regulates the expression of R gene SNC1	Li et al. (2010)
	SWR1-like group	<i>PIE1/CHR13</i>	At3G12810	Enhances resistance to <i>Pst</i> DC3000, up-regulates the expression of SA-pathway genes	March-Diaz et al. (2008)
	SNF2-like group	<i>SYD/CHR3</i>	At2G28290	Increases sensitivity to <i>B. cinerea</i> , down-regulates expression of ET/JA pathway genes (PDF1.2, VSP2, and Myc2)	Walley et al. (2008)

and *PR2* are well-defined markers for SA-mediated basal and R gene-mediated defense against biotrophic pathogens (Ward et al., 1991; Raordan and Delaney, 2002; van Loon et al., 2006). Several studies have shown that the SA-induced activation of *PR1* is tightly correlated with an increase in the level of acetylated histones at the *PR1* locus in *Arabidopsis* (Mosher et al., 2006; Koornneef et al., 2008) and tobacco (Butterbrodt et al., 2006). Additionally, HDA19 associates directly with the promoters of *PR1* and *PR2* and deacetylates histones at *PR1* and *PR2* locus. Thus, HDA19 forms a repressive chromatin environment (low histone acetylation level) under unchallenged conditions that ensures a low basal expression of defense genes as well as the

proper induction of *PR* genes without harmful overstimulation during defense responses to pathogen attacks (Choi et al., 2012).

The HDAC proteins in the Sir2 family are NAD+-dependent HDACs that play diverse roles in a variety of physiological processes, including chromatin silencing, DNA repair, the cell cycle, and apoptosis and aging in yeast and mammalian systems (Eberharter and Becker, 2002; Yamamoto et al., 2007; Etchegaray et al., 2013). Both *Arabidopsis* and rice genomes contain two Sir2 family genes (Pandey et al., 2002). Knockdown of *OsSRT1* by RNAi in rice plants enhances histone H3K9 acetylation on the promoters of HR-related genes, which leads to hydrogen peroxide accumulation, DNA fragmentation, and cell death, suggesting a

negative role of *OsSRT1* in defense (Huang et al., 2007). Highly divergent in sequence from *OsSRT1*, *AtSRT2* is down-regulated by *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) infection and negatively regulates the plant basal defense and *PR1* expression, possibly by suppressing pathogen-induced expression of *PAD4*, *EDS5*, and *SID2* and thereby regulating SA synthesis (Wang et al., 2010).

In addition to local resistance, SAR is also related to priming for stronger activation of various defense responses that are induced following an attack by microbial pathogens (van Hulsen et al., 2006). Priming of innate immunity is correlated with chromatin modification of the promoter region of WRKY TF genes (Jaskiewicz et al., 2011) and SA- and PTI-responsive genes (Luna et al., 2012; Po-Wen et al., 2013). Researchers recently showed that repetitive abiotic stress causes the priming of PTI in *Arabidopsis*, leading to enhanced resistance to bacterial pathogens. This elevated defense after repeated exposure to environmental stress is compromised in the *hac1* mutant, establishing a link between open chromatin configuration such as HAC1-dependent histone acetylation and primed *Arabidopsis* innate immunity and bacterial resistance (Singh et al., 2014a).

Histone Methylation

The *Arabidopsis* genome encodes 37 putative SET-domain group proteins, some of which have been experimentally demonstrated to harbor histone methyltransferase (HMT) activity (Thorstensen et al., 2011). For the removal of methyl residues from the methylated histones, the lysine-specific demethylase 1 (LSD1)-like proteins and Jomonji C-domain (JmjC) proteins are effective in histone demethylation in plants (Chen et al., 2011). Dynamic histone methylation and de-methylation are involved in many cellular processes such as gene imprinting and DNA methylation (Kohler et al., 2012), and in developmental events such as vernalization (Kim and Sung, 2014). Recent findings indicate that histone methylation contributes to plant immunity against both necrotrophic and biotrophic pathogens by affecting the expression of specific NBS-LRR proteins, WRKY family TFs, as well as TFs involved in defense signaling pathways.

The first study of immune responses involving histone methylation concerned *Arabidopsis* trithrox 1 (ATX1), also known as SDG27. Loss of ATX1 function affects the transcription of a subset of pathogen- and disease resistance-associated genes, including those encoding members of the TIR-NBS-LRR classes of disease resistance proteins, lectins, and heat shock proteins, as well as several WRKY family TFs (Alvarez-Venegas et al., 2006). Further findings revealed that ATX1 directly controls H3K4me3 levels at the promoter of WRKY70 and also controls the expression of WRKY70, a positive regulator of SA-mediated defense signaling against bacterial pathogens (Alvarez-Venegas et al., 2007; Saleh et al., 2008). *Arabidopsis* trithorax-related 7 (Atxr7), another histone H3K4 methyltransferase in the trithrox1 group, physically associates with the modifier of *snc1* 9 (MOS9), which is a plant-specific protein with unknown function discovered in a forward genetic screening of the *snc1* mutant. Together with MOS9, Atxr7 is required for both maintaining the

H3K4me3 levels at the promoter of the NBS-LRR genes *Snc1* and *Rpp4* and expression of these R genes residing in the RPP4 cluster (Xia et al., 2013).

In *Arabidopsis*, another important active signature of histone modification, H3K36 tri-methylation, is catalyzed by the SET domain group 8 (SDG8, also known as EFS, LAZ2, and Ashh2; Xu et al., 2008). SDG8 is a homolog of SET2 in yeast and ASH1 in *Drosophila*. Mutation in SDG8 causes pleiotropic developmental phenotypes such as early flowering time, reduced organ size, and enhanced branch shooting (Zhao et al., 2005; Dong et al., 2008; Cazzonelli et al., 2009). A recent study revealed that *sdg8* mutant plants have reduced resistance to the necrotrophic fungal pathogens *A. brassicicola* and *Botrytis cinerea*, indicating that SDG8 plays a crucial role in plant defense through H3K36me3-mediated activation of a subset of genes (including *ERF1*, *PDF1.2a*, and *VSP2*) in the JA/ET signaling pathways (Berr et al., 2010). Another study showed that SDG8 is required for both basal and R-protein-mediated resistance and that SDG8 maintains the LAZ5 locus in a transcriptionally active state by modifying its H3K36me3 level. LAZ5 is a member of an immune receptor class involved in the detection of specific pathogens and subsequent cell death (Palma et al., 2010). In a comparative analysis of three *Arabidopsis* ASH1 family mutants, loss of function of *ASHH2* and *ASHR1* resulted in more rapid HRs to both a non-pathogenic strain (*hrpA*-) and a pathogenic strain (DC3000) of *P. syringae*. In contrast, the *ashr3* mutant is more resistant to the infection than the *ashr1* and *ashh2* mutants. Furthermore, *PR1* gene expression was highest in the *ashr3* mutant, while H3K4me2 levels at the *PR1* promoter region are reduced in both the *ashr1* and *ashh2* mutants upon infection by DC3000 (De-La-Pena et al., 2012). This result demonstrates that the ASH1 group H3K4 methyltransferases have both overlapping and distinct roles in the plant defense against pathogens.

Collectively, the active H3K4 and H3K36 methylation states, which are catalyzed by SET domain protein, have been implicated in the SA- and JA-mediated plant defense in *Arabidopsis*. These markers act as permissive marks for the basal expression of the defense genes or establishing the chromatin status for prompt induction when plants are challenged. In contrast, the removal of the repressive histone H3K27me3 state by the JmjC protein JMJ705 in rice also plays important roles in defense-related gene expression. When induced by a stress signal or pathogen infection, JMJ705 is involved in the methyl jasmonate-induced removal of H3K27me3 and preferential biotic stress-responsive gene activation, supporting the hypothesis that H3K27me3 maintains the resting state of defense genes under normal conditions (Li et al., 2013). FLD, a homolog of the human LSD1, was originally discovered to promote flowering time by negatively regulating the expression of flower repressor FLC (He et al., 2003; Liu et al., 2007). A forward genetic screen revealed that *Arabidopsis* requires FLD in order to respond to the SAR signals leading to the systemic accumulation of SA; the screen also revealed that FLD influences histone modifications at the promoters of WRKY29 and WRKY6 and thereby enables a robust activation of SA signaling in response to subsequent exposure to virulent pathogens (Singh et al., 2013, 2014b).

Histone Mono-Ubiquitination

In *Arabidopsis*, histone H2B mono-ubiquitination is catalyzed by the RING E3 ligases histone mono-ubiquitinatio1 (HUB1) and HUB2, which participate in various developmental process such as the control of flowering time, the cell cycle, seed dormancy, and circadian clock (Xu et al., 2009; Lolas et al., 2010; Bourbousse et al., 2012). Additionally, HUB1 is a regulatory component of plant defense against necrotrophic fungal pathogens. *Arabidopsis* plants with mutations in the HUB1 alleles are extremely susceptible to the necrotrophic fungi *B. cinerea* and *A. brassicicola*. Consistent with the plant cell wall functioning in resistance to necrotrophic fungi by acting as a physical barrier, the thickness of epidermal cell walls is reduced in the *hub1* mutant. This suggests that HUB1 may enhance defense by increasing the thickness or otherwise modifying epidermal cell walls. Interestingly, HUB1 interacts with MED21, a subunit of the Mediator complex, in regulating the function of RNA polymerase II. *Arabidopsis* MED21 couples critical roles in disease resistance and embryo development based on the disease susceptibility and embryo-lethal phenotypes of plant lines with reduced MED21 gene expression. Thus, MED21 together with HUB1 controls critical components involved in the regulation of defense against necrotrophic fungal pathogens, suggesting a transcriptional role of Hub1-mediated histone mono-ubiquitination in defense (Dhawan et al., 2009). In contrast, responses to the bacterial

pathogen *P. syringae* are unaltered in *hub1* plants. However, a recent report showed that both Hub1 and Hub2 regulate the expression of the R genes *SNC1* and *Rpp4* (Zou et al., 2014). In the auto-immunity mutant *bon1*, which is a negative regulator of the NB-LRR-encoding R gene *SNC1* and other R-like genes (Yang and Hua, 2004; Li et al., 2007), loss of function in *HUB1* or *HUB2* reduces *SNC1* up-regulation and suppresses the *bon1* auto-immune phenotypes. Thus, HUB1 and HUB2 mediate histone 2B (H2B) mono-ubiquitination directly at the *SNC1* R gene locus to regulate its expression. This is another example of how the immune response can be fine-tuned by histone modifications at an R gene locus (Zou et al., 2014).

Chromatin Remodeling

In addition to being affected by covalent histone modifications, plant defense can also be affected by chromatin-remodeling factors that regulate R gene function and specific JA or SA pathways. The *Arabidopsis* genome encodes more than 40 ATP-dependent chromatin-remodeling factors, which can be subdivided into at least five families based on their ATPase subunits. In the broad SWI2/SNF2 protein family, DDM1 functions antagonistically to MOS1 in regulating the expression of the R gene *SNC1* (Li et al., 2010). SWR1, a component of the *Arabidopsis* SWR1-like complex that replaces the histone H2A with the histone variant H2A.Z, is

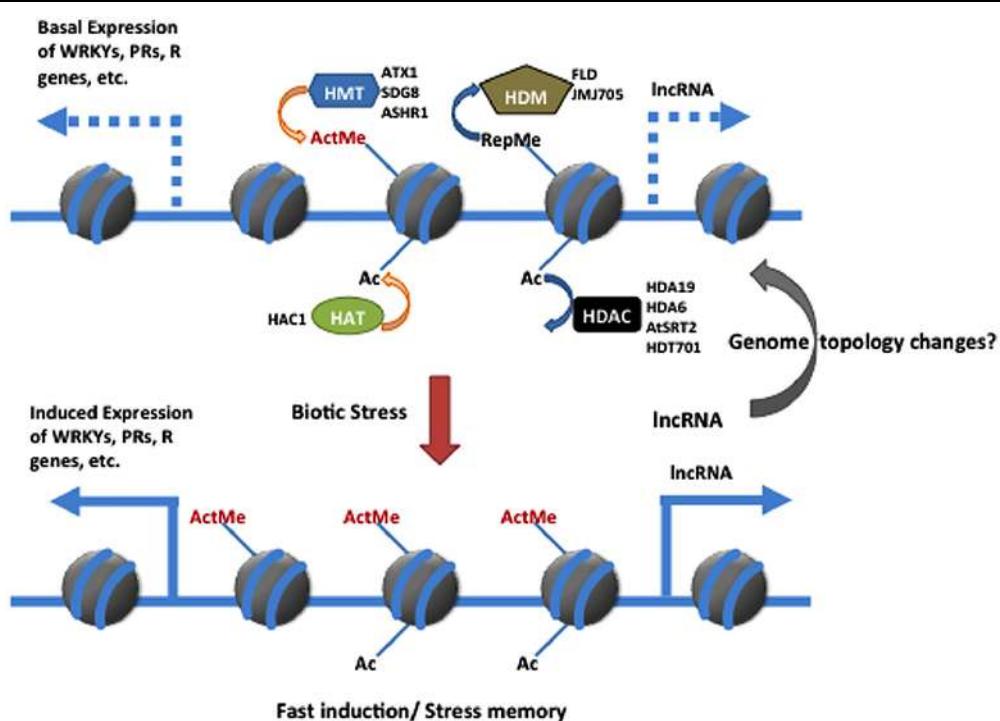


FIGURE 1 | Simplified model for participation of chromatin modification in regulating plant immunity against biotic stress. Histone modification changes in defense-related gene can be achieved through methylation/demethylation and/or acetylation/deacetylation by antagonistic interaction between HMT and HDM or HAT and HDAC. Each enzymes catalyzed different modification in regarding its roles in plant immunity is described in literature. The hypothetical involvement of the IncRNA in regulating the dynamin defense gene expression through the modulation of chromatin architecture is proposed as well. ActMe, active methylation marker; RepMe, Repressive methylation marker; HMT, histone methyltransferase; HDM, histone demethylase; HAC, histone acetylase; HDAC, histone de acetylase; IncRNA, long non-coding RNA; PR, pathogenesis-related; R, Resistance.

required for maintaining the repression of SA-dependent defense genes in unstressed plants (March-Diaz et al., 2008). SWI/SNF class chromatin remodeling ATPase SPLAYED (SYD) can be directly recruited to the promoters of selected genes, i.e., *PDF1.2a*, *VSP2*, and *MYC2*, downstream of the JA and ET signaling pathways. Therefore, SYD is required for the expression of these genes and for resistance against the necrotrophic pathogen *B. cinerea* but is not required for resistance against *P. syringae* (Walley et al., 2008).

Concluding Remarks and Perspectives

Recent research has increased our understanding of how chromatin modifications and remodeling affect defense in the model plants *Arabidopsis* and rice. Based on current evidence and as summarized in **Figure 1**, histone modifications in plant defense responses can be grouped as follows: (1) active histone marks that establish a basal expression level of the defense genes to enable an effective induction when the plant is challenged; (2) repressive histone modifications that prevent unnecessary activation of defense-related genes under normal growth conditions; (3) histone modifications that are induced after pathogen infection and that induce or reinforce the expression of defense-related genes; and (4) histone/chromatin changes that occur in response to biotic or abiotic stresses and that can be transmitted to the next generation. In the future, a combination of new genomic and proteomic approaches should be used to identify the targets of the epigenetic-related enzymes and other factors that are involved in the regulation of plant

immunity. In addition, only a few histone-modifying enzymes have been investigated. Large-scale screens and characterization of epigenetic mutants should help increase our understanding of the histone-modifying enzymes involved in the chromatin changes that occur when plants defend against pathogens. Moreover, three-dimensional structure plasticity of genomes establishes fine-tune feature in gene expression modulation rather than defined by its linear context. Emerging evidence showed that lncRNAs (long non-coding RNAs) and chromatin remodeling complexes are shaping the dynamic genome topology through chromatin loops to regulate dynamic gene expression in response to the environmental cues (Ariel et al., 2014; Jegu et al., 2014). Considering that the global genome structure is impacted in many diseases in animal systems and the participation of lncRNAs in nuclear architecture, the association between non-coding RNAs and the genome topology related to chromatin marks and organization remains an unexplored area in plant immunity.

Author Contributions

BD and G-LW wrote the manuscript.

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RNA based viral silencing suppression in plant pararetroviruses

Thomas Hohn^{1,2*}

¹ Botanical Institute, University of Basel, Basel, Switzerland, ² Friedrich Miescher Institute, Basel, Switzerland

The 35S promoter of cauliflower mosaic virus and that of other plant pararetroviruses gives rise to an RNA, which is both a pre-genome and a polycistronic mRNA. The 600 nucleotide long very structured leader of this RNA is also transcribed separately. The resulting 8S RNA is then converted to a double strand giving rise to a huge set of siRNAs, which suppress silencing. In this Mini-Review I discuss how this versatile stretch of 600 nts constitutes a masterpiece of evolution.

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Edited by:

Raúl Alvarez-Venegas,
Centro de Investigación y de Estudios
Avanzados del Instituto Politécnico
Nacional, Mexico

Reviewed by:

Peer Schenk,
The University of Queensland,
Australia
Peter Moffett,
Université de Sherbrooke, Canada

*Correspondence:

Thomas Hohn,
Botanical Institute, University of Basel,
Schoenbeinstrasse 6, CH 4056
Basel, Switzerland;
Friedrich Miescher Institute,
Maulbeerstrasse 66, CH 4058 Basel,
Switzerland
hohn@fmi.ch

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Introduction

Plants respond to virus infections mainly by RNA silencing (RS). RS is generally initiated by recognition of double stranded RNA, usually accumulating as a by product of virus replication. In addition for some cases effector-triggered immunity (ETI) to virus infections was reported (Table 1 in Zvereva and Pooggin, 2012), ETI is initiated by an interaction of viral effectors with intracellular NB-LRR proteins and leads in most cases to hypersensitive response (HR), death of the infected cells and systemic acquired resistance (SAR); Successful virus infections depend on viral counter actions mediated by suppressors (VSRs) interfering with silencing (Szittya and Burgýán, 2013) and at least in some cases on viral avirulence proteins (Avrs) blocking ETI (Zvereva and Pooggin, 2012).

Silencing is initiated by transcription of virus RNAs by viral or host RNA-dependent RNA polymerases (RDRs) to yield dsRNAs. These are cleaved by dicer-like proteins (DCLs) into 21–24 nt small RNA duplexes (siRNAs). *Arabidopsis thaliana* has four dicers. The ds RNAs derived from cytoplasmic RNA viruses are diced by DCLs 4 and 2, while those derived from viruses establishing minichromosomes, i.e., geminiviruses and caulimoviruses, are cleaved in addition by DCLs 1 and 3 (Akbergenov et al., 2006; Blevins et al., 2006; Moissiard and Voinnet, 2006). The siRNA duplexes are stabilized by methylation of the 2'OH groups at their 3'-termini. The duplexes are melted and the single-strand “guide-siRNAs” are picked up by Argonaute proteins (AGOs) to form RNA-induced silencing complexes (RISCs). These are guided to cognate virus RNA strands, where they induce RNA cleavage or inhibition of translation (Brodersen and Voinnet, 2006; Hohn and Vazquez, 2011; Pikaard and Mittelsten-Scheid, 2014).

Individual viruses use different, usually unrelated viral proteins to interfere with silencing by binding to dsRNA, inhibiting or degrading dicers, interfering with or inactivating AGO proteins or interacting with loaded RISCs (Szittya and Burgýán, 2013). Since viral RNAs are targets rather than inhibitors of silencing they have not as yet been considered as silencing suppressors. However, recent data obtained with plant pararetroviruses, such as *Cauliflower Mosaic Virus* (CaMV), and *Rice Tungro Bacilliform Virus* (RTBV) allow the extension of the list of viral suppressors to ds viral suppressor RNAs (Blevins et al., 2011; Rajeswaran et al., 2014a).

Cauliflower Mosaic Virus, a Short Warrant

Cauliflower Mosaic Virus is a plant pararetrovirus, the genome of which accumulates in the infected plant nucleus in multiple copies of an 8 kb circular minichromosome. Within virus particles the DNA circle is relaxed due to three short gaps with overhangs (**Figure 1d**) that mark the starts/ends of minus- and plus-strand DNA synthesis. The minus strand gap is located at the primer (met-tRNA) binding site, the other two at polypurine stretches. (For reverse transcription the met-tRNA primes minus strand DNA synthesis, while the polypurine stretches prime plus strand DNA synthesis).

Cauliflower Mosaic Virus encodes seven proteins (**Figure 1b**). Of special interest is the unique transactivator/viroplasmin (TAV). TAV is a multifunctional protein forming viral inclusion bodies and enabling polycistronic translation and virus assembly (reviewed in Hohn and Rothnie, 2013). TAV also acts as elicitor of innate immunity (Love et al., 2012; Zvereva and Pooggin, 2012) and as silencing suppressor, inhibiting the RDR6/DCL4-dependent 21 nt siRNA pathway (Haas et al., 2008; Shivaprasad et al., 2008; Hohn, 2013).

Cauliflower Mosaic Virus produces three primary RNAs: 35S RNA, 19S RNA, and 8S RNA (Guilley et al. (1982; **Figure 1a**). The

35S RNA covers the whole genome and is terminally redundant due to a conditional polyadenylation signal, which is passed at the first encounter with the transcription machinery, but recognized at the second (Sanfaçon and Hohn, 1990). It acts both as pregenomic and as polycistronic mRNA (Fütterer et al., 1988). Its translation depends on TAV, which is encoded by the subgenomic 19S RNA. The 8S RNA is non-coding. It coincides with the 600 nt long highly structured leader of the 35S RNA. Translation initiation from the 35S RNA depends on “shunting,” whereby the scanning ribosome bypasses the highly structured central portion of the leader (Hohn et al., 2002). Small open reading frame “A” in front of the central stem structure is required for this process (**Figure 1c**).

Complex and long leaders are not unique to CaMV. Inspection of 14 related pararetroviruses, including rod-shaped *Banana Streak Virus* (BSV) and RTBV (Pooggin et al., 1999) revealed that they all have comparable leaders with structural, but not sequence similarities. Like for CaMV, these carry several sORFs, the first of which is 5–10 nts away from the central stem structure and spatially close to the first true ORF, predicting a shunting mechanism similar to the one for CaMV. Shunting was explicitly shown also for RTBV (Pooggin et al., 2006, 2008).

In addition to these major RNAs, all size classes of CaMV-derived siRNA (21–24 nts) of both polarities have been reported,

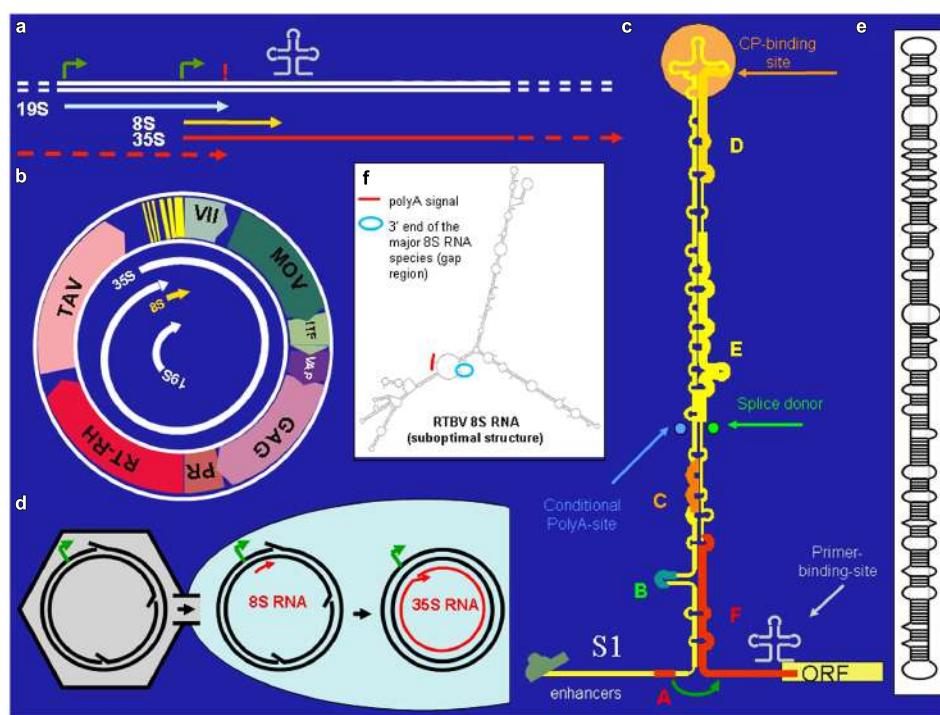


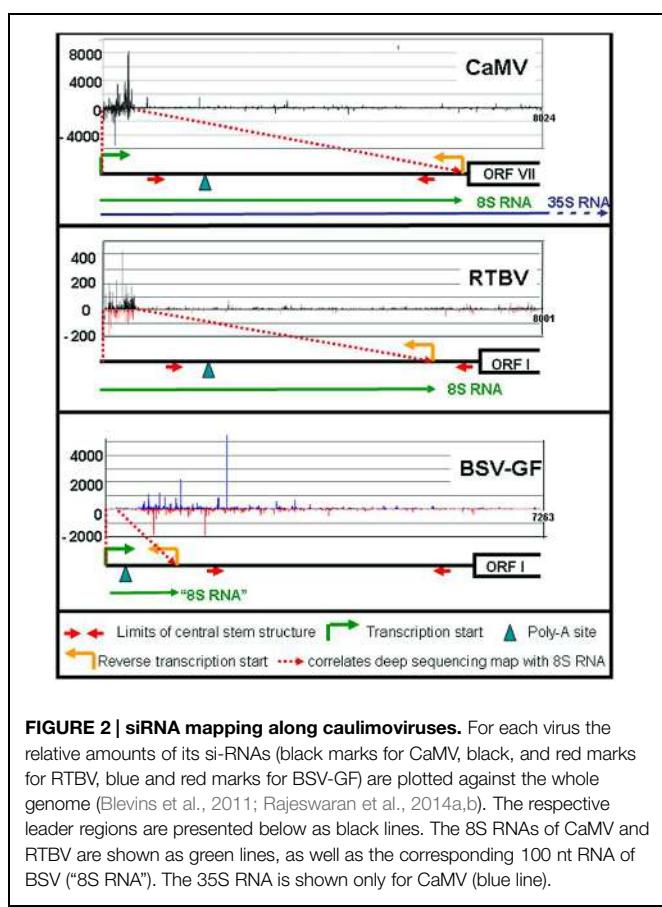
FIGURE 1 | Cauliflower Mosaic Virus (CaMV). **(a)** Positions of promoters (bent arrows), primer binding site (cloverleaf), polyadenylation signal (!) on the CaMV DNA, and the positions of the CaMV individual CaMV RNAs. **(b)** The 8 kb long circular CaMV DNA, its transcripts and its coding regions [ORF VII, no obvious function; MOV, movement protein; ITF, insect transmission factor; VAP, virion associated protein; GAG, capsid protein, PR/RT-RH, protease and reverse transcriptase fused coding region (POL);

TAV, transactivator/viroplasmin]. **(c)** The CaMV RNA leader with its compact secondary structure. Capital letters correspond to small ORFs. Special features are indicated. The roundish arrow symbolizes the shunt process. **(d)** Nuclear entry of open circular CaMV DNA. **(e)** Structure of poxviroid. **(f)** Suboptimal folding of *Rice Tungro Bacilliform Virus* (RTBV) 8S RNA revealing spatial vicinity of the facultative polyadenylation signal and the fall-off site.

making up half of the total amount of siRNAs in the infected plants. All four *Arabidopsis* DCLs including DCL1 are implicated (Blevins et al., 2006; Moissiard and Voinnet, 2006). Deep sequencing revealed that the bulk of those siRNAs are derived from the 600 nts of the 35S RNA leader region/8S RNA (82%), while siRNAs derived from the remaining 7400 nts of CaMV RNAs are rare (18%; **Figure 2**). DCLs 3, 1, and 2 are involved giving rise to species of 24 nt (47%), 21 nt (27%), and 22 nt (14%). Interestingly, the production of 21 nt long siRNAs by RDR6/DCL4/DRB4 is inhibited by TAV in its function as proteinaceous silencing suppressor (Haas et al., 2008; Shivaprasad et al., 2008).

The 8S RNA

To learn more about the preferential siRNA production, 8S RNA was isolated and characterized in detail by circularization-reverse-transcription PCR (Blevins et al., 2011). It starts at exactly the same position as the 35S RNA (**Figure 1a**), has a cap and ends at a narrow cluster of positions close to the start/end of reverse transcription and lacks a poly-A tail. Interestingly, not only sense 8S RNA (s-8S RNA) was found, but also antisense 8S RNA (as-8S RNA). The as-8S RNA starts roughly where the s-8S RNA ends and ends exactly where the s-8S RNA starts. The as-8S RNA has neither a cap nor a poly-A tail.



How is the poly-A tail-less s-8S RNA produced? Cauliflower mosaic virions are guided to nuclear pores via nuclear localization signals (Leclerc et al., 1999). Due to their large size, the virions cannot enter the nucleus, but just deliver the open circular DNA (**Figure 1d**). There must be a time window until the gaps/overhangs of nascent CaMV DNA are removed by repair enzymes and ligase and the supercoil closed. If transcription is initiated before DNA closure, the nascent RNA may fall off at the gap/overhang of the DNA minus strand or near of it as s-8S RNA. A fall-off would explain the lack of polyadenylation. The length of s-8S RNA is thus defined by the distance between start of transcription from the 35S promoter and roughly the primer binding site.

The mechanism of as-8S RNA production is not yet known. At the relevant antisense positions CaMV DNA contains neither promoter-like sequences nor polyadenylation signals, making ordinary DNA-depending antisense transcription unlikely. Although promoters lacking TATA-boxes exist in plants and other organisms (Morton et al., 2014), transcription directed by them would still produce capped, and polyadenylated transcripts. Any type of transcription using the met-tRNA as a primer is also unlikely, since its sequence or part of it is not included in as-8S RNA. Furthermore, synthesis of the as-8S RNA requires neither RNA-dependent RNA-polymerases 1, 2, or 6 nor POL IV and POL V, ruling out their involvement (Blevins et al., 2006).

A possibility would be that a DNA-dependent RNA-polymerases (POL I, II, or III) is involved (Bonfiglioli et al., 1996 for POL I; Lehmann et al., 2007 for POL II). In fact, RNA dependent RNA-polymerase activity of Pol II has been observed by several authors (Wagner et al., 2013 and references therein). For instance, pospiviroid- and *Hepatitis Delta Virus* RNAs are replicated by POL II in an α -amanitin-sensitive mode and pospiviroids apparently make use of an RNA-based promoter located on highly structured circular viroid RNA (Pelchat et al., 2002; Flores et al., 2011). Inspection of the secondary structure of s-8S RNA (Fütterer et al., 1988) reveals an interesting resemblance to viroid RNA (**Figure 1e**): both RNAs have long stretches of imperfectly matched dsRNA. This suggests that also the ds form of 8S RNA might originate from transcription of 8S RNA by POL II, perhaps using an RNA-based promoter, as in pospiviroids (Bojić et al., 2012). Future experiments will be required to test this hypothesis.

Whatever the mechanism, the as-8S RNA production on the s-8S RNA template may either lead directly to an 8S-RNA duplex or the two strands may anneal later. A nuclear involvement of POL II in as-8S RNA production and duplex formation would be in line with the high proportion of 24 nt long siRNAs produced by the nuclear dicer DCL 3 (see below).

8S-RNA Derived siRNAs and the Decoy Model

One very attractive hypothesis suggests a function for the siRNAs derived from the 8S RNA duplex: they may act as

decoys competing with the remaining siRNAs for free AGO proteins. This would also explain the very low amount of siRNAs produced from the CaMV coding region. Experiments using AGO1 antibodies indeed showed that 21 and 22 nt long siRNAs derived from the 8S RNA were bound to AGO1, while those derived from the remaining CaMV region did not (Blevins et al., 2011).

The 8S derived siRNAs on the other hand cannot efficiently target the 8S RNA itself or the leader of 35S RNA. The compact structure of these RNA sequences renders them as unfavorable targets for AGO-RISCs. A similar effect is discussed for viroids, the rod-like structures of which are perfect targets for DCLs but very poor ones for AGO/RISCs (Itaya et al., 2007; Pumplin and Voinnet, 2013).

If the decoy model is correct, the 8S RNA should also lead to large amounts of siRNAs in a chimeric context. To test this, the CaMV 8S RNA was ectopically expressed in a *Cabbage Leaf Curl (Gemini) Virus* (CaLCuV) vector, leading to s-8S like RNA, in this case in a polyadenylated version. Also in this connection large amounts of 8S RNA-derived siRNAs of both polarities were observed, the majority of which was again 24 nt long. The chimeric virus was produced in higher amounts than the empty vector or a CaLCuV vector loaded with a GUS gene, again indicating RNA-based silencing suppression (Blevins et al., 2011).

On the other hand, no substantial general reduction of host small RNAs was observed during CaMV infection. This might have to do with compartmentalization, separating virus, and host siRNAs spatially. For instance, 24 nt long siRNAs together with POL IV accumulate in Cajal bodies inside the nucleolus (Li et al., 2006; Pontes et al., 2006), while the viral ones might accumulate outside the Cajal bodies. miRNAs might evade suppression by successfully competing for AGO1 with siRNAs; including those derived from the 8S RNA.

Shunting and Decoy, Comparison with other Plant Pararetroviruses

RNA-based silencing suppression might be a general strategy of plant pararetroviruses. Sense and antisense 8S RNAs were also identified in RTBV infected rice plants (Rajeswaran et al., 2014a). In this case the s-8S RNA was more precisely terminated at the corresponding gap/overlap of the minus DNA strand than the 8S RNA of CaMV. Interestingly a minority of the RTBV s-8S RNAs had a short poly-A tail. Although no poly-A signal was found close to this polyadenylation site, inspection of an, albeit suboptimal RTBV 8S RNA secondary structure reveals a spatial neighborhood between the facultative polyadenylation signal and the fall-off site (Figure 1f). This resembles the case of *human T-cell leukemia virus* (HTLV), where a polyadenylation signal is moved over a distance of 290 nts to the facultative polyadenylation site through secondary structure (Shimotohno et al., 1984).

Like in the case of CaMV 8S RNA, huge amounts of siRNAs are produced also from the RTBV-derived 8S-RNA duplexes

(Rajeswaran et al., 2014a). Since RTBV is phloem-limited, naturally their percentage is lower (17% compared to 83% host sRNAs). Again, the majority of the 8S-derived siRNAs are 24 nt long and very few siRNAs were derived from the RTBV coding region (Figure 2).

Different results were obtained for BSV-GF (Rajeswaran et al., 2014b). For BSV-GF and most other BSV isolates only a very short stretch (~100 nts) of RNA is located between promoter and fall-off site, apparently too short for efficient asRNA and siRNA production (Figure 2).

Comparison of the three viruses confirms that 8S RNAs are produced by fall-off at the primer binding site, if properly spaced, and that it does not depend on the compact structure of the leader, which is present in all these three viruses. This compact structure of the leader, however, protects it from AGO-RISC-dependent degradation.

Conclusion 1: A Masterpiece of Evolution

The stretch of 600 nts comprising both, the leader of the CaMV pregenomic RNA and the blunt-ended 8S RNA constitutes a masterpiece of evolution. Due to its position it allows unusual fall-off transcription, due to its compact secondary structure it is resistant to AGO-mediated degradation. This secondary structure apparently leads also to an unusual replication mechanism, giving rise to antisense 8S RNA, which hybridizes with its template yielding 8S RNA duplexes as source of huge amounts of decoy siRNAs. The obvious disadvantage of such a structure: inhibition of ribosome scanning and translation is compensated by an ingenious positioning of a small open reading frame, which initiates a shunt mechanism leading the scanning ribosome directly to the start site of translation.

Conclusion 2: Implications

Analysis of the siRNA patterns in pararetrovirus-infected plants have led to the discovery of a novel silencing suppression strategy. Although mechanistic details await further experimentation, at least some players of the game are testable. Future science will reveal whether RNA-based silencing suppression is a more widely used strategy and whether host organisms have developed strategies to fight such activity.

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Overexpression of *Arabidopsis thaliana ERI*, the homolog of *C. elegans Enhancer of RNAinterference*, leads to enhanced growth

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Edited by:

Celia De-la-Peña,

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Reviewed by:

Ming-Bo Wang,

Commonwealth Scientific and Industrial Research Organization, Australia

Naeem H. Syed,
Canterbury Christ Church University, UK***Correspondence:**

Markus Kuhlmann,

Department of Molecular Genetics, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK Gatersleben), OT Gatersleben, Corrensstraße 3, 06466 Stadt Seeland, Germany
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Overexpression of *Arabidopsis thaliana ERI*, the homolog of *C. elegans Enhancer of RNAinterference*, leads to enhanced growth.*Front. Plant Sci.* 6:531.

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Rhonda C. Meyer¹, Gunnar Höning², Ronny Brandt¹, Fernando Arana-Ceballos¹, Cathleen Neitsch¹, Gunter Reuter², Thomas Altmann¹ and Markus Kuhlmann^{1*}

¹ Department of Molecular Genetics, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK Gatersleben), Stadt Seeland, Germany, ² Department of Developmental Genetics, Institute of Biology, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany

Organisms adopt a wide range of strategies to adapt to change. Gene silencing describes the ability of organisms to modulate the expression of susceptible genes at certain times at the transcriptional or the translational level. In all known eukaryotic organisms 21-nt long short interfering RNAs (siRNAs) are the effector molecules of post-transcriptional gene silencing (PTGS), while 24-nt long siRNAs are involved in PTGS in plants. Mutant studies in *Caenorhabditis elegans* lead to the identification of the enzyme ERI (Enhancer of RNAinterference) with enhanced PTGS. Although the genes involved in growth vigor and growth rate are still unknown, it becomes clearer that the population of small RNAs plays a role in the very early phase of plant development. To pinpoint the link between growth and siRNAs, the expression of *Arabidopsis* uni-gene *Enhancer of RNAi (ERI)* homolog from *C. elegans* was modulated. Increased degradation of small RNAs was achieved by ectopic *AtERI* overexpression *in planta*. Based on global small RNA analysis, *AtERI* overexpression affects mainly the population of 21 mers, excluding miRNAs. To identify target genes, *AtERI* gain-of-function mutants were analyzed, and differentially abundant small RNAs were identified. Plants with an elevated level of *AtERI* were bigger in all three light intensities analyzed, indicating an inhibitory function of particular small RNAs in plant growth, with differences in relative growth rates depending on developmental stage and light intensity. Understanding the role of these siRNAs could open new avenues for enhancing plant growth.

Keywords: ERI, Enhancer of RNAi, small RNA, *Arabidopsis*, biomass, enhanced growth

Introduction

Gene silencing is a natural genetic mechanism that allows organisms to control gene expression according to developmental stage (Boerjan et al., 1994) or viral infections (Covey et al., 1997; Ratcliff et al., 1997). In *Arabidopsis thaliana* the mechanism of gene silencing is very well understood and the most important factors are identified (Bologna and Voinnet, 2014). In these mechanisms small RNAs are the most important molecules determining the silencing target specificity by their

sequence homology. Based on their function and processing pathway, small RNAs are grouped into microRNAs (miRNAs, Axtell, 2013) and small interfering RNAs (siRNAs, Le Trionnaire and Twell, 2010). miRNAs are processed from hairpin forming, self-pairing single stranded precursor molecules. The length of miRNAs varies from 18 to 24 nt. The most abundant families are 21-nt long (Cuperus et al., 2010). siRNAs are the effector molecules of gene silencing. They are processed from double-stranded precursor RNAs. While 21-nt long siRNAs are involved in post-transcriptional gene silencing (PTGS), the presence of 24-nt long siRNAs is a typical hallmark for transcriptional gene silencing (TGS, Matzke et al., 2007) in plants. These heterochromatic RNAs are guiding the RNA-directed DNA methylation (RdDM) machinery in the nucleus to perform *de novo* DNA methylation at complementary genomic locations. An additional class of 22-nt long small RNAs, which is not related to gene silencing, is derived from degraded chloroplastic transcripts and results from the protective action of pentatricopeptide repeat (PPR) proteins against exonucleases (Rupe and Schmitz-Linneweber, 2012).

Role of Small RNAs in Development

The role of miRNAs in plant development is currently a point of investigation. The involvement of miRNAs as key regulators of flowering time (miR159, miR172, miR156, and miR171), hormone signaling (miR159, miR160, miR167, miR164, and miR393), or shoot and root development (miR164), was reviewed by (Wang and Li, 2007). During early seedling development the regulation mediated by the presence of miR165, miR166, miR164, and miR319 is of special importance for germination and developmental phase transitions (Wang and Li, 2007; Rubio-Somoza and Weigel, 2011). miR396 was identified as a regulator of the family of GRF transcription factors. Ectopic overexpression of this miRNA resulted in altered leaf shape and decreased cell number of the leaves (Liu et al., 2009a). The degradation of miRNAs was associated with the enzymatic activity of SDN1 (Small RNA degrading nuclease 1, Ramachandran and Chen, 2008). SDN1 was shown to be deterred by targets with 2'-O-methyl modification on the 3' terminal ribose of single-stranded siRNA *in vitro*.

As the class of siRNAs is more inhomogeneous with respect to their processing and function, the investigation of the involvement of particular siRNAs is more complex. The involvement of siRNAs during early plant development has been described for at least two mechanisms. The first one involves PTGS via the presence of 21-nt long small RNAs (Mallory and Vaucheret, 2010). The second mechanism involves 24-nt heterochromatic small RNAs and addresses gene regulation mediated by RNA directed DNA methylation (Matzke et al., 2007; He et al., 2011). Particular 24-nt long siRNAs might have an impact on the early growth vigor of *Arabidopsis* plants: A decrease in the amount of 24-nt long small RNAs correlated with an increase in biomass during the early growth phase [10–14 days after sowing (DAS), Groszmann et al., 2011]. This correlation implies a functional relevance of 24-nt long small RNAs for hybrid incompatibility as well as interspecific hybrids (Ng et al., 2012). An analysis of hybrid crosses between the accessions Landsberg *erecta* and C24

revealed the importance of the class of heterochromatic 24 mers that are associated with the RdDM mechanism. In a genome-wide study of 24 mers and DNA methylation, candidate genes could be identified that are differently methylated in the offspring compared to their parents upon a hybridisation event (Shen et al., 2012). From that analysis 77 genes were identified as being susceptible to differential DNA methylation in the hybrids. The same correlation of heterochromatic small RNAs and improved growth vigor could also be detected in hybrid crosses of wheat, rice (He et al., 2010) and maize (Barber et al., 2012; He et al., 2013). The analysis of the *mop1* mutant, affecting the homolog of *RDR2*, an RNA-dependent-RNA-polymerase involved in the production of silencing related small RNAs (Jia et al., 2009), revealed that in parallel to the reduced 24-nt heterochromatic small RNAs an increase of 22 and 21-nt small RNAs was detectable in maize (Barber et al., 2012). Crosses derived from *mop1* also showed better performance in corn yield.

ERI (Enhancer of RNAi) Encodes a 3'-5' Endonuclease Belonging to the Ribonuclease H-like Protein Family

Although the DICER mediated processing of small RNA is well understood, no candidate gene involved in the degradation or further processing of siRNAs has been described in plants so far. The first enzyme reported to be involved in siRNA degradation was ERI-1 (ENHANCER OF RNAi) isolated in *C. elegans* based on its effect on RNAi (Kennedy et al., 2004). The contribution of this enzyme to the antiviral defense mechanisms of *C. elegans* was demonstrated (Wilkins et al., 2005). While in *eri-1* mutant cell culture vesicular stomatitis virus accumulation was reduced, the accumulation of virus in single cells was increased. *In vitro* analyses using recombinant 3'hEXO, a human homolog of *C. elegans* ERI-1, revealed that this enzyme degrades the 3' overhangs of siRNAs, while the double-stranded region remained unaffected (Yang et al., 2006). Additional ERI-homologs are described in *Schizosaccharomyces pombe* (Gabel and Ruvkun, 2008), *Mus musculus* (Hong et al., 2005), and *Dictyostelium discoideum* (Kuhlmann et al., 2005). Based on sequence similarities the coding region of *At3g15140* was identified as *ERI-1* homolog in *A. thaliana* (Ramachandran and Chen, 2008).

We analyzed the effects of the ectopic overexpression of the *Arabidopsis* *Enhancer of RNAi* (*ERI*) homolog to verify its role in degradation of siRNAs in *Arabidopsis*, to identify target genes undergoing PTGS, and to elucidate the link between growth and siRNAs.

Materials and Methods

Plant Material

The 35S:*AtERI* lines were generated using a pCAMBIA1302 binary vector backbone which carried the *At3g15140* derived cDNA under control of the *CaMV35S* promoter. The *ERI-1* cDNA was amplified from RNA of accession Col-0 after reverse transcription (RevertAid Reverse Transcriptase, Thermo) using primers ERI full forward and rev (Supplementary Table). The cDNA was cloned via the pSC-A vector using a Strataclone

PCR-cloning kit (Agilent Technologies, cat.no. 240205) and constructed into pCAMBIA1302 using SpeI restriction site, replacing the GFP. Transformation of *A. thaliana* Col-0 by the floral dip method (Clough and Bent, 1998) was performed, using *A. tumefaciens* C59 pGV2260. The resulting plants were selected for hygromycin (20 mg/l) resistance. Single-copy T-DNA insertions were identified by segregation and Southern blot analysis. The integrity of the transferred transgene was analyzed by sequencing of PCR products. The insertion site of the line that was used as crossing partner was estimated by Genome walker 2.0 kit (Clontec). The insertion of the T-DNA was identified within a repetitive genomic region (SINE9) and did not affect any gene function. For antibiotic resistance test of plants on selective tissue culture medium, seeds were surface-sterilized (10 min, 8% NaClO) and grown under long day regime on hygromycin (20 mg/l; 35S:AtERI). To monitor PTGS the 35S:AtERI was crossed to a reporter line containing 4 copies of β -GLUCURONIDASE (GUS, Schubert et al., 2004). The homozygous plants identified by PCR and segregation analysis containing reporter line 4xGUS and 4xGUS/35S:AtERI were used for high throughput sequencing.

For the analysis of plant growth, seeds of the selected homozygous 35S:AtERI line and the wild type Col-0 accession were stratified before germination for 3 days at 4°C in the dark. Plants were cultivated on soil at 21°C under a 16 h light/8 h dark (long day) regime for seed production or under 8 h light/ 16 h dark (short day) regime to generate material for RNA and DNA analysis.

RNA Analysis

Total RNA samples for transcript measurements were extracted from seedlings grown for 1 week on soil under short day conditions and from rosette leaves of plants grown for 6–8 weeks under short day conditions. Total RNA was extracted with Qiagen RNAeasy kit and small RNA was extracted from 1 g leaf tissue with the mirVana miRNA Isolation kit (Ambion) according to manufacturer's protocol. cDNA was prepared using RevertAid H Minus M-MuLVRT (Fermentas) according to manufacturer's protocol. mRNA analysis was performed on oligo dT transcribed cDNA using specific primers (Supplementary Table 1). cDNAs were quantified by the RT-qPCR method using the iCycler (Bio-rad) and the iQ SyBRGreen Supermix (Bio-rad). Program: 1: 5' 95°C; 2: 15' 95°C; 3: 30' 65°C; 4: 30' 72°C; 5: goto 2 40x; 6: Melting curve 65°C 10' +0.5°C 60 repeats; 7: 4°C in triplicate from two biological replicates. Mean values are indicated as bar heights, standard deviation of values as error bars. PHOSPHOFRUCTOKINASE (PFK; AT4G04040) and ACTIN2 (AT3G18780, Supplementary Figure 1) was used as reference gene.

High Throughput Sequencing of Small RNA

The fraction of small RNA (mirVana) was treated according the manufacturer's protocol for Illumina TruSeq smallRNA sample prep kit. The size selection was performed on a 2% Agarose-TAE-Gel (2 h at 120V). The region of 135–170 bp was cut from the gel (mean fragment length wild-type: 136 bp, 35S:AtERI: 137 bp, reference 50 bp-Ladder ThermoScientific) and purified with

Qiagen PCR purification kit. Adapter trimming was performed via CLC Genomics Workbench (CLC Genomics Workbench 6.5.1, 2014) on the 3' end of the read with the P8 adapter 5'-TGGAA TTCTCGGTGCCAAGGAACCTCCAGTCAC-Index-ATCTCG TATGCCGTCTTGCTTG-3'. After trimming the reads were mapped on the *A. thaliana* reference genome extracted from TAIR10 (Lamesch et al., 2012) with segemehl (Hoffmann et al., 2009) using standard parameters. The resulting SAM-Files (Li et al., 2009) were processed with a custom made Perl-script (URL¹) and the extracted read count and coverage normalized with counts per million reads. All plots were produced with R (R Development Core Team, URL²).

Plant Growth Assays

Plants were grown in a mixture of 85% (v) red substrate 2 (Klasmann-Deilmann GmbH, Geeste, Germany) and 15% (v) sand in 96-well-trays (QuickPot QP 96T, HerkuPlast Kubern GmbH, Ering, Germany). After 2 days of stratification at 5°C in constant darkness, seeds were germinated and seedlings cultivated in a walk-in growth-chamber with a 16/8 h day/night regime, 20/18°C, 60/75% relative humidity. To avoid position effects, trays were rotated around the growth chamber every day. Plants were grown at 51, 101, or 187 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photosynthetically active radiation (PAR) in a lattice square design in two independent experiments with 32, respectively 24, replicates per light condition.

Determination of Leaf Area and Shoot Dry Biomass

For seed size determination, 20 *Arabidopsis* seeds were fixed with adhesive plate seal (Thermo Fisher Scientific, Loughborough, UK) on a sheet of paper displaying a 5 cm scale bar. The seed were scanned at a resolution of 1200 dpi on an Epson Expression 10000XL flatbed scanner (Seiko Epson Corporation, Suwa, Japan). The measurement of seed length, width and area was performed using the Evaluator software (developed by Dmitry Peschansky, IPK Gatersleben) according to the software instructions. The Evaluator algorithm isolates the seed area from the background based on differences in pixel intensities, creates a contour boundary and counts the pixels inside the boundary as a measure of area (Meyer et al., 2012).

Images of plants were taken until 16 DAS and whole leaf area was determined using the GrowScreen imaging system and software described in (Walter et al., 2007). Leaf area was extracted from the images using the software Bayer2Area (Meyer et al., 2010).

Shoot dry biomass was determined 20 DAS. The harvested aerial parts of the plants were placed in a vacuum oven at 80°C for 48 h. Dry biomass was measured using an analytical balance (Excellence XS205 Dualrange, Mettler Toledo, Gießen, Germany) with LabX direct Balance software. Mean shoot dry biomass in mg plant^{-1} and mean leaf areas in $\text{mm}^2 \text{ plant}^{-1}$ were estimated using a nested two-factorial ANOVA with line and light-intensity as independent variates and seed size as covariate.

¹<http://www.perl.org/docs.html>

²<http://www.R-project.org>

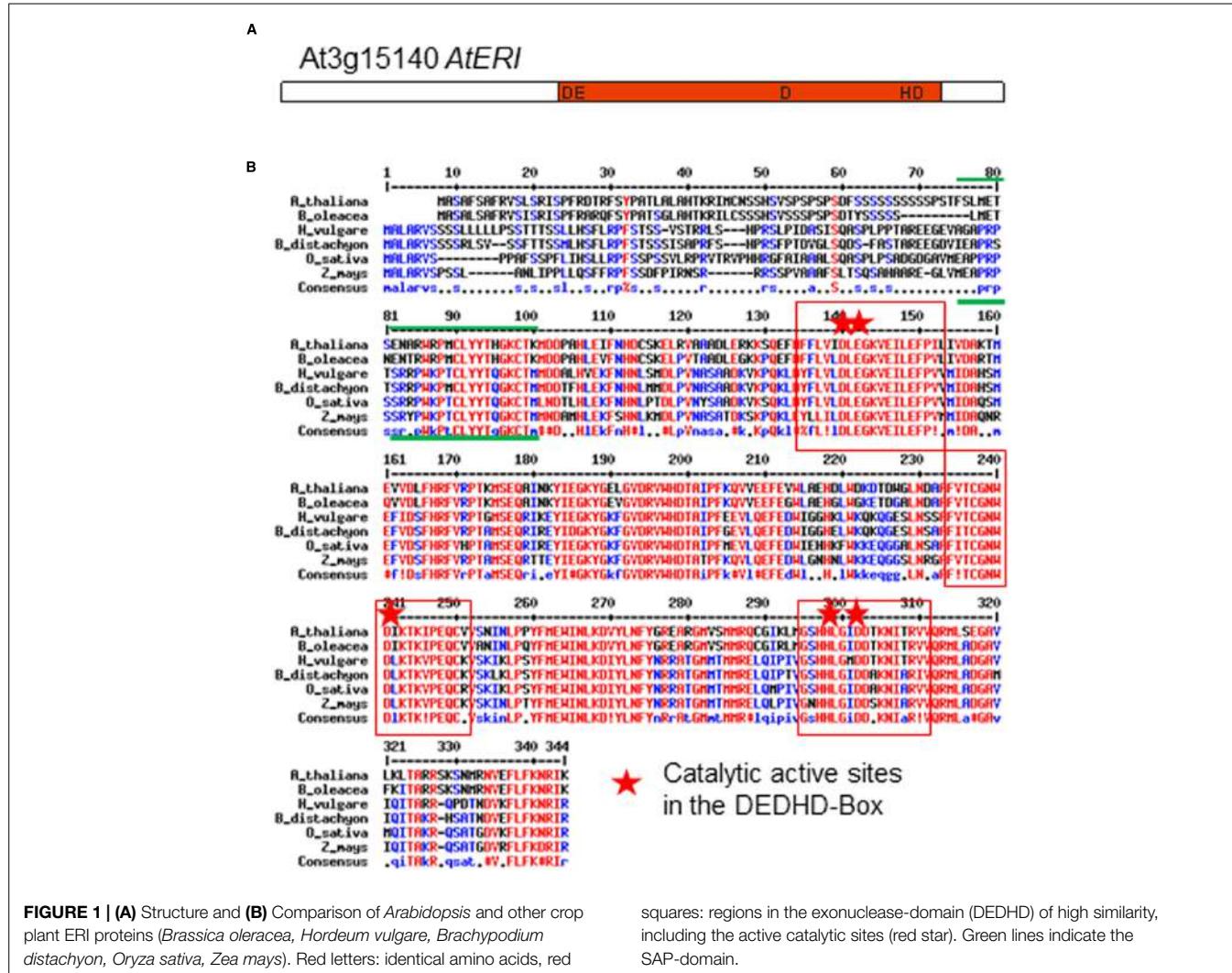


FIGURE 1 | (A) Structure and **(B)** Comparison of *Arabidopsis* and other crop plant ERI proteins (*Brassica oleracea*, *Hordeum vulgare*, *Brachypodium distachyon*, *Oryza sativa*, *Zea mays*). Red letters: identical amino acids, red

squares: regions in the exonuclease-domain (DEDHD) of high similarity, including the active catalytic sites (red star). Green lines indicate the SAP-domain.

Relative growth rates (RGRs) were calculated as $[\ln(\text{leaf area at timepoint 2}) - \ln(\text{leaf area at timepoint 1})]/(\text{timepoint 2} - \text{timepoint 1})$. Differences in RGR were tested via a two-factorial ANOVA with natural log-transformed leaf area as the dependent variable (Poorter and Lewis, 1986). Seed size was taken as leaf area at timepoint t_0 . The interaction term between line and time was partitioned using a second-order polynomial contrast for the factor time. According to Poorter and Lewis (1986), a significant linear interaction term indicates that differences in RGR are linear over time, i.e., maintained during the experiment, while the quadratic interaction term measures the extent to which differences in RGR changed with time.

Results

Identification and Characterization of the *Arabidopsis thaliana* ERI Homolog

AtERI (*At3g15140*) encodes a protein of 337 amino acids of the ribonuclease H-like superfamily. The catalytic core component of all enzymes belonging to the ERI subfamily is the DEDDH

domain. This C-terminal domain (**Figure 1A**) is responsible for the 3' overhang modifying activity on small RNA molecules. In addition the proteins from that subfamily contain a SAP domain (after SAF-A/B, Acinus, and PIAS), responsible for an interaction with nucleic acids (Aravind and Koonin, 2000). The SAP domain, which is located in the N-terminal part of the human, mice, worm and slime mold homologous protein, can also be found in the ERI-homologs from different crop plants such as *Brassica oleracea*, *Hordeum vulgare*, *Brachypodium distachyon*, *Oryza sativa*, and *Zea mays* (**Figure 1B**), indicating an evolutionary conservation in the plant kingdom. As the presence of SAP and tripartite DEDDH domains is unique to ERI proteins as compared to other exonucleases (Ramachandran and Chen, 2008), *Arabidopsis At3g15140* was identified as the only putative ERI-1 homolog.

The full-length cDNA derived from *At3g15140* contains six exons. Notably, the first exon contains a TCT-microsatellite structure (starting 226 bp after ATG) which varies in length in different *Arabidopsis* accessions. Based on sequence complementarity, it is a *miR5021*-cleavage target site (RegRNA2.0, Chang et al., 2013).

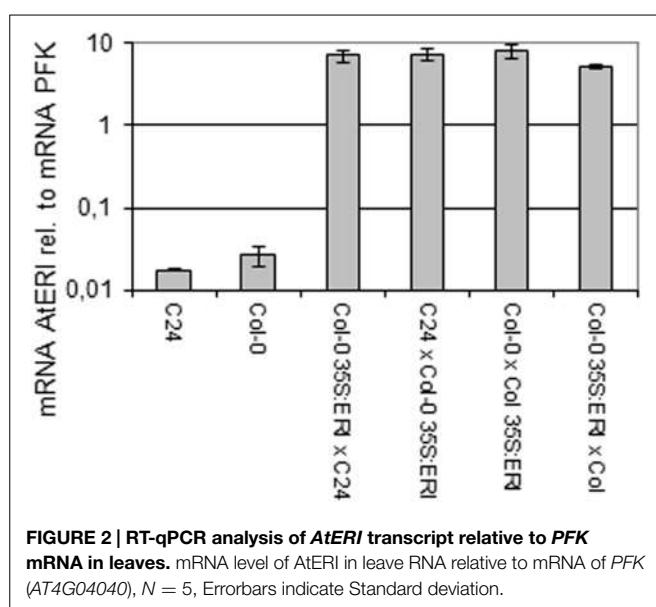


FIGURE 2 | RT-qPCR analysis of AtERI transcript relative to PFK mRNA in leaves. mRNA level of AtERI in leave RNA relative to mRNA of PFK (AT4G04040), $N = 5$, Errorbars indicate Standard deviation.

According to expression databases (Genevestigator, Zimmermann et al., 2004, and eFP browser, Winter et al., 2007), expression of *At3g15140* is relatively weak, with peaks of expression in the early development from seeds to cotyledons, and during the transition from vegetative to reproductive stage.

Plants expressing the *AtERI* cDNA under the control of the *CaMV35S* promoter were generated by *Agrobacterium*-mediated transformation. Three independent plant lines were obtained after transformation. The presence of a single T-DNA integration locus in the genome was concluded by genetic 3:1 segregation on hygromycin selection. The mRNA level of endogenous and transgenic *AtERI* was quantified by qRT PCR relative to *ACTIN2* mRNA in leaves of 6 week old soil grown plants. The homozygous plant line with the high expression (approximately 850-fold more *AtERI* transcripts in leaves than the *Col-0* wild-type plants) was selected for further studies and crossed to *Col-0* and *C24*. Expression of *AtERI* was tested in the offspring (Figure 2, Supplementary Figure 1). The *35S::AtERI* line showed no obvious phenotypical differences in morphology and onset of flowering within short and long-day regimes when compared to wild-type plants (data not shown).

Sequencing of Small RNA Reveals a Reduction of 21 mers in AtERI Overexpression Plants

Leaves of *Col-0* wild type and *35S::AtERI* overexpression plants containing a PTGS reporter system (Schubert et al., 2004) were used for RNA extraction. The enriched fraction of small RNAs was subjected to high throughput sequencing utilizing the Illumina TruSeq smallRNA-Kit and Illumina HiSeq 2000 sequencer. After trimming of adapter sequences and selecting molecules in the range from 16 to 32 nt, 5.4 million sequences from the wild type and 9.5 million sequences from the *35S::AtERI* line were used for mapping to the *Arabidopsis* genome and the miRNA database (miRBASE, Kozomara and Griffiths-Jones, 2014). For analysis of the relative abundance, obtained data were normalized

to reads per million and the relative abundance of different size classes was estimated (Figure 3). From the distribution of the read sizes, it is apparent that the class of 21-nt long reads is underrepresented in the *AtERI* overexpressing line compared to the wild type *Col-0* (17.8% versus 28.4%). This is consistent with the proposed function of *AtERI* as siRNA specific exonuclease reported from other organisms. The reads were mapped to the *Arabidopsis* genome (TAIR10) and separated according to their best match into nuclear, chloroplast and mitochondrial genome origin. No significant change in the relative abundance of small RNAs originating from the chloroplastic or the mitochondrial genome was detected (Figure 4). Neither 21 mers nor 24 mers related to gene silencing, homologous to chloroplast genes, nor the 22 mers derived from degradation events (Rupe and Schmitz-Linneweber, 2012) showed a differential abundance. Among the list of genes with differential abundant small RNAs only one gene encoded by the chloroplast genome ATCG00620 could be identified. From the results obtained and the absence of any phenotypic alteration of the leaves, a leaf specific function of *AtERI* involved in degradation of small RNAs of chloroplastic origin was excluded. Furthermore the absence of any small RNAs homologous to the *CaMV35S* promotor sequence was indicative for the absence of any trans-silencing event, based on presence of multiple promotor copies (Supplementary Figure 1B).

For analysis of miRNA, the identified reads were mapped to the list of published miRNA sequences (Kozomara and Griffiths-Jones, 2014). Reads were normalized (reads/million) and evaluated for differential abundance with CLC workbench software (QIAGEN). 159 sequences could be assigned to *Arabidopsis* miRNAs, while 34 sequences showed homologies to miRNAs from other species. Comparing the relative abundance of all miRNAs analyzed we found 8 miRNAs less abundant in the *AtERI* overexpressing plants (with miR841 showing the strongest decrease by 12-fold) and 94 miRNAs without significant influence. For miR841 no function or downstream target gene is known. 84 members of miRNA-families are more abundant in the *AtERI* overexpressing plants (Supplementary Table 2). Among all miRNA sequences analyzed, an increased abundance of miRNAs normalized to reads per million could be found in the *AtERI* overexpressing plant. Notably, miRNA396 (*ath-miR396a-3p*), reported to suppress GROWTH-REGULATING FACTOR (Liu et al., 2009b; Debernardi et al., 2012) showed a 2.8-fold increase in normalized reads compared to *Col-0*. The detected general increase of miRNAs resulted from the proportional decrease of highly abundant 21 mers in the *AtERI* overexpressing plant. No general influence of overexpressed *AtERI* was found on miRNA processing.

In order to identify target genes undergoing PTGS, the 21-nt long reads corresponding to siRNAs (Martinez de Alba et al., 2013) were mapped to the coding regions of the genes. The presence of 21 mers homologous to the coding regions is indicative of PTGS. The opposite trend compared to the global abundance of 21 mers was observed. 116 regions/genes with higher abundance in the *AtERI* overexpressing plant could be identified (Supplementary Table 3). For functional categorization of the target regions/genes with higher abundance of small RNA in the *AtERI* overexpressing line, the MAPMAN software (Thimm

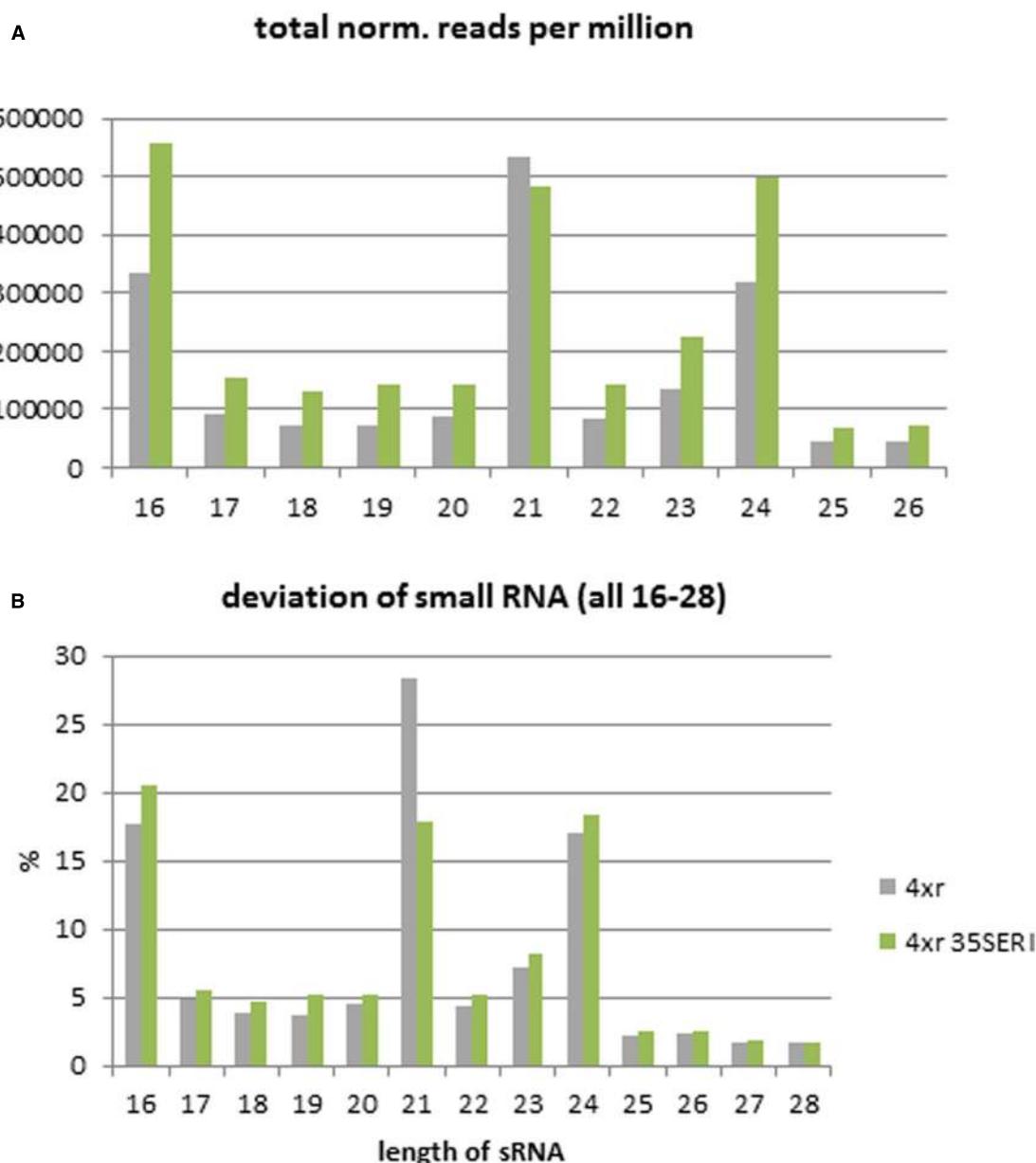


FIGURE 3 | Size distribution of small RNAs. (A) Read count obtained by high throughput sequencing normalized to reads per million. **(B)** Population of small RNAs separated in size classes given in percent of total estimated

reads from Col-0 and 35S:AtERI in 4xGUS reporter background. 4xr (gray): Col-0 with 4xGUS reporter, 4xr 35SERI (green): 35S:AtERI with 4xGUS reporter.

et al., 2004) was used. The gene ontology mapper clearly shows the high abundance of small RNAs associated with genes from the category “RNA.” The genes in this category are either encoding tRNAs or tRNA-related proteins.

In contrast, only five coding regions/genes were found to be associated with siRNAs downregulated in the *AtERI*-overexpressing line (Table 1). Targets being less subjected to PTGS include the genes encoding growth regulatory factors GRF3 (AT2G36400) and GRF4 (AT3G52910). Also a small nuclear non-coding RNA (AT1G26235, Marker et al., 2002), a gene encoding a chloroplast located pentatricopeptide repeat-containing protein

(SVR7) involved in chloroplast biogenesis via RNA binding (AT2G17033, Liu et al., 2010) and a not yet characterized gene (AT1G47389) could be identified.

Increased Growth in AtERI Overexpressing Plants

Growth differences between Col-0 and the *AtERI* overexpressing line were analyzed on two levels: static differences at harvest as biomass at 20 DAS, and dynamic differences during development (0–16 DAS) as RGR based on leaf area.

The *AtERI* overexpressing line had significantly ($p < 0.001$) higher aerial biomass under all light conditions (Table 2).

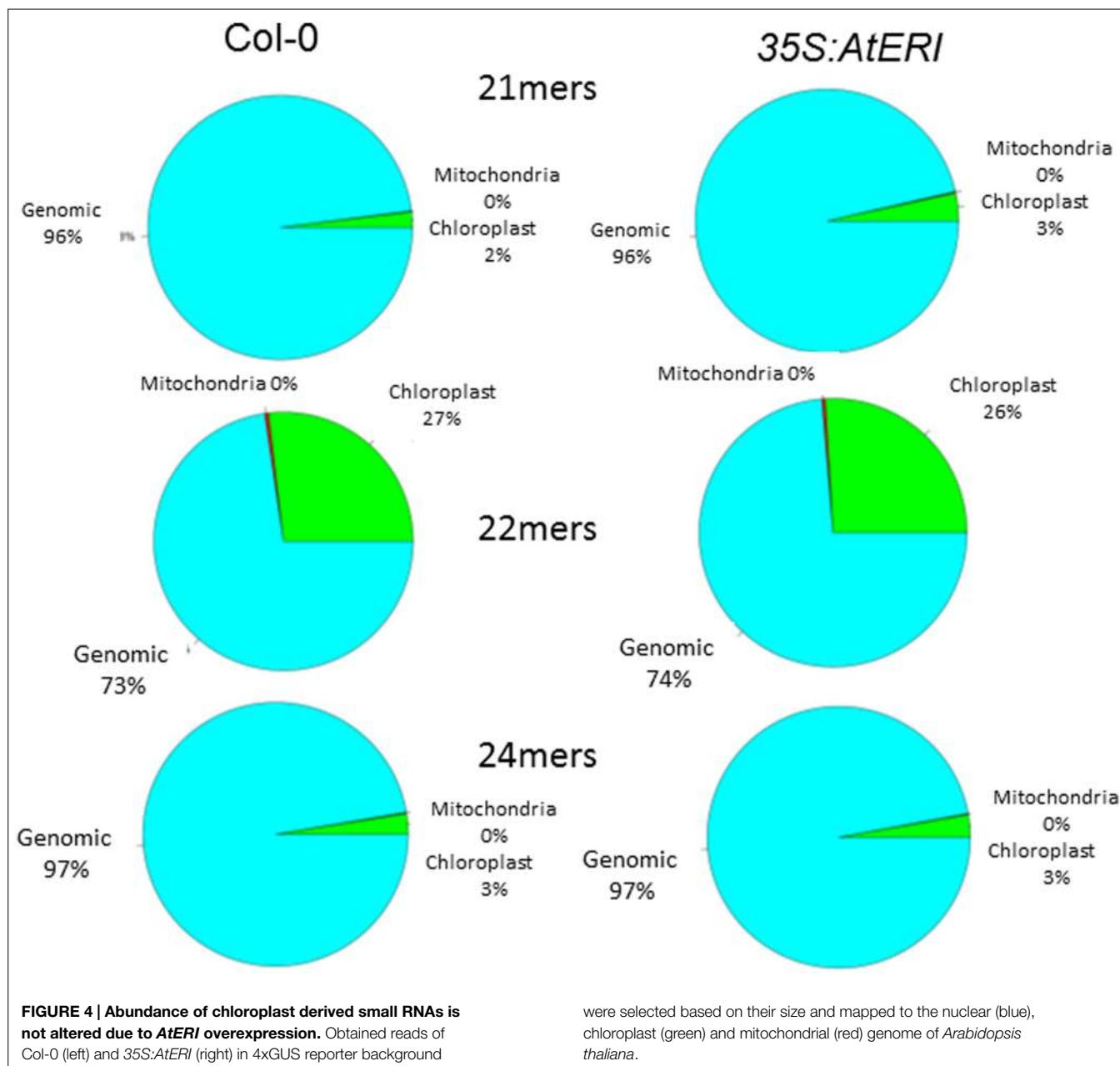


TABLE 1 | List of annotated genes with less association of 21 mers in 35S:AtERI.

Gene	logFC	logCPM	PValue	
AT2G36400	-2,02621676	3,62198957	0,00051627	GRF3 (AT2G36400) growth-regulating factor 3
AT3G52910	-2,05526714	3,61842118	0,0004839	GRF4 (AT3G52910) growth-regulating factor 4
AT2G17033	-2,26204171	2,19855313	0,00125456	Pentatricopeptide repeat-containing protein
AT1G26235	-4,79504295	0,43524652	0,00061644	ncRNA
AT1G47389	-7,5390791	0,74908175	0,00017327	Uncharacterized protein

Differences in leaf area depended on the developmental stage only at low light intensity (**Table 2**).

To detect possible differences in the developmental pattern, RGRs between 0, 6, 8, 14, and 16 DAS were determined based

on leaf area. Differences between RGRs were estimated by a two-factorial ANOVA (**Table 3**). Only the quadratic interaction term was significant, i.e., differences in RGRs changed over time (Poorter and Lewis, 1986). The overexpressing line showed overall

TABLE 2 | Biomass and leaf area of 35S:AtERI and Col-0.

Trait	Light intensity ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Col-0	35S:AtERI	p-value	Significance (Bonferroni)	ese
Biomass						
20DAS (mg plant $^{-1}$)	51 101 187	0.940 4.973 10.196	1.110 6.840 14.378	< 0.001 < 0.001 < 0.001	*** *** ***	0.2386
Leaf area						
6DAS ($\text{mm}^2 \text{plant}^{-1}$)	51 101 187	1.824 3.569 3.994	2.038 4.626 5.730	0.050 < 0.001 < 0.001	ns *** ***	0.1516
Leaf area						
8DAS ($\text{mm}^2 \text{plant}^{-1}$)	51 101 187	3.91 9.12 11.65	4.1 11.79 16.06	0.418 < 0.001 < 0.001	ns *** ***	0.3830
Leaf area						
14DAS ($\text{mm}^2 \text{plant}^{-1}$)	51 101 187	19.9 63.8 94.1	22.3 85.9 130.9	0.007 < 0.001 < 0.001	** *** ***	2.980
Leaf area						
16DAS ($\text{mm}^2 \text{plant}^{-1}$)	51 101 187	33.9 128.2 202.2	40.0 182 273.9	< 0.001 < 0.001 < 0.001	*** *** ***	6.340

Values represent mean values estimated with 2-catorial ANOVA; ese indicates the estimated standard error of means within the same light condition. ns, not significant; ** $p < 0.01$, *** $p < 0.001$.

TABLE 3 | Differences between relative growth rates (RGRs).

Source of variation ^a	s.s. ^b	d.f. ^c	p ^d
Light	396.901	2	< 0.001
Line	9.09441	1	< 0.001
Time	7859.79	4	< 0.001
Light.Line.Time: linear	0.24859	2	0.165
Light.Line.Time: quadratic	0.64553	2	0.009
Residual	99.9416	1450	
Total	8225.03	1497	

^aSources of variation comprise the independent variables "light" (the different light intensities), "line" (Col-0 and 35S:AtERI) and "time" (time points of leaf area determination), and interaction terms. Only the values for the interaction term Light.Line.Time are shown.

^bSum of squares.

^cDegrees of freedom.

^dProbability of the F test.

higher RGR than the Col-0 wild type (Figure 5). The largest differences occurred for all light intensities during early growth (RGR_{0-6}), with the overexpressing line displaying higher values. Interestingly, ectopic overexpression of AtERI also led to higher RGRs at the later developmental stage (RGR_{14-16}), except at 187 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Discussion

The Reduction of siRNAs in AtERI Overexpressing Plants Supports the Proposed Function of Degradation

The plants used for the present study are ectopically overexpressing the *A. thaliana* homolog of the 3'-5'-endonuclease Enhancer of RNAi (AtERI) under control of the promotor of the Cauliflower mosaic virus (*ProCaMV35*). The enhanced ectopic expression of AtERI was confirmed by the increased presence

of AtERI mRNA. Based on the homology and domain structure of AtERI it was concluded that the function of AtERI is similar to the already described exonucleases from human, mice, worm and slime mold. The described protein structure (Figure 1) is conserved among all crop species analyzed and is indicative of a unique and highly conserved function in the plant kingdom. As described in *C. elegans*, the function of ERI could be addressed to degradation of small RNAs by removal of the protruding 3'-nt. This shortening of siRNA led to their exclusion from the mechanism of PTGS.

The profile of small RNAs obtained by high throughput sequencing showed a reduction of sequences with the specific size of 21-nts. This is in agreement with the proposed function of AtERI as 3'-5'-endonuclease. Also the increased abundance of 16-nt long small RNA in the AtERI overexpressing plant argues for an increased degradation process accumulating the smaller products. Mapping of the small RNAs to the miRBASE and the *A. thaliana* coding regions reveal the specificity of AtERI function on 21 mer sized siRNAs. As the majority of miRNAs are unchanged in their abundance it can be concluded, in agreement with already published data (Ramachandran and Chen, 2008), that the degradation of miRNAs is performed by SDN1 and not affected by ERI.

The population of 22-nt small RNAs, mainly derived from incomplete degradation of chloroplast derived transcripts (Rupe et al., 2011), is also not affected by ectopic AtERI overexpression. The functional contribution of SVR7, found to be a target less susceptible by increased AtERI expression to the pool of chloroplast derived small RNAs is not clear from the current state of knowledge.

Despite the global reduction of 21-nt sized RNAs, an increase of siRNA associated with the coding regions was detected. This is

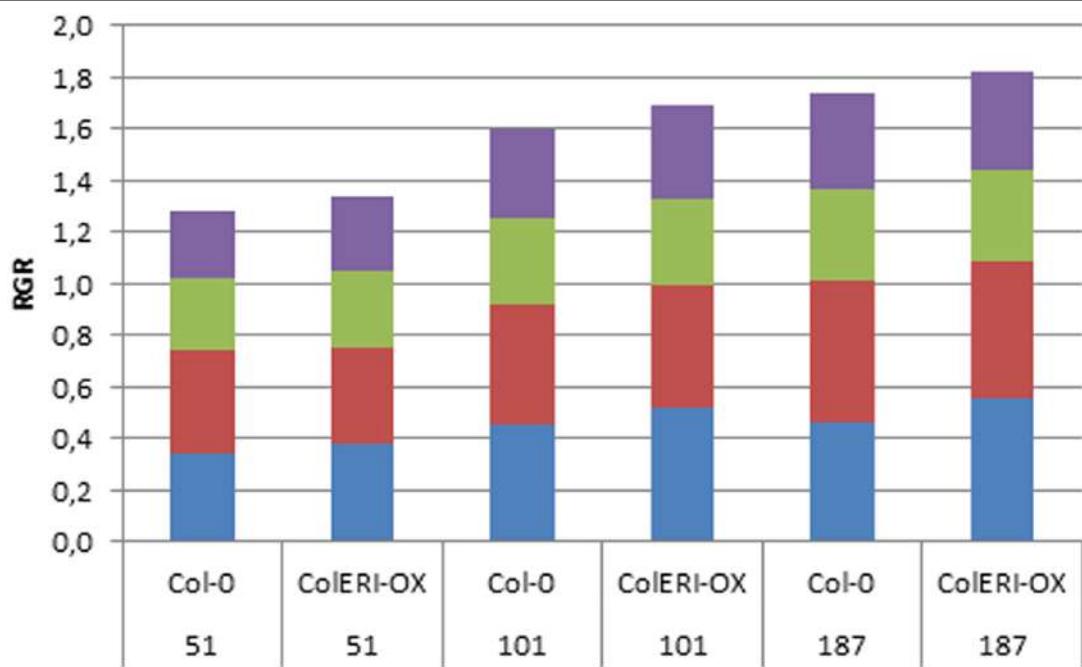


FIGURE 5 | Relative growth rates in Col-0 and 35S:AtERI at different light intensities. RGRs were estimated from seed (0 DAS) and leaf area (6, 8, 14, 16 DAS) and are given in $\text{mm}^2 \text{d}^{-1}$: blue, RGRO-6

(between 0 and 6 DAS); red, RGR6-8; green, RGR8-14; purple, RGR14-16. 51, 101, 187 indicate the light intensities ($\mu\text{mol m}^{-2}\text{s}^{-1}$) the plants were grown in.

in agreement with the fact that the majority of targets subjected to PTGS is derived from retroelements and transcribed non-coding regions (Vaucheret and Fagard, 2001).

The increased abundance of 24 mer, triggers of the TGS mechanism and associated with RNA directed DNA methylation, supports the model for an already proposed backup system of retroelement inactivation (Creasey et al., 2014) in the AtERI overexpressing plant.

Summarizing the data it can be concluded that AtERI functions as 3'-5'-endonuclease with specificity for siRNAs involved in the mechanism of PTGS in *A. thaliana*.

Overexpression of AtERI Affects the Early Growth

Plants ectopically overexpressing AtERI accumulated more biomass than the Col-0 wild type. The differences were established early during development, most likely during seedling establishment. Small increases in RGRs between lines may lead to large differences in size (Milborrow, 1998). A larger leaf area during seedling growth would allow the AtERI overexpressing lines to absorb more light than the Col-0 wild type, potentially resulting in increased photosynthetic activity per plant. The transcription factor AtGRF3, for which small RNAs homologous to the coding region were identified, is involved in cell expansion during early development (Kim et al., 2003). This is also consistent with the native expression of AtERI during early development. Interestingly, native AtERI expression shows a second peak during the transition from vegetative to reproductive growth, which might explain the increased RGRs during the later development.

Although biomass differences between AtERI overexpressing and wild type plants were observed for all three light intensities

analyzed, the temporal pattern of RGR differences varied with the light intensity. The stronger initial growth at the highest intensity was damped by reduced growth at later stages. The reasons for this behavior remain unclear. It is well known that growth differences can be influenced by different light intensities. In F1 hybrids of the *Arabidopsis* accessions Col-0 and C24, biomass heterosis was increased in higher light intensities (240 vs. 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$) due to a sustained increase in RGR (Meyer et al., 2004). Light intensity positively affected silencing initiation and spread in *Nicotiana* (Kotakis et al., 2010).

Speculation About the Function of Identified Target Genes in Early Plant Growth

The mapping of differentially abundant 21-nt sized RNA to the coding region of the *Arabidopsis* genome led to the identification of 116 genes with increased abundance of siRNAs in the 35S:AtERI plant. The presence of 21 mers as hallmark of PTGS indicates that these genes undergo increased silencing in the AtERI overexpressing plant compared to the wild type. Derived from the MAPMAN based gene ontology view, a substantial proportion of these genes are associated with RNA function. More important, several well characterized ontology classes connected with energy production and general biogenesis are strongly underrepresented. From this can be concluded that no specific enhancement of gene silencing affects physiologically important genes.

Within the list of genes with decreased abundance of siRNAs, two important growth regulating factors could be identified: GRF3 and GRF4. The encoding genes are located within QTL for biomass heterosis and vegetative growth on chromosome 2 and 3, respectively (Meyer et al., 2010).

GRF3 and GRF4 are members of a transcription factor gene family that was already described for promoting plant growth during early development (Kim et al., 2003). The expression of *AtGRF3* in leaves was restricted to the very early stages of emerging leaves, consistent with the observed differences during early development. The presence of small RNAs homologous to the coding region of GRF3 and GRF4 in the wild type plant is indicative of small RNA based regulation/silencing in the early growth phase. The absence of these small RNAs would lead to reduced silencing, therefore a stronger expression, and might thus lead to increased biomass during early development. Members of the GRF family are regulated by miR396 (Liu et al., 2009b). Ectopic overexpression of miR396 lead to reduced leaf cell number and altered leaf shape. Although we did not see a general change in the population of microRNAs in the *AtERI* overexpressing plant, sequences with similarity to miR396 were analyzed and quantified. While the abundance of 20 nt long sequences (exact miRNA sequence) are not significantly altered the number of shorter sequences is increased in the *AtERI* overexpressing plant. Therefore it can be speculated that the turnover of miR396 is increased based on increased abundance of AtERI. Such increased turnover might also have a positive effect on the GRF gene expression and thereby contributing to increased production of biomass in the early growth phase. Based on the unchanged amount of miR396 (exact sequence) an influence of

AtERI overexpression on miRNA396 and subsequent regulation of the GRF transcription factor family can be excluded.

Based on the results presented a model is proposed regulating the accumulation of early biomass and growth rate via increased expression of *GRF3* and *GRF4*. While in the wild-type situation these genes are targets of post-transcriptional regulation no small RNAs are detectable in the *AtERI* overexpressing plants. Therefore it is concluded that increased degradation of PTGS associated small RNAs lead to a deregulation of naturally suppressed target genes. The described release affects positively the accumulation of early biomass and growth rate.

Acknowledgments

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Supplementary Material

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Epigenetic silencing in transgenic plants

Sarma Rajeevkumar¹, Pushpanathan Anunanthini² and Ramalingam Sathishkumar^{2*}

¹ Molecular Plant Biology and Biotechnology Division, Central Institute of Medicinal and Aromatic Plants Research Centre, Bangalore, India, ² Plant Genetic Engineering Laboratory, Department of Biotechnology, Bharathiar University, Coimbatore, India

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Edited by:

Clelia De-la-Peña,
Centro de Investigación Científica
de Yucatán, Mexico

Reviewed by:

Gong-yin Ye,
Zhejiang University, China
Ghulam Kadir Ahmad Parveez,
Malaysian Palm Oil Board, Malaysia

*Correspondence:

Ramalingam Sathishkumar,
Plant Genetic Engineering Laboratory,
Department of Biotechnology,
Bharathiar University, Coimbatore
641 046, Tamil Nadu, India
rsathish@buc.edu.in

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Epigenetic silencing is a natural phenomenon in which the expression of genes is regulated through modifications of DNA, RNA, or histone proteins. It is a mechanism for defending host genomes against the effects of transposable elements and viral infection, and acts as a modulator of expression of duplicated gene family members and as a silencer of transgenes. A major breakthrough in understanding the mechanism of epigenetic silencing was the discovery of silencing in transgenic tobacco plants due to the interaction between two homologous promoters. The molecular mechanism of epigenetic mechanism is highly complicated and it is not completely understood yet. Two different molecular routes have been proposed for this, that is, transcriptional gene silencing, which is associated with heavy methylation of promoter regions and blocks the transcription of transgenes, and post-transcriptional gene silencing (PTGS), the basic mechanism is degradation of the cytosolic mRNA of transgenes or endogenous genes. Undesired transgene silencing is of major concern in the transgenic technologies used in crop improvement. A complete understanding of this phenomenon will be very useful for transgenic applications, where silencing of specific genes is required. The current status of epigenetic silencing in transgenic technology is discussed and summarized in this mini-review.

Keywords: homology-dependent gene silencing, post-transcriptional gene silencing, systematic acquired silencing, transcriptional gene silencing, transgenic plants

Introduction

Conventionally closely related species are easier to breed than inter species/genus due to compatibility issues, and this is considered a major limitation. Transgenic technologies have allowed gene transfer to completely unrelated organisms. All these advances have increased the global transgenic plant cultivation to 181 million hectares (James, 2014). Transgenic plants with stacked genes are gaining more importance lately. Here, different genes are expressed in one transgenic plant from a single transformation event, or in consecutive steps either by re-transformation or by conventional genetic crosses involving different transgenic lines expressing a single transgenic event (Dietz-Pfeilstetter, 2010). To date, diverse traits such as disease resistance, stress tolerance, nutritional improvement, and the use of plants as host systems to produce economically important molecules have been successfully proven (Ahmad et al., 2012). The purpose of gene transfer to plants in all the above cases was to achieve specific desirable traits, where lines that failed to meet expectations are discarded, so that the best performers can be propagated (Kohli et al., 2006, 2010). Initial reports of unforeseen low gene-expression levels or silencing

of transgenes were considered failures. Later, those minor glitches emerged as a principal factor elucidating the role of epigenetics in this emerging technology (Meyer et al., 2013).

A major prerequisite for plant expressing a transgene is stability and segregation. Several reports have documented a deviation from the Mendelian segregation ratios in transgenic plants (Shrawat et al., 2007; Weinhold et al., 2013). This revealed the existence of hitherto unknown cellular mechanisms which regulate expression of transgenes. In the last three decades, many reports on transgene instabilities as well as the reasons behind these events were the main focus (Charrier et al., 2000; Graham et al., 2011; Stroud et al., 2013). The explanation for inactivation/silencing of transgene activity was a lack of transcription due to methylation of the promoter along with condensation of chromatin, or degradation of transcripts by different mechanism (Fagard and Vaucheret, 2000; Table 1).

Epigenetics

The British developmental biologist Conrad H. Waddington coined the term “epigenetics”. Epigenetics deals with studies related to interactions of genes and their products, which determine the phenotype of a system (Waddington, 1942). During the course of an organism’s development, cell fate is determined by genes and by other (epigenetic) factors, which underlies the notion of “epigenesis”. Modern biology has redefined as a phenomenon in which a gene’s activity is modulated by modifications of nucleic acids or the physical packaging of the chromatin in which it is embedded.

Two main classes of transgene-silencing phenomena have been reported to date. The first concerns position effects, in which the expression of a foreign gene is negatively regulated by flanking host DNA or chromosomal location (Matzke et al., 2000). The expression of a gene integrated into a region of euchromatin is also influenced by regulatory sequences of host genes (Kohli et al., 2006). Transgene integration into heterochromatic regions also leads to silencing (Grewal and Elgin, 2002).

The second class of silencing phenomena is based on epigenetic regulation and is a type of inactivation mechanism that can arise when multiple copies of the same or homologous sequence are introduced in a genome. Since interactions between homologous nucleic acid sequences are responsible for these silencing, it is also called homology-dependent gene silencing (HDGS) (Meyer and Saedler, 1996). Over the years, it has become clear that HDGS occurs through distinct processes, frequent one being involvement of inverted DNA repeats (IRs) and dsRNA. T-DNA integration at the same chromosomal site leads either to ‘head-to-tail’ direct repeats (DR) or to ‘head-to-head’ or ‘tail to tail’ inverted repeats (IR). T-DNAs that are arranged as IRs are often shown to have low basal expression (Mishiba et al., 2005). IRs have the ability to interact with homologous sequences elsewhere in the genome leading to chromatin remodeling. They can also induce a sequence-specific RNA degradation process, possibly via the formation of dsRNAs (Figure 1A).

Homology Dependent Gene Silencing

A major breakthrough in understanding epigenetic silencing in transgenic plants was first identified in transgenic tobacco, where interaction between two homologous promoters led to DNA methylation and silencing (Matzke et al., 1989). Two types of HDGS are known based on the stage at which it occurs, called transcriptional gene silencing (TGS), which is coupled with transcription or by promoter modification, and post-transcriptional gene silencing (PTGS), which occurs after the formation of mRNA (Jauvion et al., 2012). In TGS, interacting genes that share homology in promoter regions are highly methylated. PTGS involves sequence-specific transcript turnover in the cytosol, which further requires high homology between interacting genes. Potential factors influencing HDGS are degree of homology between the transgene and endogenous gene, the complexity of the host genome, the genomic position of two transgenes, etc. A transgene locus with a complex structure with multiple scrambled T-DNAs has been reported to have strong silencing activities in tobacco, implicating transgene complexity; and vector DNA also decides the efficiency of HDGS (Fu et al., 2000). Complexity of T-DNA structure and integrated vector sequences have been shown to regulate transgene expression in grapevine (Gambino et al., 2010). An increase of endogenous transcript levels above a critical threshold induces specific degradation of homologous transcripts.

Transcriptional Gene Silencing (TGS)

Transgenes silenced at the transcriptional level acquire metastable epigenetic status that is associated with altered methylation patterns. Transgenes are frequently methylated in cytosine residues that are located within CG, CNN, or CNG sequences. *De novo* DNA methylation can be highly sequence-specific for a specific transgene (Matzke et al., 2007). Fungi or plants expressing foreign genes also exhibit non-symmetrical methylation leading to silencing of endogenous genes. Factors responsible for non-symmetrical methylation are still obscure. Non-symmetrical methylation patterns are aided by RNA-chromatin mechanism (McGinnis et al., 2006).

Methylation in promoter regions, histones, or in coding regions influence gene expression at both the transcriptional (Huettel et al., 2006) and post transcriptional level (Regulski et al., 2013; Tsuchiya and Eulgem, 2013). Chromatin remodeling is involved in maintenance of silenced status and also in transmission of non-symmetrical methylation patterns (Meyer, 1999). Another interesting fact about TGS in transgenic plants is the association of DNA methylation along with structural changes, as methylated and silenced transgenes were less susceptible to endonucleases, reflecting an increased level of chromatin condensation (Van Blokland et al., 1997). Hence, TGS-based silencing might also involve structural changes similar to heterochromatinization, which could be the cause of these structural changes. The responsiveness of TGS of transgenes in response to environmental change was confirmed (Meyer et al., 1992; Meyer, 2015).

TABLE 1 | Reports of epigenetic silencing in transgenic plants.

Target plant	Gene (s)	Transgene effects	Reference
<i>Arabidopsis thaliana</i>	Selectable marker genes (<i>nptI/hpt</i>)	Repeated sequence of target gene at same loci lead to repeat-induced gene silencing (RIGS).	Assaad et al., 1993
<i>Nicotiana tabacum</i>	Selectable marker gene (<i>nptI</i>)	<i>De novo</i> methylation mediated silencing of <i>nptII</i>	Ingelbrecht et al., 1994
<i>Petunia hybrida</i>	Flavonoid hydroxylase gene, maize <i>A1</i> gene	Hypermethylation of 35S promoter directed <i>A1</i> gene expression lead to variegated flower pigmentation in transgenic <i>Petunia</i> lines	Meyer et al., 1994
<i>Avena sativa</i>	<i>bar</i> and <i>gusA</i>	Direct DNA–DNA interaction between multiple transgene copies resulted in silencing of <i>bar/gusA</i> gene to different levels.	Pawlowski and Somers, 1998
<i>Oryza sativa</i>	<i>bar</i> gene	Methylation of Ubi1 promoter lead to silencing of <i>bar</i> gene and bialaphos sensitivity in transgenic rice	Kumpatla and Hall, 1998
<i>Saccharum officinarum</i>	sorghum mosaic potyvirus strain SCH coat protein (CP) gene	Reduced transcript level lead to post-transcriptional gene silencing (PTGS) of CP gene in transgenic sugarcane.	Ingelbrecht et al., 1999
<i>Oryza sativa</i>	<i>GUS</i> gene	Reintroduction of <i>GUS</i> gene in <i>GUS</i> transformed rice lead to suppression of <i>GUS</i> expression due to PTGS	Kanno et al., 2000
<i>N. tabacum</i>	<i>GUS</i> gene	Gene silencing through DNA methylation lead to reduced expression of <i>GUS</i> gene in transgenic tobacco lines	Day et al., 2000
<i>Petunia</i>	<i>CHS</i> gene	White-flowering phenotype due to chalcone synthase transgene-induced silencing as a result of altered methylation in promoter	Kanazawa et al., 2007
<i>A. thaliana</i>	Phytochrome A/DNA methyl transferase I gene	Exonic methylation can lead to chromatin modification further resulting in altered gene expression mediated through reduction in the transcription rate.	Chawla et al., 2007
<i>N. tabacum</i>	<i>nptII</i>	Target gene was silenced by PTGS based on the loci of intergration	Khaitova et al., 2011
<i>Gentiana verma</i>	CaMV35S promoter	<i>De novo</i> methylation of the enhancer region of CaMV 35S promoter silencing is triggered by histone H3 deacetylation.	Yamasaki et al., 2011
<i>A. thaliana</i>	<i>A. thaliana</i> repressor of silencing1 mutant	Mutants treated with sulfamethazine exhibited reduced levels of DNA methylation and released transgene silencing. Exogenous application of p-Aminobenzoic acid restored transcriptional gene silencing (TGS) in SMZ-treated mutants	Zhang et al., 2012
<i>N. tabacum</i>	CaMV35S promoter	DNA methylation and heterochromatic histone marks were studied in different epialleles of 35S promoter driven tobacco transgenic calli. Transient loss of euchromatin modifications lead to <i>de novo</i> DNA methylation further leading to formation of stable repressed epialleles with recovered eukaryotic marks	Křížová et al., 2013
<i>N. tabacum</i>	<i>A. thaliana</i> a repressor of silencing gene (ROS1)	Transgenic lines over-expressing <i>At ROS1</i> showed higher level of demethylation in promoter as well as coding region of various genes involved in flavonoid biosynthesis and antioxidant defense response	Bharti et al., 2015

Transcriptional gene silencing can be further divided into two classes:

Transcriptional *cis* Inactivation

In plants, transgenes integrate into the genome at random positions by illegitimate recombination; hence, copy number, their integration site, and local arrangement differ in each transformation event. Also, an inverse relation between transgene copy number and gene expression suggests that multicopy integration can lead to silencing. Integrated foreign genes can undergo TGS in *cis* when multicopy T-DNA is integrated at a locus adjacent to hypermethylated regions of the host genome (Mishiba et al., 2005). More rarely, single copy transgene integration at a hypomethylated locus can lead to *cis* inactivation (Meyer and Heidmann, 1994; Elmayan and Vaucheret, 1996). A maize *A1* gene involved in floral pigmentation when overexpressed in *Petunia* led to silencing of *A1*; however, it was not silenced when *Gerbera dihydroflavonol-*

4-reductase was over expressed in *Petunia* suggesting that the transgene also influenced the silencing process. Hence, some degree of difference in DNA composition of the transgene and surrounding host genomic sequences can be recognized by the cellular machinery as foreign non-compatible DNA, leading to specific methylation and silencing (Elomaa et al., 1995). It is believed that *cis* TGS occurs as a result of pairing between closely associated copies of transgenes or endogenous genes, which leads to the formation of secondary DNA structures which are sites for DNA methylation (Vaucheret and Fagard, 2001). Cytosine methylation at CpG and CpNpG sites of transgene and the 35S promoter were also detected in transgenic grapevine transformed with Grapevine fanleaf virus (GFLV) coat protein gene (Gambino et al., 2010).

Transcriptional Trans-Inactivation

Transcriptional gene silencing can result from unidirectional effects of one transgene on another transgene or homologous

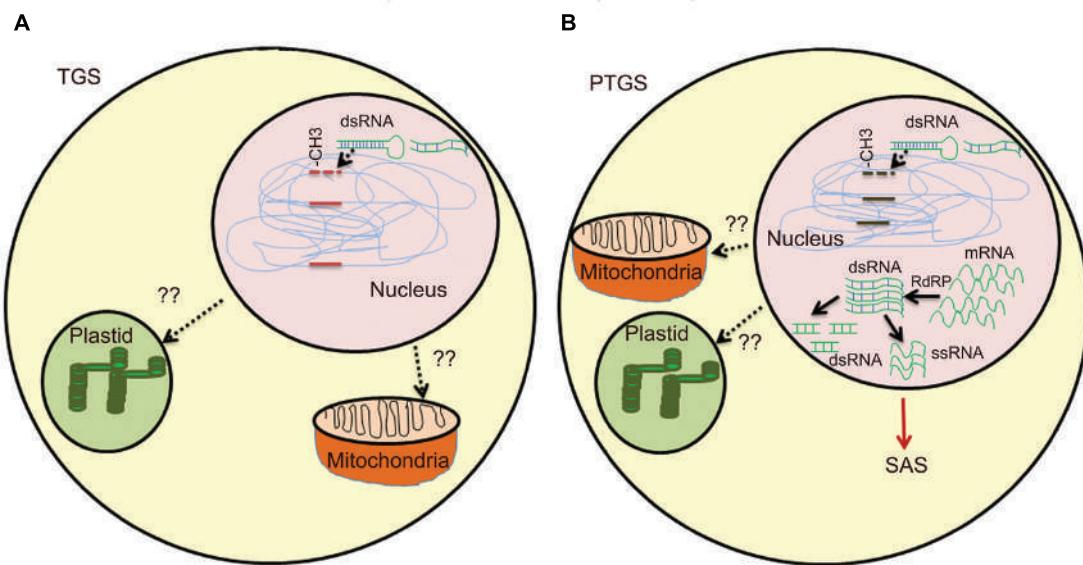


FIGURE 1 | Overview of gene silencing in transgenic plants. **(A)** Transcriptional gene silencing (TGS)- DNA methylation induces dsRNA by endogenous gene or multiple copies of transgenes. Presence of multiple copies of transgene induces formation of dsRNA. Single copy transgene loci could also lead to formation of dsRNA due to high RNA turnover. Methylation of CG, CNG, or CNN region in promoter by different methyltransferases that leads to TGS. Methylation in heterochromatin region also lead to TGS. T-DNA with transgene integrated as direct or inverted (IR) repeats are inactivated by DNA methylation. Cruciform structures formed by IRs act as substrate for DNA methyltransferases. **(B)** Post-transcriptional gene silencing (PTGS)- Methylation in coding region and high RNA turn over lead to production of dsRNA, aberrant RNAs, cRNAs. RdRP uses these aberrant RNAs as templates and convert them into a double-stranded RNA, which is further degraded by different dsRNases yielding small dsRNAs and/or ssRNAs. The ssRNAs and/or dsRNAs act as systemic silencing signals, which are transported all over the plant and trigger PTGS in adjacent cells. SAS in mitochondria and plastids are still under study.

endogenous gene. A transgene can be methylated and silenced when it is crossed with a plant in which the homologous gene is in a silenced state (Meyer et al., 1993). *De novo* methylation of one transgene is mediated by a second transgene under control of the same promoter leading to TGS in trans (Fagard and Vaucheret, 2000). Experiments using dsRNA-containing promoter sequences initiated TGS and subsequently *de novo* DNA methylation of the corresponding transgene or endogenous gene, implying a role of an RNA intermediate in TGS (Meyer, 2000). Vaucheret and Fagard (2001) reported the role of different genes, including *ddm1* and *ddm2* in TGS in *Arabidopsis* transgenic lines. Yamasaki et al. (2011) reported methylation of asymmetric cytosine in the enhancer region of 35S promoter in transgenic gentian.

Post-Transcriptional Gene Silencing

Post-Transcriptional Gene silencing is a condition where transcripts do not accumulate in spite of continuous transcription (Vaucheret et al., 2001). PTGS can silence both transgenes and endogenous genes if both are homologous. An endogenous gene could be switched off, when a plant is transformed with another copy of the same gene. When genes involved in pigmentation, such as *chalcone synthase A* in *Petunia*, were overexpressed, many transgenic lines partially or completely lost activity of both transgene and endogenous gene (Napoli et al., 1990; Van

der Krol et al., 1990). This was later called ‘co-suppression’, which was a result of degradation of mRNA of both transgene and endogenous gene. Analysis of degradation products in tobacco expressing β -1,3-glucanase revealed that RNAs are first cleaved by endonucleases, which are further degraded by various exonucleases (Van Eldik et al., 1998). Silencing of two endogenous genes in *Arabidopsis thaliana* was triggered by the antisense and hpRNA transgenes, and silencing in this case was dependent on ploidy level, as it was less pronounced in 4n compared to 2n *Arabidopsis*. Studies indicated that transgenes were more methylated in 4n than 2n *Arabidopsis* suggesting transgenes are transcriptionally repressed in 4n plants, thus resulting in reduced expression levels compared to diploid plants (Finn et al., 2011).

Transgene-induced viral resistance, recovery from infection and proteins encoded by viruses that counteract PTGS suggested it as a potential defense response to check viral infections (Brigneti et al., 1998; Kasschau and Carrington, 1998; Dalmay et al., 2000). It is speculated that the concentration of specific RNAs derived from both transgene and endogenous gene is critical to activate PTGS. dsRNAs are one of the potential candidates, as they are formed between RNAs transcribed from IR and gene homologues. dsRNA is used as a template by RNA-directed RNA polymerase (RdRP) and transcription of dsRNA by RdRP would result in antisense RNAs, which ultimately could target complementary transcripts for degradation by dsRNA-specific RNases (Bond and Baulcombe, 2015; **Figure 1B**).

Post-Transcriptional *cis*-Inactivation

Post-transcriptional gene silencing *cis*-inactivation is observed when foreign genes like β -Glucuronidase, neomycin phosphotransferase, etc., were driven under strong 35S promoter (Dehio and Schell, 1994; Ingelbrecht et al., 1994; Elmayan and Vaucheret, 1996). When a 35S promoter with a double enhancer was used, more transformants showed PTGS (Elmayan and Vaucheret, 1996; English et al., 1996). Initially, perceptions about PTGS were driven by higher transcript abundance above a threshold level, which ultimately triggered degradation of transgenic RNA. Later, it was found that the level of transcription was not always found to be significantly higher in silenced plants. The presence of IR at transgene locus of silenced lines was proposed to play a crucial role in *cis* inactivation (English et al., 1996). In same year, different models for PTGS were proposed considering RNA abundance and IRs (Baulcombe, 1996). Transgene RNA could be specifically degraded when tagged with specific molecules; these tag molecules were later named small complementary RNA (cRNA). RdRP catalyzed synthesis of cRNA using transgene RNA as template (Dougherty and Parks, 1995). They could also be internal fragments generated from transgene RNA by pairing between aberrant mRNA and normal transgene RNA due to the presence of internal sequence complementarily (Metzlaff et al., 1997). cRNA can interact with mRNA forming dsRNA, which are the target for the cellular enzymes like double-strand RNase. DNA-DNA interactions can lead to methylation, which can further interfere with transcription, ultimately producing aberrant RNA. These aberrant RNAs or higher transcript abundance were owing to the use of a strong promoter that triggered methylation of the coding sequence of the respective transgene (Wassenegger et al., 1994). Interestingly, Kanazawa et al. (2007) reported conversion of PTGS to TGS in *Petunia* transgenic lines as a consequence of the transgene homologous to an endogenous gene in host genome.

Post-Transcriptional Trans-Inactivation

Post-transcriptional gene silencing was originally reported as coordinated silencing of both transgenes as well as endogenous genes, which is generally termed ‘co-suppression’ (Napoli et al., 1990). Since then, several studies revealed transgenes encoding part of, or the entire transcribed sequence of, host genes have been shown to trigger co-suppression of endogenous genes (Depicker and Van Montagu, 1997). By then it was evident from studies in transgenic *Petunia* lines expressing a chalcone synthase, where efficiency of co-suppression correlated with the strength of the promoter, that there was an effect of transgene dose on co-suppression (Que et al., 1997). Besides, the efficiency of co-suppression is delayed when endogenous host genes are not expressed or when genes are transferred to a mutant devoid of functional gene homologues (Smith et al., 1990; Vaucheret et al., 1997). Hence, it can be concluded that co-suppression cannot be considered as the unidirectional silencing effect of transgenes, rather it is a synergistic phenomenon in which interaction or presence of host genes and transgenes aids aberrant RNA and/or cRNA leading to PTGS.

Systemic Acquired Silencing

A hallmark of PTGS in plants is that it systemically transmitted in a sequence-specific manner known as systemic acquired silencing (SAS). Remarkable and recurrent features in silencing patterns during developmental stages revealed propagation of a silencing message across the plant (Vaucheret et al., 1998; Kalantidis et al., 2008). Co-suppression of endogenous and transgenes of nitrate reductase, nitrite reductase and SAM synthase in tobacco led to chlorotic or necrotic phenotypes (Boerjan et al., 1994; Palauqui et al., 1996). The non-clonal patterns were observed in all transgenic lines silenced for a specific gene and a sequence-specific message was involved in the control of PTGS. Later, grafting experiments revealed that transgene-specific effector molecules were involved in propagation of *de novo* PTGS over long distances by a phenomenon called SAS (Palauqui et al., 1997). Transgenic tobacco overexpressing *A. thaliana AtMYB90* involved in anthocyanin biosynthesis showed siRNA-mediated silencing as a result of SAS (Velten et al., 2012). A SAS PTGS of transgenes in *N. benthamiana* was initiated in localized regions of the plant when a transgene-homologous DNA was introduced (Voinnet et al., 1998). The silencing signal molecules are degraded RNA, which travels through phloem across cells through plasmodesmata (Kalantidis et al., 2008). The recipient cell can also act as a source for generating secondary signals. It has been reported that sense, antisense, and ill-defined aberrant RNAs can give rise to dsRNA which can transmit signals, ultimately leading to silencing of both transgene and endogenous gene, albeit to different levels (Figure 1B).

Small RNAs as Silencing Signals in Transgenic Plants

RNA was the driving factor for the establishment of DNA methylation patterns (Wassenegger et al., 1994) and acts a signaling agent for inducing silencing. Potato spindle tuber viroid (PSTV) in transgenic tobacco lines led to autonomous viroid RNA replication in the nucleus and induced DNA methylation in the T-DNA (Wassenegger et al., 1994). The evidence from above study clearly indicates the critical role of RNA in initiating *de novo* DNA methylation at homologous regions. Until then, DNA/RNA hybrids were believed to play a role in generating a target for *de novo* methylation. *chsA* co-suppression studies in *Petunia* led to the identification of mobile RNAs as potential candidates responsible for the induction of co-suppression (Napoli et al., 1990; Van der Krol et al., 1990). The initiation of transgene silencing has been thought to involve the generation of dsRNA. It is still under debate about factors triggering initiation of silencing even in the case of transgenes that lack unusual DNA structures.

In plants, micro RNAs (miRNAs) are produced from hairpin-like precursor RNA, which is essential for biogenesis of trans-acting siRNAs (ta-siRNAs). miRNAs are involved in regulation of gene expression by base-pairing with target RNAs further leading to their cleavage in plants. *Physcomitrella patens* transgenic

lines expressing different levels of artificial miRNA (amiRNA) revealed transcript-dependent silencing of miRNA target. Thus, a crucial regulatory role of miRNAs might be conserved in other plants also, which are under investigation. siRNAs are another class of small RNAs that are involved in epigenetic modification (Miki and Shimamoto, 2008). Endogenous siRNAs can induce DNA methylation at CpG nucleotides leading to chromatin modification and silencing. Human *H1* and *Arabidopsis* 7SL RNA promoters driving *GUS* specific short hairpin RNA resulted in the efficient silencing of *GUS* at both transcript and protein level, indicating a significant role of siRNAs in epigenetic regulation. However, transgenes are generally more sensitive against RNA silencing than endogenous genes in plants.

Transgene Silencing as Part of the Host Defense Mechanism?

Silencing cannot be considered as a mechanism that evolved to regulate transgene expression; it is a part of natural plant processes. TGS and PTGS can be considered as host defense responses against ‘foreign invading’ viruses. Hence, transgenes or their products can be equated to cellular invaders triggering defensive reactions leading to silencing of “trans” gene. PTGS recruits cellular components acting against foreign DNA that replicates extra-chromosomally in the nucleus, or RNA in the cytoplasm. A clear connecting link between PTGS and viral resistance was established after the discovery and characterization of various viral proteins that suppress PTGS (Kasschau and Carrington, 1998; Beclin et al., 2002). TGS may use cellular components acting against invading DNA that integrates into the genome. The involvement of DNA methylation can also be considered as a part of cellular defense mechanism against transposable elements. The probable function of dsRNA in initiating methylation can be correlated to retro-elements that produce RNAs with intricate secondary structures.

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Strategies to Prevent Transgene Silencing (Depicker et al., 2005)

- (1) Selection of transgenic lines with single T-DNA insert
- (2) Organelle targeting/transformation
- (3) Selection of favorable/unique integration sites
- (4) Reactivation of silent transgenes
- (5) Use of Scaffold Matrix Attachment Regions in silencing mutant host system to prevent silencing.

Concluding Remarks

The last three decades have seen immense progress and better understanding of epigenetic effects and silencing mechanisms; transgenic technologies have played a pivotal role for these achievements. Common phenomena behind different types of silencing and recent finding of involvement of siRNAs/miRNA continue to inspire efforts of scientific community to formulate comprehensive models, which also explain the silencing mechanism from an evolutionary view point. Our understanding of the influence of various factors on stability of transgene expression is improving rapidly. We cannot control or predict integration of gene into a recipient genome, nor predict the number of copies or integrity of a transgene. Hence, a comprehensive knowledge of underlying mechanisms in integration process and the influence of chromatin remodeling leading to transgene regulation are crucial. Finally, it might be useful to keep in mind that epigenetic silencing was an unexpected phenomenon; it is still hard to foresee overcoming epigenetic related silencing in transgenic system. Nevertheless, transgenic research will continue as a platform to discover new aspects of epigenetic silencing.

Author Contributions

SR and PA prepared the manuscript and RS revised it.

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