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REGULATION OF CELL FATE DETERMINATION IN PLANTS

Topic Editors
Shucai Wang and John Schiefelbein

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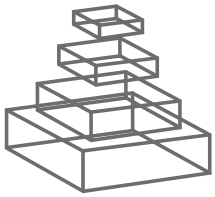

frontiers

ISSN 1664-8714
ISBN 978-2-88919-324-0
DOI 10.3389/978-2-88919-324-0

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REGULATION OF CELL FATE DETERMINATION IN PLANTS

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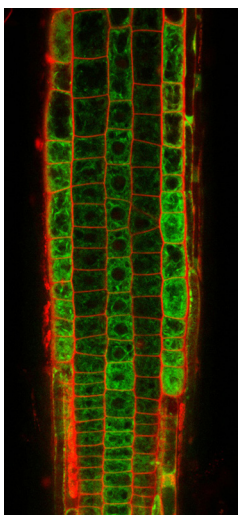

An image of an Arabidopsis root expressing the green fluorescent protein under control of the WEREWOLF epidermal promoter (WER::GFP). The differentiating non-hair cells of the root epidermis preferentially express this transgene. Green=GFP, Red=cell wall stained with propidium iodide.

"Plants are made up of a large number of distinct cell types that originate from a single fertilized egg cell. How the diversity of cell types arise in appropriate places is one of the most fascinating and attractive research areas of plant biology. During the past several decades, due to the development of new molecular techniques and tools, advances in optical microscopy, and availability of whole genome information and mutants in the model plant Arabidopsis and other plants, great advances have been made in understanding the mechanisms involved in cell fate determination in plants.

Multiple mechanisms are used to generate cellular diversity. Asymmetric cell division is one of the primary mechanisms. As an example, asymmetric cell division enables one stem cell to generate a stem cell daughter and a daughter with a distinct identity. Initially equivalent cells can also differentiate to generate different cell types. This mechanism has been clearly demonstrated in the formation of multiple cell types during epidermis development in the shoot and root. Cell fate determination is influenced by both intrinsic factors, i.e, developmental regulators, as well as extrinsic signals, i.e., environmental stimuli. By using model systems like stomata, trichome, root hair and shoot and root apical meristem cells, ligands, receptors and transcription factors have been found to regulate cell fate determination. However, the details of signaling cassettes responsible for cell fate determination remain largely unknown.

This research topic contains 12 collected articles, including 2 Opinion Articles, 5 Reviews, 4 Mini Reviews, and 1 Original Research Article. Hopefully, these articles will expand our understanding of the regulation of cell fate determination in plants."

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frontiers in
PLANT SCIENCE

EDITORIAL
published: 28 July 2014
doi: 10.3389/fpls.2014.00368

Regulation of cell fate determination in plants

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Edited and reviewed by:
Simon Gilroy, University of Wisconsin - Madison, USA

Keywords: cell fate determination, trichome, root hair, stomata, xylem, cotton fiber, protein lipid modification, Arabidopsis

Building a multicellular organism, like a plant, from a single cell requires the coordinated formation of different cell types in a spatiotemporal arrangement. How different cell types arise in appropriate places and at appropriate times is one of the most intensively investigated questions in modern plant biology. Using models such as trichome formation, root hair formation, and stomatal development in Arabidopsis, scientists have begun to discover some of the answers, including the importance of transcriptional regulatory networks, intrinsic signals such as plant hormones, and extrinsic signals such as environmental stimuli. This research topic aimed to summarize the research progress in cell fate determination in plants.

Thanks to the efforts of many people, including the authors who responded enthusiastically to the call to contribute to this research topic, as well as the peer reviewers who provided essential critical comments to ensure the highest quality and up-to-date information in the articles, a total of 12 articles were published in this research topic, including Opinion, Mini Review, Review and Original Research Articles. These articles focused on cell fate determination of different cell types as well as different aspects of a certain cell type in plants.

The specification of distinct cell types in plants is accomplished largely via the establishment of different gene expression, primarily, transcription factor gene expression. For example, recent studies have revealed that certain members of the HD-ZIP class IV homeodomain transcription factors are likely to be master regulators of specification of the shoot epidermal cell layer in plants. Takada and Iida (2014) summarized in their Mini Review the roles of these regulatory genes that are involved in epidermal cell fate specification and discussed the possible mechanisms that limit the expression and/or activity of the HD-ZIP class IV homeodomain genes to the outermost cell layer during the development of plant shoots.

Trichomes and root hairs are specialized epidermal cells whose formation is regulated through a combination of endogenous developmental programs and external signals. In Arabidopsis, trichome and root hair specification is controlled by the interplay of single-repeat R3 MYBs and several other transcription factors including the WD40-repeat protein TTG1, the R2R3 MYB transcription factor GL1 or WER, the bHLH transcription factor GL3 or EGL3, and the homeodomain protein GL2. The TTG1, GL1, or WER, and GL3 or EGL3 proteins are proposed to form a TTG1–GL3/EGL3–GL1/WER activator complex to regulate the expression of GL2, which is required for trichome formation in shoots and non-hair cell specification in roots. On the other hand, the R3 MYB proteins negatively regulate trichome formation and non-hair cell specification by competing with GL1 or WER for binding GL3 or EGL3, thus blocking the formation of TTG1–GL3/EGL3–GL1/WER activator. The expression of component genes of the transcriptional activator complex is regulated by other transcription factors, plant hormones, microRNAs, as well as the 26S proteasome.

Several of the Review and Mini Review articles were devoted to the trichome or root hair cell determination systems. Pattanaik et al. (2014) summarized the gene regulator networks controlling trichome development in Arabidopsis, including the activator complex, their regulation by plant hormones, microRNA and the proteasome system. Hauser (2014) focused on current progress on the molecular basis of the natural variation in various Arabidopsis ecotypes as well as in different plant species with emphasis on plant hormones and environmental stimuli on trichome patterning. Schiefelbein et al. (2014) described the regulatory network and the importance of the multiple feedback loops in root hair cell specification in Arabidopsis, with focus on the mechanisms that lead to the accumulation of the WER-bHLH-TTG1 activate complex in non-hair cells. Tominaga-Wada and Wada (2014) described their findings that tomato and Arabidopsis likely use similar transcription factors for root hair cell differentiation, and that a CPC-like R3 MYB may be a key common regulator of plant root-hair development. In addition, Wang and Chen (2014) focused on the roles of single-repeat R3 MYB transcription factors in the regulation of cell fate determination in Arabidopsis.

In their Original Research Article, Zhou et al. (2014) described the identification and characterization of poplar single-repeat R3 MYB transcription factors in trichome formation. They found a total of eight genes in poplar encoding R3 MYB transcription factors, and all these R3 MYB genes negatively regulate trichome formation when expressed in Arabidopsis.

Stomata are also specialized epidermal cells, and like the formation of trichomes and root hairs, stomatal development is controlled by both an intrinsic genetic regulatory network and environmental stimuli. In Arabidopsis, stomatal precursor cells undergo at least one asymmetric division and a symmetric division. Le et al. (2014) described in their Mini Review the roles of the ER/TMM signal transduction pathway in the regulation

of stomata formation and cell cycle, and the role of different plant hormones in stomata development. In her Opinion article, Serna (2003) focused on the regulation of the choice between meristemoid cell self-renewal, in which one of daughters of a dividing meristemoid retains the properties of the parent cell, and its transition through guard mother cell (GMC) fate to produce stomata.

Xylem, phloem, and procambial/cambial cells are differentiated in a spatiotemporally organized manner during vascular development. Various key regulators for xylem cell patterning and differentiation have been discovered, such as the plant hormones auxin and cytokinin, the peptide hormone CLE, microRNAs, and the transcription factor HD-ZIPIIIs, VNDs, SHR, and AHLs. Recent studies revealed that xylem cell fate determination is controlled by functional interactions among these key regulators. Kondo et al. (2014) reviewed the networks of various regulators underlying xylem cell fate determination in root vascular development.

MYBMIXTA transcription factors are involved in the regulation of epidermal cell differentiation in different plant species, including cell shape specification in petals, trichome initiation and branching in shoots and fiber initiation in seeds. MYBMIXTA-like (MML) transcription factors from the subgroup 9 of R2R3-MYBs were first characterized in *Antirrhinum majus*. Bedon et al. (2014) provided evidence in their Opinion article that members of the MML transcription factors regulate the initiation of fiber development in cotton seeds.

Post-translational modifications of proteins are often important for regulation of their functions. One of the key modifications is the attachment of a lipid group to certain amino acids in the proteins, which typically facilitates subcellular targeting and/or protein–protein interactions. Running (2014) summarized in his Review the progress of three known types of intracellular protein lipid modifications, and their roles in many plant-specific aspects of developmental processes, including cell differentiation.

In a summary, we hope the articles in this research topic will provide readers a snapshot of current molecular and genetic investigations of cell fate determination in plants.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 July 2014; accepted: 09 July 2014; published online: 28 July 2014.
Citation: Wang S and Schiefelbein J (2014) Regulation of cell fate determination in plants. Front. Plant Sci. ***5****:368. doi: 10.3389/fpls.2014.00368*
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 25 February 2014
doi: 10.3389/fpls.2014.00049

Specification of epidermal cell fate in plant shoots

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Land plants have evolved a single layer of epidermal cells, which are characterized by mostly anticlinal cell division patterns, formation of a waterproof coat called cuticle, and unique cell types such as stomatal guard cells and trichomes. The shoot epidermis plays important roles not only to protect plants from dehydration and pathogens but also to ensure their proper organogenesis and growth control. Extensive molecular genetic studies in *Arabidopsis* and maize have identified a number of genes that are required for epidermal cell differentiation. However, the mechanism that specifies shoot epidermal cell fate during plant organogenesis remains largely unknown. Particularly, little is known regarding positional information that should restrict epidermal cell fate to the outermost cell layer of the developing organs. Recent studies suggested that certain members of the HD-ZIP class IV homeobox genes are possible master regulators of shoot epidermal cell fate. Here, we summarize the roles of the regulatory genes that are involved in epidermal cell fate specification and discuss the possible mechanisms that limit the expression and/or activity of the master transcriptional regulators to the outermost cell layer in plant shoots.

Keywords: epidermal cell differentiation, positional signal, HD-ZIP class IV transcription factor, cuticle, endosperm, receptor-like kinase, calpain-like cysteine protease, *Arabidopsis thaliana*

INTRODUCTION

The shoot epidermis is a single layer of surface cells that are morphologically characterized by anticlinal cell division patterns. The outer surface of the shoot epidermis is covered with a hydrophobic structure called a cuticle, which prevents water loss, pathogen attacks, and post-genital fusion of organs (Yeats and Rose, 2013). Besides basic pavement cells, leaf epidermis contains specialized cell types such as hair cells (trichomes) and stomatal guard cells, which function to cope with dehydration and pathogen attacks. In addition to its protective function, the epidermis plays roles in the regulation of organ growth and shoot stem cell maintenance (Savaldi-Goldstein et al., 2007; Knauer et al., 2013; Nobusawa et al., 2013). In addition, protodermal cells in the shoot meristem and the embryo are necessary for the production and transport of the phytohormone auxin, which drives embryonic axis formation and lateral organ primordia initiation (Reinhardt et al., 2003; Kierzkowski et al., 2013; Robert et al., 2013; Wabnik et al., 2013).

Determination of shoot epidermal fate relies on a "position" rather than a cell "lineage," as clonal analyses has shown that there is no strict lineage restriction in developing leaves; cells can flexibly change their fates, and only the cells finally located at the surface of the organ develop into epidermis (Stewart and Dermen, 1975). However, positional cues that determine shoot epidermal cell fate remain largely unknown. This review describes recent advances in the studies of epidermal cell specification in the shoots, focusing mainly on the regulation of key transcription factors.

HD-ZIP CLASS IV TRANSCRIPTION FACTORS ARE KEY REGULATORS FOR EPIDERMAL CELL SPECIFICATION

ARABIDOPSIS THALIANA MERISTEM L1 LAYER (*ATML1*) was identified as an epidermis-specific homeobox gene that belongs to the HD-ZIP class IV family (Lu et al., 1996). *ATML1* expression is first detected in the embryos as early as the one-cell stage and its expression is restricted to the outermost cells around the 16-cell stage (dermatogen stage) after the embryos have undergone tangential cell divisions to generate outer protodermal cells and inner cells (Lu et al., 1996; Sessions et al., 1999; Takada and Jürgens, 2007; **Figures 1A–E**).

Mutations in *ATML1* and its closest homolog *PROTODERMAL FACTOR2* (*PDF2*) caused severe phenotypes associated with defects in epidermal cell specification (Abe et al., 2003). Strong mutant alleles of *atml1;pdf2* showed embryo-lethal phenotypes with irregular division patterns of the protoderm, whereas weak mutant alleles of *atml1;pdf2* produced a few leaves that lack an epidermis (Abe et al., 2003; San-Bento et al., 2014; Supplementary Table 1). *ATML1* homologs have been isolated in several species and most of them are preferentially expressed in the epidermis (Ito et al., 2003; Nakamura et al., 2006; Javelle et al., 2011). Several of these genes are implicated in epidermis-related functions, not only for the initial specification of surface cell fate but also for the generation of distinct cell types within the epidermis (Rerie et al., 1994; Roeder et al., 2012; Peterson et al., 2013; Takada, 2013).

HD-ZIP class IV transcription factors may function as transcriptional activators or repressors (Ohashi et al., 2003; Yu et al., 2008; Javelle et al., 2010; Depège-Fargeix et al., 2011; Peterson et al., 2013). ATML1 and PDF2 were shown to bind *in vitro* to an 8-bp sequence called the L1 box (Abe et al., 2001, 2003). Considering that an L1 box is often found in the promoters of epidermis-specific genes including *ATML1* and *PDF2*, ATML1 and PDF2 have been proposed to positively regulate the expression of epidermis-specific genes (Abe et al., 2001, 2003). In fact, expression of epidermis-related genes was decreased in *atml1;pdf2*

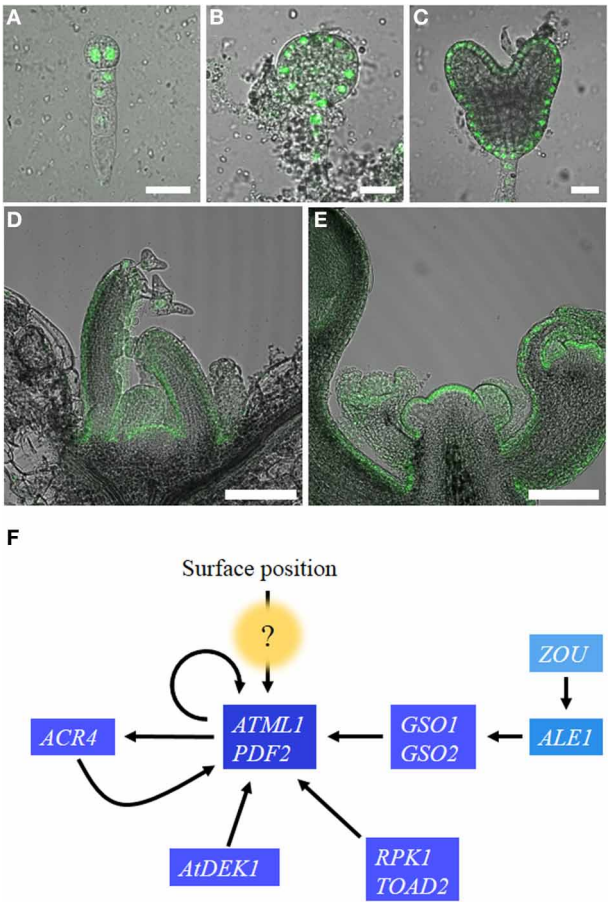

FIGURE 1 | Regulation of *ATML1* expression in the embryos and shoot apices. *ATML1* promoter activity visualized using a nuclear-localized GFP reporter in the developing embryos **(A–C)** and the shoot apices **(D,E)**. Two-cell stage **(A)**, early globular stage **(B)**, and heart stage **(C)** embryos are shown. *GFP* expression is restricted to the outermost cell layer after the 16-cell stage. Epidermis-specific expression is observed in vegetative **(D)** and inflorescence **(E)** shoot apices. **(F)** Genetic interactions among genes involved in epidermal cell fate specification during *Arabidopsis thaliana* embryogenesis. Arrows indicate positive interactions. The question mark indicates currently unknown components. For details, please see the main text. Scale bars, 20 μm in **(A–C)**; 100 μm in **(D,E)**.

(Abe et al., 2003; Takada et al., 2013). Moreover, gain-of-function experiments suggest that *ATML1* activates expression of several epidermis specific genes during the initiation of new epidermal cell fate, but may also function as a negative regulator in maintaining expression levels (Takada et al., 2013; San-Bento et al., 2014). Notably, overexpression of *ATML1* was sufficient to induce differentiation of epidermal cells such as stomata and trichomes in the inner tissues of leaves (Takada et al., 2013; Supplementary Table 1). These results are consistent with the idea that ATML1 is a master transcriptional regulator for epidermal cell specification in shoots.

Importantly, expression of *ATML1* and its putative orthologs depends on a "surface" position, irrespectively of epidermal cell identity or cell lineage, as indicated by the presence of *ATML1* promoter activity at the surface mesophyll cells of *atml1;pdf2* leaves (Takada et al., 2013). In addition, expression of *RICE OUTERMOST CELL-SPECIFIC GENE1* (*ROC1*), a rice HD-ZIP class IV gene, was induced on the cut surface during callus regeneration (Ito et al., 2002). In the maize *extra cell layers1* (*xcl1*) mutant, which develops multilayered epidermis by amplification of differentiated epidermal cells, expression of an HD-ZIP class IV gene was detected only in the outermost epidermal layer (Kessler et al., 2002). These reports suggest that identification of upstream regulators that determine the outermost cell-specific expression of *ATML1* homologs would be an effective strategy for identifying positional signals that specify shoot epidermal cell fate.

POSITIONAL SIGNALS THAT SPECIFY EPIDERMAL CELL FATE

Deletion and mutational analyses of an *ATML1* promoter revealed the involvement of several positive regulators in the protoderm-specific activation of *ATML1* (Takada and Jürgens, 2007). MicroRNAs and phytohormone auxin, two major components that are known for morphogen-like activity, appeared not to be involved in the outermost cell-specific expression of *ATML1* (Takada and Jürgens, 2007; Nodine and Bartel, 2010). Below, we discuss the candidate molecules or genes that may provide positional cues for shoot epidermal cell specification (**Figure 1F**).

CUTICLE

Cuticle is a hydrophobic lipid layer formed on the surface of the shoot epidermis. In cuticle-deficient plants, trichome numbers, stomatal density, and regular anticlinal cell division of the epidermis are impaired, suggesting that cuticular components and/or their precursors are required for the patterning of epidermis (Yephremov et al., 1999; Gray et al., 2000; Sieber et al., 2000). Cuticle can be observed in the zygote and is maintained only in the outermost cells of the embryo even before a layer of protoderm is visible (Bruck and Walker, 1985). Therefore, the presence of cuticle or cuticle biogenesis may be instructive for epidermal cell specification and/or maintenance. In fact, expression of *ROC1* was reduced in a rice mutant defective in the biosynthesis of very-long-chain fatty acids (VLCFAs), which serve as precursor of cuticular wax (Tsuda et al., 2013). Although it has been shown that *ATML1* and other HD-ZIP class IV genes positively regulate expression of cuticle biosynthesis genes and facilitate cuticle deposition, these results suggest that cuticle also functions to maintain epidermal cell identity as a positive feedback mechanism (Javelle et al., 2010; Wu et al., 2011; Takada, 2013; Takada et al., 2013). VLCFAs or its derivatives produced in the epidermis have been recently suggested to function as non-cell autonomous signals that promote cell proliferation in internal tissues (Nobusawa et al., 2013). Therefore, some intermediates or byproducts formed during cuticle biosynthesis may play roles in pattern formation in plants.

SIGNALING FROM THE ENDOSPERM

In the angiosperms, the embryo is surrounded by endosperm tissues, which provide nutrients to the developing embryo. Recent studies show that signaling from the endosperm is necessary for epidermal cell differentiation during embryogenesis. *abnormal leaf shape1* (*ale1*) and *zhoupi* (*zou*) mutants are defective in cuticle

formation in organs generated during embryogenesis (Tanaka et al., 2001; Yang et al., 2008; Supplementary Table 1). *ZOU* encodes an endosperm-specific transcriptional regulator that promotes the expression of *ALE1* and other genes required for the breakdown of the endosperm (Yang et al., 2008; Supplementary Table 1; **Figure 1F**). *ALE1* encodes a subtilisin-like serine protease, and its expression in the endosperm is sufficient to rescue cuticle-deficient phenotypes of *ale1* and *zou*, suggesting that *ALE1* non-cell-autonomously promotes epidermal cell differentiation (Tanaka et al., 2001; Xing et al., 2013; Supplementary Table 1; **Figure 1F**). Considering that subtilisin-like proteases are involved in the processing of peptide hormone precursors in animals, the simplest scenario would be that ALE1 produces ligands promoting epidermal cell specification in the endosperm and the outermost cells of the embryos that receive those ligands differentiate into the epidermis (Steiner, 1998; Tanaka et al., 2001). GASSHO1 (GSO1) and GSO2 are candidate receptor-like kinases that receive signals produced by ALE1. GSO1 and GSO2 are preferentially expressed in the embryo, and *gso1;gso2* shows severe cuticle-deficient phenotypes epistatic to those of *ale1* (Tsuwamoto et al., 2008; Xing et al., 2013; Supplementary Table 1; **Figure 1F**).

It is not certain whether ALE1 and GSO1/GSO2 are generally required for the initiation of epidermal cell fate or specifically required for the cuticle formation on the surface of the embryo. In fact, epidermal cell specification can occur in the absence of the endosperm (such as during organ regeneration from calli, somatic embryogenesis, and aerial organ initiation in post-embryonic development). Plant embryos may require ALE1 and GSO1/GSO2-mediated signaling for efficient deposition of cuticle on the surface of the protodermal cells that develop in close physical contact with surrounding endosperm cells.

CRINKLY4

crinkly4 (*cr4*) is a maize mutant with defects in the development of leaf epidermis and aleurone layer (Becraft et al., 1996). *CR4* encodes a plant-specific receptor-like kinase, and mutations in a homolog of *CR4* in *Arabidopsis* [*ARABIDOPSIS THALIANA HOMOLOGUE OF CRINKLY4* (*ACR4*)] cause phenotypes defective in epidermal cell differentiation, lateral root initiation, and root initial cell maintenance (Gifford et al., 2003; Watanabe et al., 2004; De Smet et al., 2008; Stahl et al., 2009, 2013; Supplementary Table 1). Although *acr4* shows a mild effect on epidermal cell differentiation, *acr4;ale1* double mutants show severe phenotypes with reduced *ATML1* expression in the embryo, suggesting that *ACR4* acts in parallel with the "endosperm pathway" to positively regulate epidermal cell differentiation upstream of *ATML1* (Tanaka et al., 2007; Supplementary Table 1; **Figure 1F**).

However, expression of *ACR4* is restricted to the epidermis of the embryos and shoots, and its epidermis-specific expression depends on an L1 box in the promoter, suggesting that *ACR4* is a downstream target of HD-ZIP class IV transcription factors (Tanaka et al., 2002; Gifford et al., 2003; San-Bento et al., 2014; Supplementary Table 1; **Figure 1F**). *ACR4* expression consistently began later than *ATML1* expression during embryogenesis (Tanaka et al., 2002; Gifford et al., 2003). Moreover, ATML1 and PDF2 were associated with an *ACR4* promoter *in planta*, and *ACR4* expression was reduced in *atml1;pdf2* (Abe et al., 2003; San-Bento et al., 2014).

ACR4 is localized to the basal and lateral membranes of the epidermis, suggesting that it is involved in intercellular communication between the same and different layers (Gifford et al., 2003, 2005; Watanabe et al., 2004). ACR4 and CR4 were shown to localize to plasmodesmata (PD), pores connecting plant cells, in the aleurone cells and the cotyledon epidermis (Tian et al., 2007; Stahl et al., 2013). Pore sizes of the PD connecting aleurone cells were wider than those connecting aleurone and underlying starchy endosperm cells (Tian et al., 2007). These observations may suggest that CR4 is involved in the modulation of the size of PD pores and facilitates intercellular communication between the same layer, maintaining epidermal/aleurone cell fate. Considering together, these results suggest that ACR4 is possibly more involved in the maintenance of epidermal cell fate downstream of *ATML1* than in the perception of positional signals for epidermal cell specification (**Figure 1F**).

LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASE IN THE EMBRYO

RECEPTOR-LIKE PROTEIN KINASE1 (RPK1) and TOADSTOOL2 (TOAD2) are leucine-rich repeat receptor-like kinases redundantly required for epidermal cell differentiation in the embryo (Nodine et al., 2007). *rpk1;toad2* shows embryo-lethal phenotypes with disorganized cell division patterns particularly in the basal half of the embryo proper (Nodine et al., 2007; Supplementary Table 1). *ATML1* mRNA was detected in the outer cell layer of *rpk1;toad2* at the dermatogen stage but disappeared after the early globular stage, suggesting that these receptor-like kinases are necessary for the maintenance but not for the initial specification of epidermal cell fate (Nodine et al., 2007; Supplementary Table 1; **Figure 1F**). Because of the embryonic lethality, the roles of RPK1 and TOAD2 in shoot epidermal cell differentiation in the post-embryonic development are unknown.

DEFECTIVE KERNEL 1: A CALPAIN-LIKE PROTEASE

DEFECTIVE KERNEL 1 (DEK1) is a calpain like cysteine protease that is conserved among land plants (Lid et al., 2002; Liang et al., 2013). Downregulation of *DEK1* expression in several species causes phenotypes associated with defects in epidermal differentiation such as reduced deposition of cuticle, disorganization of cell division planes, and ectopic differentiation of mesophyll plastids (chloroplasts) in the surface cells (Becraft et al., 2002; Ahn et al., 2004; Johnson et al., 2005; Tian et al., 2007; Supplementary Table 1). Strong mutant alleles of *dek1* cause embryo-lethal phenotypes in *Arabidopsis*, maize, and rice, and expression of *ATML1* homologs disappears in these embryos, implying that *DEK1* is necessary for the initiation of epidermal fate in the early embryo (Lid et al., 2002, 2005; Johnson et al., 2005; Hibara et al., 2009; Supplementary Table 1; **Figure 1F**).

DEK1 mRNA is expressed ubiquitously, suggesting that its activity is regulated post-translationally (Wang et al., 2003; Johnson et al., 2005; Lid et al., 2005; Hibara et al., 2009). DEK1 is composed of an N-terminal membrane-spanning region and a C-terminal cytosolic region that includes the calpain cysteine protease (Lid et al., 2002; Tian et al., 2007). It has been hypothesized that upon binding of epidermis-promoting ligands to the

N-terminal region, DEK1 is cleaved by its autocatalytic activity to release the C-terminal region, an active form of the calpain (Wang et al., 2003; Tian et al., 2007; Johnson et al., 2008). In animals, calpain-like proteases are involved in the activation/inactivation of several signaling molecules, suggesting that DEK1 transduces signals for epidermal specification (Storr et al., 2011). However, overexpression of active truncated forms of DEK1 in *Arabidopsis* was not sufficient to upregulate the expression of *ATML1* (Johnson et al., 2008; Supplementary Table 1). Moreover, modulation of *DEK1* activity affected cell division and growth also in internal tissues, suggesting that the action of DEK1 is not epidermis-specific (Johnson et al., 2008; Supplementary Table 1). Johnson et al. (2008) proposed that DEK1 controls mainly cell division in developing leaves and that epidermal cells respond more sensitively to the amount of DEK1; reduction in cell division rate in *dek1* may cause discontinuity and abortion of the epidermis (Johnson et al., 2008). In this scenario, DEK1 is possibly more involved in the maintenance rather than the initiation of the epidermal cell layer.

POST-TRANSCRIPTIONAL REGULATION OF HD-ZIP CLASS IV ACTIVITY

Localization of some HD-ZIP class IV transcription factors was not limited to nuclei of heterologous cells (Zhang et al., 2010; Yang et al., 2011). The HD-ZIP class IV transcription factor GLABRA2 (GL2), a positive regulator of trichome formation, was restricted to the nuclei only in trichome cells and not in internal cells, suggesting a cell-type-specific regulation of nuclear transport (Szymanski et al., 1998). HD-ZIP class IV transcription factors contain a putative lipid/sterol binding domain (START) and a dimerization motif (ZLZ), implying a regulation of their activities by dimerization and binding of lipid/sterol ligands (Schrick et al., 2004). In fact, sterol and VLCFA biosynthesis-deficient mutants are defective in proper distribution of stomata and trichomes, respectively, (Yephremov et al., 1999; Qian et al., 2013). However, to date the roles of START domains have not been investigated, except for an observation showing that an N-terminal part of a START domain can function as a transcriptional activation domain in yeast and maize suspension cells (Depège-Fargeix et al., 2011).

ATML1 was shown to heterodimerize with PDF2 *in planta* and it is possible that dimerization with other HD-ZIP class IV proteins changes the activity of ATML1 in a cell-type dependent manner, although ectopic expression of *ATML1* alone was sufficient to induce epidermal cell fate in inner tissues (Takada et al., 2013; San-Bento et al., 2014). In related transcription factors, DNA-binding was inhibited by oxidation of Cys residues in the ZLZ motif, suggesting that redox signals are also involved in the regulation of ATML1 activity (Tron et al., 2002).

REPRESSION OF INNER CELL FATE

Several lines of evidence show that acquisition of epidermal cell fate is associated with a loss of mesophyll or internal cell fate. First, epidermis-deficient *atml1;pdf2* and *DEK1* knockdown lines showed ectopic differentiation of mesophyll cells on the surfaces of leaves and cotyledons, respectively, (Abe et al., 2003; Johnson et al., 2005). Second, *rpk1;toad2* embryos exhibited ectopic subepidermal marker expression in the outermost cell layer (Nodine et al., 2007; Supplementary Table 1). Third, overexpression of *ATML1* decreased differentiation of green mesophyll cells in leaves (Takada et al., 2013). Moreover, *cr4* and *dek1*, which are defective in "surface" aleurone layer differentiation in the maize endosperm, cause ectopic differentiation of "inner" starchy endosperm cells on the surface of the endosperm (Becraft et al., 1996; Becraft and Asuncion-Crabb, 2000). Therefore, repression of internal or "default" cell fate may be a general requirement for surface cell differentiation in plants. It is not possible, however, to test whether or not mesophyll cell differentiation represses epidermal cell fate because no positive regulators of inner cell fate are available at present. Chloroplast development itself appears not to exert a negative effect on epidermal cell differentiation, considering that stomatal guard cells possess chloroplasts and no ectopic epidermal cell differentiation has been reported in the mesophyll tissues of albino plants (Stewart and Dermen, 1975).

Mesophyll cells possibly represent a primitive state of leaf cells, considering that ancestral aquatic algae are composed mainly of mesophyll-like cells. Land plants may have repressed mesophyll cell differentiation to evolve an epidermis on the surface. Evolutionary studies, including comparative genomics, may be useful for identifying molecular components that promote epidermal cell formation (Zalewski et al., 2013).

FUTURE PERSPECTIVES

Despite the extensive molecular genetic studies in model plants, positional signals that specify shoot epidermal cell fate remain unknown (**Figure 1F**). Most of the receptor-like kinases, characterized by their roles in shoot epidermal cell differentiation, are possibly involved in the maintenance than in the specification of epidermal cell fate. This appearance may be because of the difficulties in distinguishing between phenotypes associated with "specification" and those related to "maintenance" of epidermal cell fate in forward genetic screens.

The cuticle-bearing outermost cells should have distinct mechanical properties compared with inner cells. Moreover, cells located at the surface are unique, in that they are in constant contact with the environment. These unique properties could influence the differentiation of epidermal cells. Attempts to directly isolate epidermis-promoting biomolecules and to identify physical/environmental constraints influencing epidermal cell fate may shed new light on the issue.

AUTHOR CONTRIBUTIONS

Shinobu Takada wrote the main manuscript text and Hiroyuki Iida prepared **Figure 1** and Supplementary Table 1. All authors reviewed the manuscript.

ACKNOWLEDGMENTS

This work was supported by the grants from the Japan Society for the Promotion of Science [20657012, 22687003, and 23657036 to Shinobu Takada].

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00049/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 December 2013; paper pending published: 19 January 2014; accepted: 01 February 2014; published online: 25 February 2014.

*Citation: Takada S and Iida H (2014) Specification of epidermal cell fate in plant shoots. Front. Plant Sci. **5**:49. doi: 10.3389/fpls.2014.00049*

This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 05 June 2014
doi: 10.3389/fpls.2014.00259

An overview of the gene regulatory network controlling trichome development in the model plant, *Arabidopsis*

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Trichomes are specialized epidermal cells located on aerial parts of plants and are associated with a wide array of biological processes. Trichomes protect plants from adverse conditions including UV light and herbivore attack and are also an important source of a number of phytochemicals. The simple unicellular trichomes of *Arabidopsis* serve as an excellent model to study molecular mechanism of cell differentiation and pattern formation in plants. The emerging picture suggests that the developmental process is controlled by a transcriptional network involving three major groups of transcription factors (TFs): the R2R3 MYB, basic helix-loop-helix (bHLH), and WD40 repeat (WDR) protein. These regulatory proteins form a trimeric activator complex that positively regulates trichome development. The single repeat R3 MYBs act as negative regulators of trichome development. They compete with the R2R3 MYBs to bind the bHLH factor and form a repressor complex. In addition to activator–repressor mechanism, a depletion mechanism may operate in parallel during trichome development. In this mechanism, the bHLH factor traps the WDR protein which results in depletion of WDR protein in neighboring cells. Consequently, the cells with high levels of bHLH and WDR proteins are developed into trichomes. A group of C2H2 zinc finger TFs has also been implicated in trichome development. Phytohormones, including gibberellins and jasmonic acid, play significant roles in this developmental process. Recently, microRNAs have been shown to be involved in trichome development. Furthermore, it has been demonstrated that the activities of the key regulatory proteins involved in trichome development are controlled by the 26S/ubiquitin proteasome system (UPS), highlighting the complexity of the regulatory network controlling this developmental process. To complement several excellent recent relevant reviews, this review focuses on the transcriptional network and hormonal interplay controlling trichome development in *Arabidopsis*.

Keywords: gene regulation, microRNA, ubiquitin/26S proteasome, trichome

INTRODUCTION

Trichomes are epidermal protuberances that, depending on species, are located on the aerial parts of plants such as the leaves, stems, petioles, petals, and seed coat. They are generally classified into two types: simple, or non-glandular, and glandular secreting trichomes (GSTs; Wagner et al., 2004). Trichomes play an important role in plant growth and development by protecting them from UV light, insect predation, and excess transpiration. The phytochemicals secreted by GSTs provide protection against pathogens and pests, and also attract pollinators (Wagner, 1991; Wagner et al., 2004; Schilmiller et al., 2008). GSTs can also be considered "chemical factories" as they synthesize and secrete many economically important compounds (Schilmiller et al., 2008). The unicellular non-glandular trichomes of *Arabidopsis* serve as an excellent experimental system to study molecular mechanism of cell differentiation and pattern formation in plants.

The production and distribution of trichomes is spatially and temporally controlled. During the early vegetative phase, trichomes are present only on the adaxial side of the rosette leaves, whereas they are found on both adaxial and abaxial surfaces in the adult vegetative phase. During the reproductive stage, the trichome number gradually decreases on the main inflorescence stem and flowers (Telfer et al., 1997). Trichomes originate from the protodermal cells of the developing leaf primordia. The protodermal cells destined to become trichomes cease to divide and enter an endoreduplication cycle in which DNA replication continues in the absence of nuclear and cellular division. The mature trichome has a stalk with two to three branches and an average DNA content of 32C, suggesting that trichome cells undergo at least four rounds of endoreduplication during development (Hulskamp et al., 1994; Schnittger and Hulskamp, 2002). Over 30 different genes are known to control the developmental processes (Schellmann and Hulskamp, 2005). Extensive genetic and molecular analyses suggest that a network of transcription factors (TFs), belonging to three major groups: the R2R3 MYBs, the basic helix-loop-helix (bHLH) factors, and the WD40 repeat (WDR) protein, plays a crucial role in trichome development. These three groups of TFs form a trimeric activator complex, MYB-bHLH-WDR (MBW) that positively regulates the expression of downstream targets, which, in turn, induces trichome formation. The single repeat R3 MYBs act as negative regulators of trichome development. They compete with the R2R3 MYB to bind the bHLH factors and form

a repressor complex (Serna and Martin, 2006; Ishida et al., 2008; Yang and Ye, 2013; Wang and Chen, 2014). Additionally, a group of C2H2 zinc finger TFs has been implicated in trichome formation on inflorescence stems and flowers in *Arabidopsis* (Gan et al., 2006, 2007a; Zhou et al., 2011, 2013).

Phytohormones, including gibberellins (GA), cytokinins(CK), and jasmonic acids (JA), are involved in numerous developmental processes in plants. These phytohormones also play a crucial role in trichome development in *Arabidopsis* (Chien and Sussex, 1996; Perazza et al., 1998; Dill and Sun, 2001; Traw and Bergelson, 2003; Gan et al., 2007a,b; Maes et al., 2008; Yoshida et al., 2009; Qi et al., 2011, 2014). In *Arabidopsis*, microRNAs have been shown to be involved in trichome development by controlling the expression of key regulatory genes (Yu et al., 2010). Recently, a posttranslational control mechanism has been implicated in trichome development (Patra et al., 2013a,b). Here, we focus on transcriptional regulatory network involved in the development of *Arabidopsis* trichomes. Furthermore, we discuss the influence of different phytohormones and their interactions on gene expression affecting trichome formation. The role of miRNA and 26S/ubiquitin proteasome system (UPS) in trichome development is also briefly discussed.

TRANSCRIPTION FACTOR COMPLEX IN TRICHOME DEVELOPMENT

In *Arabidopsis*, trichome development is regulated by a transcriptional network involving several groups of TFs, namely, the MYB, bHLH, WDR, and C2H2 zinc finger proteins. The R2R3 MYB family in *Arabidopsis* is comprised of 126 members. Based on the conservation of the DNA binding MYB domain and the amino acid motifs in C-terminal domain, the R2R3 MYBs are divided into 25 sub-groups (Stracke et al., 2001; Dubos et al., 2010). The R2R3 MYBs belonging to subgroup 15, MYB0/GLABROUS1 (GL1) and AtMYB23/MYB23, are involved in trichome development (Oppenheimer et al., 1991; Kirik et al., 2001). These two proteins are functionally equivalent during trichome initiation but not during trichome branching (Kirik et al., 2005). Recently, a newly characterized R2R3MYB, AtMYB82, that does not belong to sub-group 15, has also been shown to regulate trichome development (Liang et al., 2014). Expression of *AtMYB82* under the control of the *GL1* promoter complements the trichome defect of the *gl1* mutant, suggesting that both GL1 and AtMYB2 are functionally equivalent.

The bHLH TF family is one of the largest known groups of TFs in *Arabidopsis* with more than 160 members divided into 12 subgroups (Heim et al., 2003). GLABROUS3 (GL3) and ENHANCER OF GLABROUS3 (EGL3), members of subgroup IIIf, are involved in trichome development in a partially redundant manner (Payne et al., 2000; Zhang et al., 2003). Mutation in the *GL3* locus results in fewer trichomes and reduced branching. The nuclei in *gl3-1* mutants undergo three, rather than four, rounds of endoreduplication, and this correlates with reduced trichome branching observed in this mutant. The *EGL3* locus has a moderate effect on trichome number. However, *gl3 egl3* double mutants have a glabrous phenotype. In addition to GL3 and EGL3, TRANSPARENT TESTA 8 (TT8) and AtMYC1, other members of the bHLH subgroup IIIf, have also been shown to affect trichome development (Maes et al., 2008; Symonds et al., 2011; Zhao et al., 2012). TT8 controls anthocyanin and proanthocyanidin (PA) biosynthesis in vegetative tissues and the seed coat (Nesi et al., 2000; Baudry et al., 2004). Maes et al. (2008) have demonstrated that TT8 also controls trichome development on leaf margins in *Arabidopsis*. *AtMYC1* mutants have less trichomes, compared with the wild type, indicating AtMYC1 acts as a positive regulator of trichome initiation (Zhao et al., 2012).

WD40 repeat proteins contain highly conserved 40–43 amino acid tandem repeats usually ending with Trp-Asp (WD). They are involved in the regulation of a number of processes, including cell cycle, cell fate determination, and cell signaling (Neer et al., 1994). In *Arabidopsis*, the WDR gene, *TRANSPARENT TESTA GLABRA 1* (*TTG1*), is a single copy gene (Walker et al., 1999). The *ttg1* mutant has pleiotropic phenotype, which is glabrous and deficient in anthocyanin accumulation (Walker et al., 1999).

In addition to the R2R3 MYBs, a group of seven R3 MYBs, that include TRIPTYCHON (TRY; Schnittger et al., 1999; Schellmann et al., 2002), CAPRICE (CPC; Wada et al., 1997), ENHANCER OF TRY, and CPC 1 (ETC1, ETC2 and ETC3; Kirik et al., 2004a,b; Wester et al., 2009), and TRICHOMELESS 1 (TCL1 and TCL2; Wang et al., 2007; Gan et al., 2011), are also involved in trichome development. Analyses of loss-of-function mutants reveal that these R3 MYBs act as negative regulators. Loss-of-function mutation in *CPC* causes increased trichome density (Schellmann et al., 2002) whereas mutation in *TRY* results in a trichome clustering phenotype (Schnittger et al., 1999; Schellmann et al., 2002). Mutation in *ETC1* does not dramatically affect the trichome phenotype whereas mutation in *ETC2* or *ETC3* results in increased trichome numbers. The higher order *ETC* mutants (*etc1 etc3* and *etc1 etc2 etc3*) exhibit significantly higher numbers of trichome compared to the respective single or double mutants, suggesting a redundant function by these regulators in trichome development (Kirik et al., 2004a,b; Wester et al., 2009). Loss-of-function mutations in the *TCL1* or *TCL2* locus result in ectopic trichome formation on inflorescence stems and pedicels (Wang et al., 2007; Gan et al., 2011). The number of trichomes on inflorescence stems and pedicels increase significantly in the *cpc tcl1* double mutant. The higher order *cpc etc1 etc3 tcl1* quadruple mutant, exhibits more trichomes on internodes and pedicels, compared to the *tcl1* or *cpc tcl1* double mutant, suggesting a role of CPC, ETC1 and ETC3 in trichome formation on inflorescence stems and pedicels (Wang et al., 2007, 2008).

The MYB (GL1)-bHLH (GL3/EGL3)-WDR (TTG1) proteins form a trimeric MBW complex that activates the expression of the homeodomain protein, GLABROUS2 (GL2; Rerie et al., 1994), which, in turn, induces trichome formation. GL3 contains three different protein-protein interaction domains: the N-terminal MYB-interacting region (amino acid 1-97) that interacts with GL1/CPC/TRY, the middle portion that includes the transactivation domain (amino acid 212-401) interacting with TTG1, and the C-terminal bHLH and ACT-like domain (amino acid 400-637) that homo/heterodimerize (Payne et al., 2000; Zhang et al., 2003). Recent studies demonstrate that the C-terminal domain of GL3/EGL3 also interacts with a number of factors involved in phytohormone signaling and protein degradation (Qi et al.,

2011, 2014; Patra et al., 2013b). The MYB TFs contain a conserved amino acid signature motif, $[D/E]Lx_2[R/K]x_3Lx_6Lx_3R$, that is crucial for interaction with the bHLH proteins (Zimmermann et al., 2004). Physical interaction between GL1 and TTG1 has not been demonstrated. These findings suggest that the bHLH factors act as docking sites for a number of regulatory proteins, including the MYB and WDR proteins in the MBW complex. AtMYC1, like GL3/EGL3, has also been shown to interact with GL1 and TTG1, but does not dimerize. An arginine (Arg173) residue in AtMYC1 is found to be critical for its interaction with GL1. This Arg residue is conserved in GL3 and EGL3, and is essential for interaction with MYB partners (Zhao et al., 2012). The R3 MYBs typically lack the activation domain and compete with the R2R3 MYB, GL1, to bind GL3/EGL3, and form a repressor complex, thereby affecting the expression of downstream targets. There is a marked difference in the ability of these single repeat MYBs to disrupt the GL1-GL3 interaction. Using yeast three hybrid assay, CPC has been demonstrated as the most potent inhibitor of this activation complex, followed by ETC1, TRY, ETC2, and ETC3 (Wester et al., 2009). In a protoplast assay, TCL1 has been shown to be stronger than CPC in binding to GL3 (Wang et al., 2008). Using YFP-tagged CPC and ETC3, it has been demonstrated that these R3MYBs move from cell-to-cell and the strong binding of CPC to GL3 affects the mobility of CPC (Wester et al., 2009). Although the cell-to-cell movement signature motif (WxM) is conserved in all R3MYBs (Wang and Chen, 2014), the movement of TCL1 has yet to be experimentally demonstrated. Whether the strong interaction of TCL1 with GL3 affects its movement and biological activity still remains to be elucidated.

TRICHOMELESS 1 binds the *GL1* promoter in a chromatin immunoprecipitation (ChIP) assay and negatively regulates *GL1* expression (Wang et al., 2007). TCL1 probably acts as a negative regulator of trichome development by affecting the expression of *GL1*, as well as competing with GL1 for binding to GL3. Although the expression of most single repeat MYBs appears to be regulated by the MBW complex (Morohashi et al., 2007; Morohashi and Grotewold, 2009), it is unclear whether MBW also controls *TCL1* (Wang et al., 2007; Wang and Chen, 2014).

The C2H2 zinc finger proteins constitute one of the largest families of regulatory proteins, with 176 members in *Arabidopsis*, and are involved in numerous developmental and physiological processes in plants (Englbrecht et al., 2004). GLABROUS INFLORESCENCE STEMS (GIS), GIS2, ZINC FINGER PROTEIN 8 (ZFP8; Gan et al., 2006, 2007a), ZFP5 (Zhou et al., 2011, 2012), and ZFP6 (Zhou et al., 2013), which encode C2H2 zinc finger TFs, are involved in trichome formation in inflorescence stems and floral organs. Gene expression analyses of knockout mutants reveal a transcriptional hierarchy with ZFP6 acting upstream of ZFP5 which regulates the expression of *GIS*, *GIS2,* and *ZFP8*. GL1 and GL3, key TFs in the MBW complex, function further downstream of GIS2 and ZFP8 (Zhou et al., 2013).

Collectively, these findings suggest that a regulatory loop involving a group of activators and repressors fine tunes the expression of downstream gene targets and ultimately trichome formation. The activator complex (GL3/EGL3-GL1-TTG1) induces the expression of genes encoding the repressors (TRY/CPC) which can move into neighboring cells to form a repressor complex (GL3/EGL3-CPC/TRY-TTG1) and inhibit function of the activators. In addition to an activator–repressor mechanism, a depletion mechanism has also been proposed to operate, in parallel, during trichome development (Bouyer et al., 2008; Balkunde et al., 2011). In the depletion process, GL3 traps TTG1, resulting in depletion of TTG1 protein in neighboring cells. Consequently, cells with high levels of GL3 and TTG1 proteins are developed into trichomes. The depletion model is derived from the following findings: (a) TTG1 protein moves between cells, (b) TTG1 protein is preferentially accumulated in trichome initials and depleted in surrounding cells, and (c) depletion of TTG1 protein in neighboring cells, and its accumulation in trichome initials, is lost in the *gl3* mutant. Supporting this model, Balkunde et al. (2011) show that GL3 controls TTG1 movement, and interaction between GL3 and TTG1 is necessary for intracellular movement and epidermal distribution.

PHYTOHORMONES AND TRICHOME DEVELOPMENT

Phytohormones, including GA, JA, and CK, play pivotal roles in controlling a wide array of biological processes in plants. Accumulating evidences suggest that these phytohormones are also crucial in trichome development. Here, we discuss the influence of different phytohormones and underlying molecular mechanisms, which control trichome formation in *Arabidopsis*.

Gibberellin is known to regulate a number of developmental processes in plants including seed germination, hypocotyl elongation, flowering, and trichome development. Evidence for the involvement of GA in trichome development comes from the analyses of several mutants in GA biosynthesis and signaling pathways in *Arabidopsis*. The GA biosynthesis mutant *ga1-3* has completely glabrous leaves, and application of exogenous GA to these plants restores trichome development. Additionally, GA stimulates the expression of *GL1*, and relative to wild type plants, the *ga1-3* mutant contains less *GL1* transcripts (Perazza et al., 1998). *SPINDLY* (*SPY*) is a repressor of GA signaling in *Arabidopsis*. Mutation in the *SPY* locus results in increased trichome formation (Chien and Sussex, 1996; Perazza et al., 1998). The *Arabidopsis* DELLA proteins are inhibitors of GA signaling and encoded by a family of five genes: *GIBBERELLIC ACID INSENSITIVE* (*GAI*), *REPRESSOR OF ga1-3* (*RGA*), and three *RGA-LIKE* genes (*RGL1*, *RGL2,* and *RGL3*). Among the five DELLA proteins, RGA, and GAI play significant roles in trichome formation. Mutations in *RGA* and *GAI* restore trichome initiation in the *ga1-3* mutant (Dill and Sun, 2001). Consistent with this, the expressions of several trichome regulators, including *GL1* and *GL3*, are up-regulated in *DELLA*-defective *ga1-3* mutants, whereas conditional over-expression of *RGA-GR* (RGA fused to rat glucocorticoid receptor) in these mutants reduces the expression of these trichome regulators (Gan et al., 2007b). Maes et al. (2008) have demonstrated that GA stimulates trichome formation through up-regulation of key TF genes. Expression of *GL1*, *MYB23*, *GL3* and *EGL3* are induced, whereas expression of *TRY*, *ETC1,* and *ETC2* are reduced, in response to GA treatment. *TTG1* expression is not significantly affected following GA treatment (Maes et al., 2008). Taken together, these findings suggest that GA regulates trichome formation by modulating the expression

of key regulatory genes. These conclusions are further substantiated by the elegant demonstrations that individual TFs in the MBW complex are direct targets of DELLA proteins (Qi et al., 2014). RGA or RGL2 physically interact with GL3, EGL3, and GL1 to repress the transcriptional function of the MBW complex. Furthermore, analysis of the DELLA mutant, *penta* (*gai-t6 rga-t2 rgl1-1 rgl2-1* wild type for *RGL3*), *gl3 egl3* double mutant, and *penta gl3 egl3* mutants, suggest that the MBW complex acts downstream of DELLA proteins. Based on these findings, it is proposed that upon perception of the GA signal, protein–protein interactions between DELLA-GL1/GL3/EGL3 are disrupted as DELLA proteins are recruited to the SCF^{SLY} complex, and subsequently degraded by the 26S proteasome system. GL3, EGL3, and GL1 are consequently released to form a complex with TTG1 and activate the expression of GL2, which, in turn, mediates trichome formation.

Recent studies suggest that TFs other than MYB and bHLH proteins, also operate in the GA signaling pathway to regulate trichome development in inflorescence stems and flowers. The expression of C2H2 zinc finger TF genes, *GIS, GIS2, ZFP8, ZFP5, and ZFP6*, is stimulated in response to exogenous application of GA (Gan et al., 2006, 2007a,b; Zhou et al., 2013).

Jasmonic acid and its derivatives, collectively known as jasmonates (JAs), act as key signaling molecules that regulate numerous developmental processes in plants. JA biosynthesis is triggered in response to a variety of signals including wounding. Traw and Bergelson (2003) have shown that mechanical wounding and JA significantly induce trichome development in plants. The *Arabidopsis aos* mutant, deficient in JA biosynthesis due to a knockout mutation in the *ALLENE OXIDE SYNTHASE* (*AOS*) gene, produces less trichomes compared to wild type plants and this defect can be rescued by JA treatment (Yoshida et al., 2009). Maes et al. (2008) have also demonstrated that JA stimulates trichome development by modulating the expression of several regulatory genes. In *Arabidopsis*, the F-box protein CORONATINE INSENTIVE1 (COI1), along with ASK1/ASK2, Cullin1, and Rbx1, form the SCF^{COI} complex that mediates JA signal transduction (Xie et al., 1998; Devoto et al., 2002). JA induction of trichome formation is attenuated in the *coi1-2* mutant, which is defective in JA signaling and produces less trichomes compared with wild type plants (Qi et al., 2011). Collectively, these findings show that JA plays an important role in trichome formation. Recent findings by Qi et al. (2011) elucidate the molecular mechanism underlying this process. They demonstrate that the MBW complex is involved in JA-induced trichome development in *Arabidopsis*. The expression of *GL3* and *GL1* is significantly induced in response to JA treatment in wild type plant, but severely weakened in the *coi1-2* mutant. Moreover, GL3 and GL1 physically interact with the JA-ZIM domain (JAZ) proteins in yeast, as well as plant cells. The JAZ proteins are known negative regulators of JA signaling. They are recruited to the SCF^{COI} complex upon perception of the JA signal and are subsequently degraded by the 26S proteasome system (Chini et al., 2007; Thines et al., 2007). These observations suggest that, in the absence of JA, the JAZ proteins bind to GL3, EGL3, and GL1 and inhibit the formation of the MBW complex. Upon perception of the JA signal, the JAZ proteins are degraded by the 26S proteasome system, thereby releasing GL3, EGL3, and GL1 to form the complex with TTG1, and activate the downstream targets to promote trichome formation.

Cytokinin (6-benzylaminopurine, BAP) acts as a positive regulator of trichome development in *Arabidopsis* (Maes et al., 2008). Plants treated with BAP produce more trichomes per leaf; however, the trichomes are shorter and nuclear DNA content is less than in untreated plants, indicating that BAP affects endoreduplication in trichomes. The expression of *GL1*, *MYB23*, *GL3*, and *EGL3*, is also stimulated following BAP treatment. The expression of *GIS2*, *ZFP5*, *ZFP8*, and *ZFP6*, that regulate trichome formation on inflorescence stems, is also influenced by cytokinins (Gan et al., 2007a; Zhou et al., 2013).

Trichome formation is also affected by brassinosteroids (BR), ethylene (ET), and salicylic acid (SA). The *Arabidopsis bls1* mutant, impaired in BR response, develops fewer trichomes on both abaxial and adaxial surfaces of the leaf, indicating a possible involvement of BR in trichome development (Laxmi et al., 2004). The ET receptor mutant *etr2-3*, has completely unbranched trichomes. Through epistatic and gene expression analysis, it has been shown that the ET signaling cascade involves CHROMATIN ASSEMBLY FACTOR 1 (CAF1) and TRY to control trichome branching, and is independent of the GL3, GL2 pathway (Plett et al., 2009). Application of SA significantly reduced the number and density of trichomes in different cultivars of *Arabidopsis*, indicating its negative regulatory role in trichome development (Traw and Bergelson, 2003).

PHYTOHOMONE CROSS-TALK AND TRICHOME DEVELOPMENT

Phytohormones are known to act synergistically as well as antagonistically to regulate different developmental processes in plants. The antagonistic action of GA and JA regulate hypocotyl elongation, root growth, and flowering (Hou et al., 2010, 2013; Yang et al., 2012), whereas their synergistic action regulates stamen development and trichome formation (Traw and Bergelson, 2003; Song et al., 2013; Qi et al., 2014). Trichome density and number are significantly higher in plants treated with a combination of GA and JA compared with plants treated with only JA. Consistent with this, the expression of *GL2* and *MYB23* are found to be significantly up-regulated by combined treatment of GA and JA. Moreover, the GA biosynthetic inhibitor, paclobutrazol, represses JA-induced trichome formation and expression of *GL2* and *MYB23*. JA-induced trichome formation is also attenuated in the GA biosynthesis mutant *ga1-3*. Similarly, the *coi1-1* mutant, defective in JA signaling, inhibits GA-induced trichome formation. Recent studies by Qi et al. (2014) reveal the molecular mechanism underlying the GA-JA synergy in trichome development. Both JAZ and RGA (DELLA protein) bind to trichome regulators, GL3, EGL3, and GL1. GA and/or JA signals control the level of these repressor proteins via 26S proteasome-dependent proteolysis and maintain the stable transcription of the activators that induce trichome formation.

The positive regulatory role of GA and CK on trichome development is well documented. A recently identified C2H2 zinc finger TF, ZFP6, seems to function as an integrative hub of GA and CK signals in promoting trichome formation in *Arabidopsis*. *ZFP6*

expression is induced in response to GA and CK treatment. Moreover, GA- and CK-induced expression of the downstream targets of ZFP6, *ZFP5*, *ZFP8,* and *GL1*, is significantly affected in *zfp6* mutant (Zhou et al., 2013).

The negative cross-talk between JA and SA-dependent pathways in *Arabidopsis* is well documented. These phytohormones act antagonistically to regulate trichome development. *Arabidopsis* plants treated with a combination of JA and SA produce lower numbers of trichomes compared with plants treated with JA alone (Traw and Bergelson, 2003).

microRNA AND TRICHOME DEVELOPMENT

microRNAs (miRNAs) are small endogenous non-coding RNAs of 20–22 nt in length and present in plants, animals, and protozoa. miRNAs modulate the expression of their target genes at the posttranscriptional level and thus control many aspects of cellular functions (Voinnet, 2009; Fabian et al., 2010). Recent studies indicate that miRNAs regulate trichome development by modulating the expression of *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes (Yu et al., 2010). SPLs are a group of plant-specific TFs that share a highly conserved SBP DNA-binding domain first identified in a protein that binds to the promoter of the *SQUAMOSA* gene of *Antirrhinum majus* (Cardon et al., 1999). SPLs regulate numerous fundamental aspects of plant growth and development, including phase transition, trichome distribution and flowering (Chen et al., 2010). In *Arabidopsis*, SPLs are negative regulators of trichome development in inflorescence stem and floral organs. The *SPL* gene family has 17 members, 10 of which are targeted by miRNA156 (Rhoades et al., 2002). Expression of miRNA156 and *SPLs* (*SPL3*/*SPL9*) are temporally regulated (Wu and Poethig, 2006; Yu et al., 2010). miRNA156 levels are highest in young plants and decline as the plant ages. On the contrary, expression of *SPLs*, targets of miRNA156, is low in young plants and increase gradually during the reproductive stage. The expression pattern fits well with the gradual loss of trichomes on stem and floral organs. Consistent with this, plants expressing a mimicry target of miR156 accumulate significant *SPL* transcripts and show a reduction in trichome density on stems. Similar results are found when miR156-resistant forms of *SPL3*, *SPL10*, *SPL13,* and *SPL9* are over-expressed in plants. By comparison, transgenic *Arabidopsis* plants constitutively expressing miRNA156 produce ectopic trichomes on stem and floral organs. Moreover, SPL9 directly binds to the promoters of *TCL1* and *TRY*, negative regulators of trichome development, and activates their expression in a GL1-independent manner, leading to reduced trichome formation (Yu et al., 2010). Together, these observations suggest that the temporal control of trichome development in *Arabidopsis* is regulated by the miR156-targeted SPL TFs.

UBIQUITIN/26S PROTEASOME SYSTEM AND TRICHOME DEVELOPMENT

The 26S proteasome is a multi-subunit ATP-dependent protease complex assembled from two particles: the 20S core particle (CP) and the 19S regulatory particle (RP; Zwickl et al., 1999). The UPS-dependent proteolysis is the most elaborate and complex regulatory mechanism controlling activities of short-lived proteins in eukaryotes. Over 1300 genes in the *Arabidopsis* genome are associated with the 26S proteasome pathway (Vierstra, 2003). Loss-of-function mutations in one of the RP components, RPT2a, result in several physiological abnormalities including aberrant trichome development. The *rpt2a* mutant has larger trichomes with increased branch number. Additionally, trichomes of *rpt2a* plants have larger nuclei compared with the wild type, suggesting RPT2a is involved in regulation of endoreduplication in trichomes (Sonoda et al., 2009).

26S/ubiquitin proteasome system comprises E1, E2, and E3 enzymes that act coordinately to conjugate ubiquitin moieties to the target proteins and pave the way for subsequent degradation by the 26S proteasome. E3 enzymes determine substrate specificity by recognizing a single or small group of proteins and in plants, are divided into two subgroups, RING/U boxes and HECT ubiquitin ligases (Smalle and Vierstra, 2004). In *Arabidopsis*, mutation in the UPL3/KAK locus, which encodes a HECT domain E3 ligase, results in trichomes with increased branch number and higher nuclear DNA content, suggesting that UPL3 regulates of ploidy level in trichomes by controlling the activities of proteins that normally promote the endoreduplication cycle (Downes et al., 2003; El Refy et al., 2003). GL3 is a potential target of UPL3 because it is a positive regulator of endoreduplication in trichomes, and the supernumerary trichomes in *upl3*/*kak* seedlings is suppressed in *kak gl3* double mutant background (Sako et al., 2010). Our recent demonstration, that GL3 and EGL3 are short-lived proteins, supports the hypothesis that GL3/EGL3 are targets of UPL3. UPL3 physically interacts with GL3 and EGL3, and mediates degradation via UPS (Patra et al., 2013b). Additionally, we have also shown that TTG1 and TT8, the regulator of trichome formation on leaf margin, are targets of UPS. However, the specific E3 ligase that mediates this degradation remains to be identified (Patra et al., 2013a). Endoreduplication cycles in *Arabidopsis* trichomes are also controlled by another class of ubiquitin ligases, the RBX1-containing Cullin-RING E3 ubiquitin ligases (CRLs). Of five known cullin genes, *CUL1*, *CUL3A,* and *CUL4* are strongly expressed in young trichomes. *CUL1* and *CUL3A* loss-of-function mutants are phenotypically indistinguishable from wild type. However, a knock-down line of *CUL4* produces small trichomes with less nuclear DNA content. The CUL4-CRL complex modulates cyclin-dependent kinase (CDK) activity presumably by mediating the degradation of a class of CDK inhibitors during endoreduplication cycles (Roodbarkelari et al., 2010).

CONCLUDING REMARKS

During the past decade, substantial and significant progress has been made in delineating the elaborate gene regulatory network that controls trichome development in *Arabidopsis*. Multiple lines of evidence suggest that a number of transcriptional activators and repressors act in concert to fine tune the spatial and temporal distribution of trichomes. Additionally, phytohormones such as JA, GA, and CK, act synergistically or antagonistically to modulate the expression of genes encoding these regulators. Recent findings, pertaining to miRNAs and 26S/UPS-dependent regulation of TFs in trichome development, highlight the complexity of the regulatory network. Whether other regulators such as GL1/MYB23 and R3 MYBs are targets of miRNA and/or 26S/UPS remains to be elucidated.

Our current knowledge about the gene regulatory network is largely limited to the unicellular non-glandular trichomes in the model plant, *Arabidopsis*. Very little is known about the regulatory network that controls the development of glandular secretory trichomes. This type of trichomes is found in many plants, including tobacco, tomato, basil, and mint, and is thus an important source of phytochemicals. Over-expression of *GL1* in tobacco does not augment trichome formation, suggesting that different regulatory mechanisms control trichome development in *Arabidopsis* and tobacco (Payne et al., 1999). Recently, an integrated genomic database, TrichOME (www.planttrichome.org), has been developed. The database hosts more than a million EST sequences from both trichome and corresponding non-trichome tissues from 13 species, including tobacco, basil, and mint, and provides a potential source for genes involved in development of glandular and non-glandular trichomes (Dai et al., 2010). Understanding the regulatory network that controls the development of multicellular trichomes will aid our efforts to engineer trichomes to produce commercially important phytochemicals.

ACKNOWLEDGMENTS

Research in the Ling Yuan lab is supported by grants from the US Department of Energy (DE-AR0000204) and the Kentucky Tobacco Research and Development Center.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 April 2014; paper pending published: 05 May 2014; accepted: 21 May 2014; published online: 05 June 2014.

*Citation: Pattanaik S, Patra B, Singh SK and Yuan L (2014) An overview of the gene regulatory network controlling trichome development in the model plant, Arabidopsis. Front. Plant Sci. **5**:259. doi: 10.3389/fpls.2014.00259*

This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 03 July 2014
doi: 10.3389/fpls.2014.00320

Molecular basis of natural variation and environmental control of trichome patterning

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Trichomes are differentiated epidermal cells on above ground organs of nearly all land plants. They play important protective roles as structural defenses upon biotic attacks such as herbivory, oviposition and fungal infections, and against abiotic stressors such as drought, heat, freezing, excess of light, and UV radiation. The pattern and density of trichomes is highly variable within natural population suggesting tradeoffs between traits positively affecting fitness such as resistance and the costs of trichome production. The spatial distribution of trichomes is regulated through a combination of endogenous developmental programs and external signals. This review summarizes the current understanding on the molecular basis of the natural variation and the role of phytohormones and environmental stimuli on trichome patterning.

Keywords: trichome development, natural variation, QTL, GWAS, abiotic, biotic, defense

INTRODUCTION

Plants have evolved sophisticated morphological and chemical systems to cope with biotic and abiotic challenges. Differentiated epidermal cells such as leave hairs or trichomes represent one of these systems. Trichomes develop on above ground organs including seeds and fruits and occur in a large variety of morphologies. They can be single-celled or multicellular, branched or unbranched, and glandular or non-glandular, characteristics often used for species identification (Luckwill, 1943; Beilstein et al., 2006, 2008). Trichomes have a range of protective functions however producing them is costly, depend on plant resource availability and can have negative impacts on plant growth and vigor (Wilkens et al., 1996). Therefore trichome production often underlies qualitative and quantitative variation in most plant species.

TRICHOMES AND ABIOTIC FACTORS

In adverse environments, trichomes are beneficial because they influence the water balance, protect photosynthesis and reduce photoinhibition. Their density is negatively correlated with the rate of transpiration (Choinski and Wise, 1999; Benz and Martin, 2006) and that of carbon dioxide diffusion (Ehleringer et al., 1976; Galmés et al., 2007). The pubescent nature of plants growing in extreme alpine and Mediterranean habitats correlates with trichome's ability to protect the underlying tissue from sunlight by increasing the reflectance and reducing the heat load. Many plants accumulate also UV-absorbing compounds such as flavonols in trichomes which further protect the underlying photosynthetic tissues from damaging amount of UV-A and UV-B radiations (Karabourniotis et al., 1995, 1998; Karabourniotis and Bornman, 1999; Tattini et al., 2000, 2007; Morales et al., 2002; Liakopoulos et al., 2006; Yan et al., 2012). Evidence that trichomes are structural adaptations to low temperature and enhance tolerance to freezing came from studies on birch where frost increased rapidly the density of glandular trichomes (Prozherina et al., 2003). Some heavy metal tolerant plants accumulate heavy metals in trichomes serving for detoxification purposes (Choi et al., 2001, 2004; Marmiroli et al., 2004; Freeman et al., 2006; Harada and Choi, 2008; Sarret et al., 2009; Quinn et al., 2010). Heavy metal loaded trichomes might contribute to elemental defense strategies (Boyd, 2012; Cheruiyot et al., 2013; Kazemi-Dinan et al., 2014).

TRICHOMES AND BIOTIC CHALLENGES

Many studies show that trichomes serve as physical barrier against biotic stressors such as insects, herbivores, fungal infections, and even parasitic plants (Peiffer et al., 2009; Runyon et al., 2010; Tian et al., 2012). Solanaceous species such as tomato and related species produce a variety of trichomes. The long multicellular type I trichomes that have a small glandular vesicle at the tip on hypocotyls for example effectively hinder the infection of tomato (*Solanum lycopersicum*) with its parasite *Cuscuta pentagona* (Runyon et al., 2010). Recent studies in several tomato wild relatives found that the presence, density, longevity and size of type I and the shorter multicellular type IV glandular trichomes correlates with resistance against whitefly (Firdaus et al., 2012, 2013). Oviposition and feeding experiments with the specialist moth *Plutella xylostella* on different *Arabidopsis thaliana* accessions showed that oviposition varied significantly among populations and could partly be explained by a negative relationship between trichome density and egg number, and a positive relationship between plant size and egg number (Handley et al., 2005). Experiment with glabrous and hairy *Arabidopsis lyrata* morphs and larvae of *Plutella xylostella* show that trichomes increased resistance to leaf damage and reduced oviposition in adult plants (Sletvold et al., 2010). However, in young plants that develop fewer trichomes this effect was not significant (Puentes and Ågren, 2013). The larvae of the crucifer-feeding beetle, *Phaedon brassicae,* grew slower on hairy leaves of *Arabidopsis halleri*. Hairy leaves were less damaged when

glabrous leaves were abundant in free choice experiments (Sato et al., 2014).

While non-glandular trichomes can be seen as structural defenses, glandular trichomes are also a source of highly interesting biomolecules (Shepherd et al., 2005; Liu et al., 2006; Kang et al., 2010). Apart from the above mentioned flavonols, glandular trichomes synthesize and/or store other highly valuable secondary metabolites such as terpenoids, phenylpropenes, methyl ketones (Fridman et al., 2005; Ben-Israel et al., 2009), acyl sugars (Schilmiller et al., 2012; Stout et al., 2012; Xu et al., 2013), and proteinase inhibitors (Tian et al., 2012) and thus contribute to the chemical repertoire of defense strategies. Given that trichomes provide both structural and chemical defense systems against herbivores and pathogens they are appealing targets for breeding (Gruber et al., 2006; Glas et al., 2012).

Controversial is the effect of trichomes for fungal infections: While damaged trichomes are often the starting point for colonialization with powdery mildew (*Erysiphe necator*) on grapevine buds (Rumbolz and Gubler, 2005), *Botrytis cinerea* on harvested tomato (Charles et al., 2008), *Phoma clematidina* on clematis (Van De Graaf et al., 2002) or *Beauveria bassiana* on poppy (Landa et al., 2013), glandular trichomes are often able to secrete exudates with antifungal activity as shown in a wild potato species (*Solanum berthaultii*) and its resistance to *Phytophthora infestans* (Lai et al., 2000). The disease incidence correlated negatively with the density and polyphenol-oxidase activity of short type A trichomes that have a four-lobed membrane-bound gland at their tips (Lai et al., 2000). In the infection of chickpea (*Cicer arietinum*) with *Ascochyta rabiei* the concentration of a highly acidic trichome exudate is crucial. At low concentrations the exudate promotes germination of *Ascochyta rabiei* conidia while at high concentrations germination is inhibited (Armstrong-Cho and Gossen, 2005). Also glandular trichomes of tobacco (*Nicotiana tabacum*) produce a potent inhibitor, T-phylloplanin, which inhibits germination of the oomycete *Peronospora tabacina* (Kroumova et al., 2007). The effect of trichomes is specific for the resistance to fungi. For example, while in *Arabidopsis thaliana* the infection with the soil-borne pathogen *Rhizoctonia solani* is not affected by trichome density, *gl1* mutants were more resistant and the *try* mutant with clustered trichomes had an enhance colonialization with *Botrytis cinerea* (Calo et al., 2006). However, *Arabidopsis thaliana* transgenes expressing the antifungal α-1,3-glucanase of *Trichoderma harzianum* in trichomes were more resistant to *Botrytis cinerea* demonstrating that trichomes can be engineered to increase resistance to fungal pathogens (Calo et al., 2006).

REGULATION OF TRICHOME DENSITY IN *Arabidopsis* AND OTHER BRASSICACEAE

Classical molecular genetic approaches of the model plant *Arabidopsis thaliana* identified major regulators of trichome development on leaves, stems and petiols. They fall into two classes: positive (mutants develop less trichomes) and negative regulators (mutants develop more and/or clusters of trichomes; for reviews see Balkunde et al., 2010). The positive regulators belong to three protein classes: a WD40 protein TRANSPARENT TESTA GLABRA1 (TGG1; Galway et al., 1994; Walker et al., 1999), three R2R3 MYB-related transcription factors GLABRA1 (GL1, MYB23, MYB5; Oppenheimer et al., 1991; Kirik et al., 2005; Song et al., 2009; Tominaga-Wada et al., 2012) and four basic helix-loop-helix (bHLH)-like transcription factors GLABRA3 (GL3; Payne et al., 2000), ENHANCER OF GLABRA3 (EGL3), TRANSPARENT TESTA (TT8; Zhang et al., 2003), and MYC-1 (Zhao et al., 2012). They act partially redundantly and form a multimeric activator complex, also known as MYB-bHLH-WD40 (MBW) complex which binds the promoter of *GLABRA2 (GL2)*. *GL2* encodes a homeodomain protein required for subsequent phases of trichome morphogenesis such as endoreduplication, branching, and maturation of the cell wall. The negative regulators are seven partially redundant single-repeat MYBs such as CAPRICE (CPC), TRIPTYCHON (TRY), ENHANCER OF TRY AND CPC 1, 2, 3 (ETC1, ETC2, ETC3), and TRICHOMELESS1 and 2 (TCL1, TCL2; Wester et al., 2009; Edgar et al., 2014; Wang and Chen, 2014). For most of them it has been shown that they act in a non-cell-autonomous manner. The single-repeat MYBs lack the C-terminal activation domain and inhibit the activator complex by replacing the R2R3 MYB-related transcription factors and thereby suppress trichome initiation in adjacent cells. While some of the positive and negative regulators are specific for trichome patterning others are also involved in root hair development, anthocyanin biosynthesis (Nemie-Feyissa et al., 2014), and seed coat mucilage production (Zhang et al., 2003; Song et al., 2009).

HETEROBLASTY AND HORMONAL CONTROL OF TRICHOME DENSITY

Trichome density is developmentally regulated. For example, *Arabidopsis* rosette leaves have trichomes only on the adaxial side and the number increases with the age of plants so that early leaves develop fewer and later more trichomes. On the other hand cauline leaves develop mainly abaxial and lack adaxial trichomes. This heteroblasty varies in different accessions (Larkin et al., 1996; Telfer et al., 1997; Gan et al., 2006; Hilscher et al., 2009) and is influenced by the photoperiod (Chien and Sussex, 1996).

Moreover hormones such as gibberellin (GA) promote trichome initiation and morphogenesis (Telfer et al., 1997; Perazza et al., 1998; Gan et al., 2006) by inducing *GL1* expression. The original observation was that the GA biosynthesis mutant, *ga1-3,* develops less adaxial trichomes on leaves (Chien and Sussex, 1996) and application of GA restored and induces trichome production. Furthermore GA regulates also later stages in trichome development since mutants of the SPINDLY repressor of GA signaling not only develop glabrous sepals but also over-branched leaf trichomes (Perazza et al., 1998; Silverstone et al., 2007). For the effect of GA on trichome initiation on inflorescence organs four redundantly acting C2H2 transcription factors have been identified: GLABROUS INFLORESCENCE STEMS (GIS, GIS2), ZINC FINGER PROTEIN 8 and 5 (ZFP8, ZFP5). They act upstream of GL1 and are involved in the action of cytokinin on trichome initiation (Gan et al., 2006, 2007a,b; Zhou et al., 2013).

As mentioned above trichomes can be induced by wounding and insect attack (Larkin et al., 1996; Yoshida et al., 2009) and the plant hormones involved in signaling these stresses

are jasmonic acid (JA) and salicylic acid (Traw and Bergelson, 2003). Recently is has been shown that the JA receptor, CORONATINE-INSENSITVE1 (COI1), is involved in JA induced trichome production in tomato and *Arabidopsis* (Li et al., 2004; Qi et al., 2011) and that several repressors of JA signaling, JAZ1, 2, 5, 6, 8, 9, 10, 11 are able to interact with components of the activator complex such as EGL3, GL3, TT8, MYB75, GL1 (Qi et al., 2011).

The positive effect of JA on trichome production is antagonized by salicylic acid. Reduced trichome development was observed after salicylic acid treatment or on mutants with elevated salicylic acid levels such as the *CONSTITUTIVE EXPRESSION OF PR GENE* (*cpr)* mutants (Bowling et al., 1997; Traw and Bergelson, 2003; An et al., 2011).

NATURAL VARIATIONS AS SOURCE OF NOVEL TRICHOME REGULATORS

Molecular analyses of natural variations of morphological and developmental traits have been a powerful approach to identify new genes important for adaptation to different environments (Assmann, 2013). For example the analyses of natural sequence variations of *GL1* show that in particular the 3′ end is responsible for the glabrous phenotype of the *Arabidopsis thaliana* accession Mir-0, Br-0, Fran-3, PHW-2, 9354, Wil-2, Est as well as for hairless *Arabidopsis lyrata, Arabidopsis halleri, Brassica rapa, Brassica oleracea, Brassica napus,* and radish (*Raphanus sativus)* lines (Hauser et al., 2001; Kärkkäinen and Ågren, 2002; Kawagoe et al., 2011; Li et al., 2011, 2013; Bloomer et al., 2012). Larkin et al. (1993) has experimentally tested the importance of the non-coding 3′

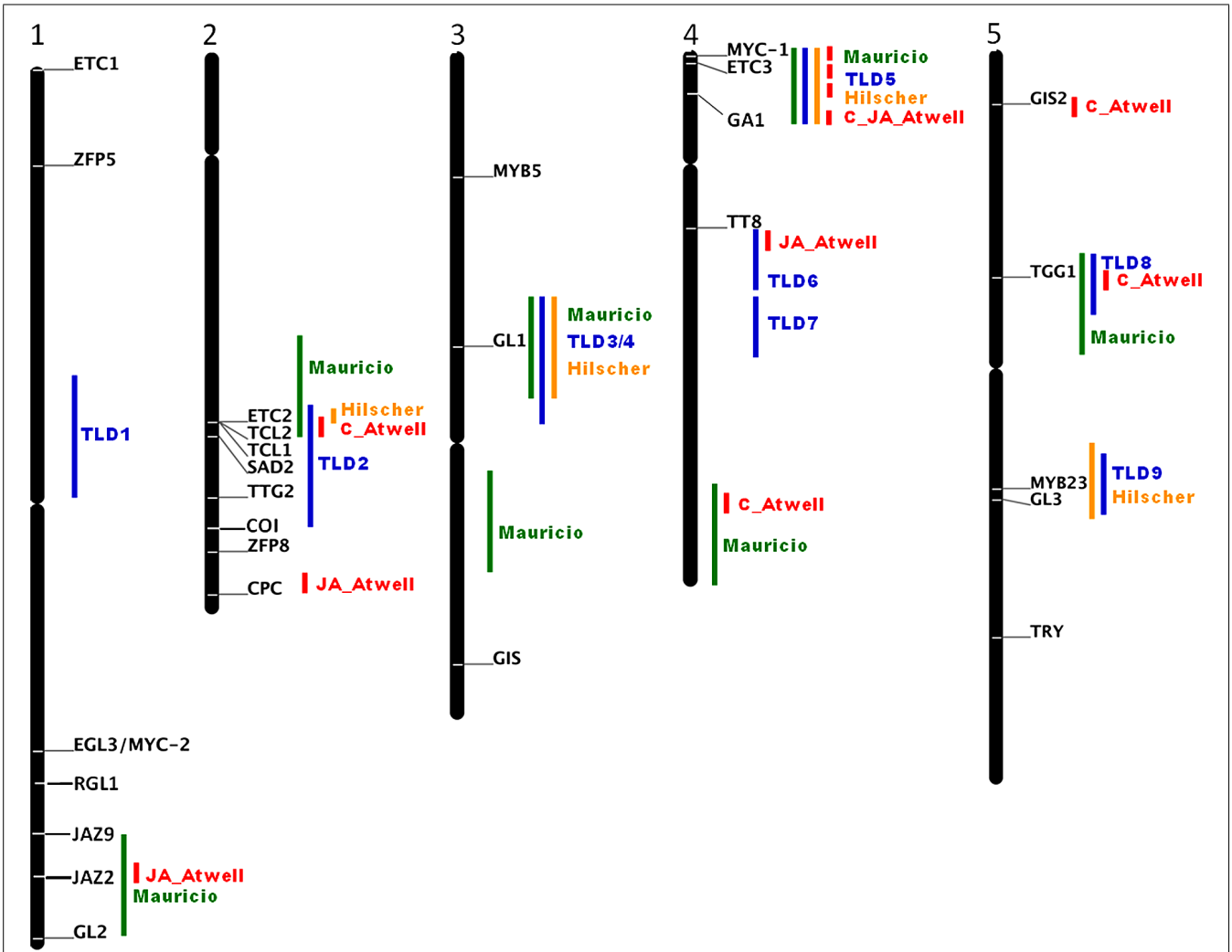

FIGURE 1 | Schematic representation of the loci influencing trichome density and number on rosette leaves from QTL analyses with different accessions (green; Mauricio, 2005), (blue; Symonds et al., 2005), (orange; Hilscher et al., 2009), and GWAS (red; Atwell et al., 2010). Indicated are the candidate genes that have been shown to influence trichome pattering and as bars the position of the QTL and GWAS loci. For GWAS only regions are indicated that contain the 200 most significant SNPs. AT3G27920 *GL1*; AT5G40330 *MYB23*; AT3G13540 *MYB5*; At5g41315 *GL3*; At1g63650 *EGL3/MYC-2*; At4g00480 *MYC-1*; At5g24520 *TGG1*; At2g37260 *TTG2*; At1g79840 *GL2*; AT2G30420 *ETC2*; AT2G30424 *TCL2*; AT2G30432 *TCL1*; AT2G46410 *CPC*; AT5G53200 *TRY*; AT1G01380 *ETC1*; AT4G01060 *ETC3*; AT3G58070 *GIS*; AT5G06650 *GIS2*; AT2G41940 *ZFP8*; AT2G31660 *SAD2*; AT1G10480 *ZFP5*; AT4G09820 *TT8*; AT1G66350 RGL1; AT1G70700 JAZ9; AT1G74950 JAZ2; AT2G39940 COI.

end and postulated that an enhancer downstream of the coding region is essential for the precise expression and function of *GL1* in *Arabidopsis*. However, major phenotypic variations are rarely the effect of only one gene and its natural alleles, more frequently phenotypic variations in natural accessions depend on several partially interacting loci with quite small contributions. Their analysis needs statistical approaches such as quantitative trait locus (QTL) mapping. The first QTL analysis discovered a major locus, named *REDUCED TRICHOME NUMBER (RTN)* in *Arabidopsis thaliana* and used recombinant inbred lines (RIL) derived from a cross between the low trichome density Ler and the medium density Col-accessions (Larkin et al., 1996). This locus was identified in all further QTL analyses with combination of different accessions (Mauricio, 2005; Symonds et al., 2005; Bloomer et al., 2014) and even in a genome wide association study (GWAS; Atwell et al., 2010) as major regulator of trichome density. Hilscher et al. (2009) finally revealed *RTN* as *ETC2* and the K19E amino acid substitution to be responsible for low trichome densities in natural *Arabidopsis thaliana* accessions. However, *ETC2* is not the only gene responsible for trichome density variations. Mauricio (2005) and Symonds et al. (2005) identified each nine QTLs for trichome density in four recombinant inbred mapping populations of *Arabidopsis thaliana*. Most of the identified QTLs regions contain or are in close proximity of known trichome initiation regulators such as *GL2, ETC2, TCL2, TCL1, SENSITIVE TO ABA AND DROUGHT2* (*SAD2*; Gao et al., 2008), *TTG2, CPC, GL1, MYC-1, ETC3, GA1, TT8, GIS, TTG1, MYB23* and *GL3*. For *MYC-1* a non-synonymous substitution was identified in few accession which however did not correlate with trichome density (Symonds et al., 2011). While the sequence variation of *ETC2, TCL2, TCL1,* and *GL1* have been studied the other candidate genes as well as genomic regions without candidate genes such as the loci TLD1 on chromosome 1, TLD6 and TLD7 in the middle of chromosome 3 and others identified with GWAS await closer examinations (**Figure 1**). With the availability of the 1001 *Arabidopsis* genomes association studies of the remaining candidate genes are now straight forward (Ossowski et al., 2008; Cao et al., 2011).

OUTLOOK AND POTENTIAL OF UNDERSTANDING THE BASIS OF NATURAL VARIATIONS AND ENVIRONMENTAL INFLUENCES ON TRICHOME DENSITY REGULATION

Although the major players of trichome density regulation have been identified in the model plant *Arabidopsis* they are still not sufficient to explain all the naturally occurring variations in this plants species. Great potential for the identification of still missing regulators will come from next generation sequencing possibilities in combination with classical genetic, population genetic and comparative approaches using different plant species. There are already several examples where trichome patterning regulators from wild relatives or even crops and distantly related species such as cotton, tomato and hop have been successfully and functionally studied in the model plant *Arabidopsis* (Wang et al., 2004; Guan et al., 2011; Kocábek and Matoušek, 2013; Tominaga-Wada et al., 2013). However, there are further needs for research determining the molecular basis of the patterning of different types of glandular trichomes and in particular of pharmaceutical and agronomically interesting plant species. Since trichomes serve as morphological, and in cases of glandular trichomes as chemical defense barriers against many abiotic stresses and biotic attacks, increasing their density has great potential to improve broad-spectrum pest and pathogen resistance in crops.

ACKNOWLEDGMENTS

Funding for this work was provided by grants from the Austrian Science Fund (FWF). The author thanks the anonymous reviewers for helpful comments.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 May 2014; paper pending published: 06 June 2014; accepted: 17 June 2014; published online: 03 July 2014.

Citation: Hauser M-T (2014) Molecular basis of natural variation and environmental control of trichome patterning. Front. Plant Sci. ***5****:320. doi: 10.3389/fpls.2014.00320*

This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 17 February 2014
doi: 10.3389/fpls.2014.00047

Regulation of epidermal cell fate in Arabidopsis roots: the importance of multiple feedback loops

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The specification of distinct cell types in multicellular organisms is accomplished via establishment of differential gene expression. A major question is the nature of the mechanisms that establish this differential expression in time and space. In plants, the formation of the hair and non-hair cell types in the root epidermis has been used as a model to understand regulation of cell specification. Recent findings show surprising complexity in the number and the types of regulatory interactions between the multiple transcription factor genes/proteins influencing root epidermis cell fate. Here, we describe this regulatory network and the importance of the multiple feedback loops for its establishment and maintenance.

Keywords: root hairs, transcription factors, pattern formation, feedback loops, *Arabidopsis thaliana*

EPIDERMAL CELL PATTERNING IN THE ARABIDOPSIS ROOT

The specification of root hair cells and non-hair cells in the Arabidopsis root is a well-studied model for understanding cell fate decisions in plants (Schiefelbein et al., 2009; Tominaga-Wada et al., 2011; Grebe, 2012). Newly formed epidermal cells located outside the cleft separating two adjacent underlying cortical cells (the "H" cell position) differentiate into root-hair cells, whereas epidermal cells not located over the cleft (the "N" cell position) develop into non-hair cells, due to differential cell-type-specific gene expression (**Figure 1**) (Cormack, 1962; Berger et al., 1998; Bruex et al., 2012). Genetic and molecular studies over the past 20 years have now provided a fairly clear picture of the transcriptional regulators responsible for establishing this differential cell-type gene expression. What has been surprising is the large number of regulatory mechanisms and interactions by these transcription factors in the process of root epidermal cell specification. In this mini-review, we describe the basic transcription factor components and then we outline the many categories of regulatory mechanisms and their roles in establishing the epidermal cell fates.

THE BASIC COMPONENTS OF THE NETWORK

At its core, cell fate in the root epidermis is dependent on the relative abundance of a transcription factor complex consisting of three types of proteins: the Myb-domain protein WEREWOLF (WER) (Lee and Schiefelbein, 1999), two redundantly acting bHLHs GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Payne et al., 2000; Bernhardt et al., 2003, 2005; Simon et al., 2013), and the WD-repeat TRANSPARENT TESTA GLABRA (TTG1) (Galway et al., 1994). Differentiating epidermal cells that generate a significant amount of this WER-bHLH-TTG complex express the downstream HD-ZIP gene *GLABRA2* (*GL2*), which represses transcription of the hair-cell promoting bHLH *ROOT HAIR DEFECTIVE 6* (*RHD6*) (Masucci and Schiefelbein, 1994; Menand et al., 2007), leading to the expression of dozens of non-hair-cell-specific genes and the non-hair cell fate (**Figure 1**) (Masucci et al., 1996; Bruex et al., 2012). Differentiating cells that do not accumulate a significant amount of the WER-bHLH-TTG complex are able to express *RHD6*, and as a result, initiate transcription of hundreds of root-hair-cell-specific genes (Cvrckova et al., 2010; Bruex et al., 2012). Given the key role of the WER-bHLH-TTG transcriptional complex for the cell fate decision, there has been great interest in defining and understanding the mechanisms that regulate its accumulation in the two cell types. Recent research has uncovered an array of intra- and intercellular mechanisms responsible for controlling the abundance of this key complex.

REGULATORY MECHANISMS IN THE NETWORK

LATERAL INHIBITION

The activity of the WER-bHLH-TTG complex is inhibited by a set of small, one-repeat Myb proteins, which includes CAPRICE (CPC), TRIPTYCHON (TRY), and ENHANCER OF TRY AND CPC1 (ETC1) (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004; Simon et al., 2007). These proteins are able to bind to the GL3/EGL3 bHLHs, competitively inhibiting WER binding and generating a non-functional complex (Lee and Schiefelbein, 2002). Accordingly, these proteins accumulate in the H-position cells, where they promote the hair cell fate (Kurata et al., 2005; Kang et al., 2013). Unexpectedly, the transcription of these one-repeat Mybs was found to occur predominately in the N cells, due to positive regulation by the WER-bHLH-TTG complex itself, and the proteins appear to move through plasmodesmata to accumulate in the H cells (Kurata et al., 2005; Kang et al., 2013). The ability of cells adopting the non-hair fate (accumulating WER-bHLH-TTG) to generate diffusible molecules (CPC/TRY/ETC1) that prevent adjacent cells from adopting the same fate (via inhibition of the WER-bHLH-TTG action) effectively represents a kind of lateral inhibition mechanism, a general strategy widely employed by multicellular organisms to establish distinct cell

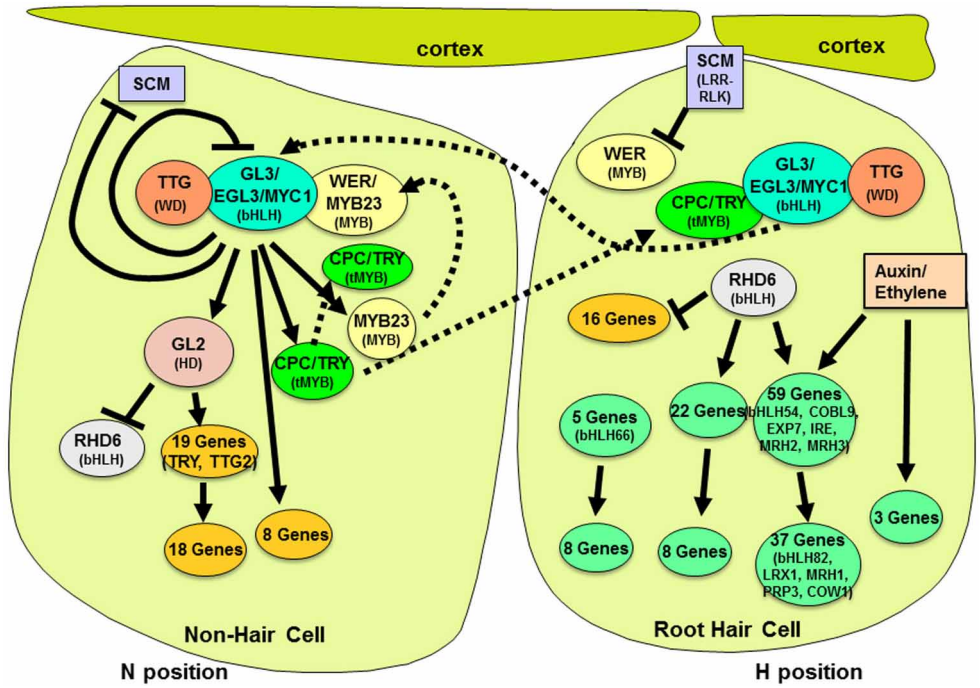

FIGURE 1 | Molecular genetic regulation of cell fate specification in the Arabidopsis root epidermis. Root-hair specific gene expression occurs in differentiating epidermal cells located in a cleft between adjacent cortical cells (right-most cell). Gene regulatory activity is indicated by solid lines with arrows (positive transcriptional effect) or bars (negative transcriptional effect). Dotted lines represent the movement of proteins within or between cells. The downstream genes shown here are taken from Bruex et al. (2012) and defined as hair or non-hair genes based on their accumulation in the root epidermis in either *cpc try* (non-hair) mutants vs. *ttg1*, *wer myb23*, or *gl3 egl3* (hairy) mutants.

identities in an initially equivalent field of cells (Meinhardt and Gierer, 2000). What is unusual about the lateral inhibition used here is its direct nature; the molecule produced by the inhibiting cell is used as both the signal and the inhibitor of the recipient cell.

FEEDBACK AT MULTIPLE DEVELOPMENTAL TIMES

Although the *CPC*, *TRY*, and *ETC1* genes are all positively regulated by the WER-bHLH-TTG complex, the effect on *TRY* is indirect because it is downstream of the N-cell regulator GL2 (**Figure 1**) (Simon et al., 2007). This means that TRY production will be developmentally delayed, relative to CPC and ETC1. Since the CPC/ETC1 and TRY proteins are members of different subtypes and appear to vary in their properties (Pesch and Hulskamp, 2011), this regulatory organization may generate different ratios of subtypes during epidermis development important for pattern establishment.

POSITIVE FEEDBACK

The MYB23 protein is the Arabidopsis MYB most closely related to WER, and MYB23 is capable of substituting for WER in root hair development (Kang et al., 2009). Further, the *MYB23* gene is under the positive transcriptional regulatory control of the WER-bHLH-TTG complex. Taken together, these data indicate that the *MYB23* gene participates in a positive feedback loop in the N cells (**Figure 1**), which apparently is used to maintain relatively high levels of the complex, due to MYB23's presumed participation in the complex (Kang et al., 2009). The identification of a positive feedback loop affecting the WER-bHLH-TTG complex was satisfying, because theoretical models of lateral inhibition in pattern formation typically require such self-promoting loops to create stable peaks of activator accumulation even in the presence of high inhibitor levels (Meinhardt and Gierer, 1974, 2000). Accordingly, in the root epidermal system, mutants lacking *MYB23* function are less able to adopt appropriate cell fate decisions (Kang et al., 2009).

MUTUAL REINFORCING LOOPS

The *GL3* and *EGL3* bHLH genes were found to be preferentially transcribed, and to have their transcripts preferentially accumulate, in the H cells rather than the N cells of the developing root epidermis, due to negative transcriptional regulation of these genes by the WER-bHLH-TTG complex (Bernhardt et al., 2005). This was an unexpected finding, since the GL3 and EGL3 proteins are required for the proper function and differentiation of the N cells. Based on the use of GFP-tagged proteins, the GL3/EGL3 gene products were found to preferentially accumulate in the N cells, implying movement of these proteins from H to N cells (**Figure 1**). The opposite movement of the hair-promoting CPC/TRY/ETC1 (N to H cells) vs. the non-hair-promoting GL3/EGL3 (H to N cells) has been proposed to represent an intercellular mutual reinforcing loop that exists to provide robustness to the patterning system (**Figure 1**) and this view has received support from theoretical

modeling studies (Savage et al., 2008; Benitez and Alvarez-Buylla, 2010).

MOLECULAR TRAPPING

The observed preferential accumulation of CPC (and presumably TRY and ETC1) in the H cells is believed to be necessary for robust pattern formation, though the mechanism responsible for causing these mobile factors to accumulate in H cells has long been a mystery. A possible explanation has recently been provided by the finding that this accumulation is EGL3 dependent (Kang et al., 2013). A CPC-GFP fusion protein, expressed under control of the *CPC* or *SHORTROOT* (*SHR*) promoter, lacked preferential H-cell accumulation in the *gl3 egl3* mutant and exhibited reduced movement in GL3/EGL3 overexpression lines (Kang et al., 2013). One possibility is that EGL3 serves to "trap" the CPC inside the H cells by relatively strong binding (Kang et al., 2013). This is at least superficially similar to the proposed nuclear trapping of TTG1 by GL3 in the trichome specification system (Balkunde et al., 2011).

FEEDBACK ON POSITIONAL SIGNALING

The position-dependent pattern of hair and non-hair cells is dependent on signaling through the SCRAMBLED (SCM) receptor-like kinase (Kwak and Schiefelbein, 2007). SCM action leads to reduced *WER* transcription, and since this appears to preferentially occur in the H cell position, it explains how SCM signaling helps generate the cell-type pattern. Interestingly, the preferential SCM action in the H cells is likely due to differential accumulation of SCM. This differential SCM accumulation is achieved by a negative feedback loop between the WER-bLHLH-TTG complex and the *SCM* gene expression, ensuring reduced SCM signaling in the N position (Kwak and Schiefelbein, 2008). It is proposed that this mechanism helps to "lock in" the cell fate decision, by amplifying the differential SCM signaling ability of the two cell types.

REGULATION BY HORMONES

Root hair development is affected by several plant hormones, most commonly reported for auxin and ethylene (Masucci and Schiefelbein, 1996; Pitts et al., 1998). In general, the hormones appear to promote root hair formation, because addition of exogenous hormone typically leads to increased hair length or number whereas inhibition of hormone production/action tends to reduce hair length or number. Indeed, transcriptome assays show that a majority of the downstream root hair genes, but not the non-hair genes, are responsive to addition of auxin (IAA) or ethylene (ACC) (Bruex et al., 2012). Thus, the accumulation and activity of hormone pathway elements provide the opportunity for regulation of root-hair pattern at a relatively late stage, perhaps allowing for environmental influence of root hair formation.

REGULATION BY HISTONE MODIFICATION

The expression of the patterning genes and the arrangement of the root cell types are influenced by histone acetylation. Treatment of roots with trichostatin A (histone deacetylase inhibitor) or mutations in the histone deacetylase gene *HDA18* cause N-position cells to become root hair cells (Xu et al., 2005). Because the HDA18 protein does not directly bind to the patterning gene promoters (Liu et al., 2013), there may be an intermediate set of histone-regulated genes responsible for this level of control.

THOUGHTS ON THE COMPLEXITY OF THE NETWORK

In this minireview, we have highlighted the multitude of regulatory mechanisms that are employed to control the relative abundance of the critical transcription factors in epidermal cell specification. Considering these many components and interactions (**Figure 1**), it is appropriate to wonder why this system has evolved such complexity to control a seemingly simple case of cell fate specification. One possible explanation is that the complex regulatory interactions reflect a requirement for robustness; to ensure that once a cell fate decision is made, that this decision is fully adopted and is not allowed to be altered at any step (Barkai and Leibler, 1997; Benitez and Alvarez-Buylla, 2010). Another possibility for the existence of multiple regulatory mechanisms may be that they provide opportunities for the modification/adjustment of the cell fate decision at many points in the process, perhaps enabling it to respond to the many known internal and external factors that influence root hair development (Forde and Lorenzo, 2001; Datta et al., 2011). Future studies on the control of root epidermal cell fate in Arabidopsis and other species will likely yield additional insight into the importance of the many components and interactions in this complex regulatory network.

ACKNOWLEDGMENTS

We thank members of our research laboratory for helpful discussions. We apologize to those whose work could not be cited due to space constraints. Research in our group is supported by grants from the National Science Foundation (IOS-0723493 and IOS-1121602).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 December 2013; paper pending published: 14 January 2014; accepted: 30 January 2014; published online: 17 February 2014.

Citation: Schiefelbein J, Huang L and Zheng X (2014) Regulation of epidermal cell fate in Arabidopsis roots: the importance of multiple feedback loops. Front. Plant Sci. ***5****:47. doi: 10.3389/fpls.2014.00047*

This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 13 March 2014
doi: 10.3389/fpls.2014.00091

Regulation of root hair cell differentiation by R3 MYB transcription factors in tomato and *Arabidopsis*

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CAPRICE (*CPC*) encodes a small protein with an R3 MYB motif and regulates root hair and trichome cell differentiation in *Arabidopsis thaliana.* Six additional CPC-like MYB proteins including TRIPTYCHON (TRY), ENHANCER OF TRY AND CPC1 (ETC1), ENHANCER OF TRY AND CPC2 (ETC2), ENHANCER OF TRY AND CPC3/CPC-LIKE MYB3 (ETC3/CPL3), TRICHOMELESS1 (TCL1), and TRICHOMELESS2/CPC-LIKE MYB4 (TCL2/CPL4) also have the ability to regulate root hair and/or trichome cell differentiation in *Arabidopsis*. In this review, we describe our latest findings on how CPC-like MYB transcription factors regulate root hair cell differentiation. Recently, we identified the tomato *SlTRY* gene as an ortholog of the *Arabidopsis TRY* gene. Transgenic *Arabidopsis* plants harboring *SlTRY* produced more root hairs, a phenotype similar to that of *35S*::*CPC* transgenic plants. CPC is also known to be involved in anthocyanin biosynthesis. Anthocyanin accumulation was repressed in the *SlTRY* transgenic plants, suggesting that *SlTRY* can also influence anthocyanin biosynthesis. We concluded that tomato and *Arabidopsis* partially use similar transcription factors for root hair cell differentiation, and that a CPC-like R3 MYB may be a key common regulator of plant root-hair development.

Keywords: *Arabidopsis*, MYB, root-hair, tomato, transcription factors

BRIEF BACKGROUND

Cell fate determination is a critical step in plant development. In growing roots, epidermal cells differentiate into two cell types, root-hair cells, and non-hair cells in a file-specific manner. In *Arabidopsis* roots, epidermal cells in eight symmetrically positioned files differentiate into root-hair cells, and the cells of the other files become non-hair cells. Morphological analysis has shown the positional relationship between cortical cells and epidermal cells. Epidermal cells in contact with the junction of two underlying cortical cells differentiate into root-hair cells, whereas the cells in contact with only one cortical cell differentiate into non-hair cells (Dolan et al., 1993, 1994; Galway et al., 1994; Berger et al., 1998). Several regulatory factors are involved in root-hair or non-hair cell differentiation. The *glabra 2* (*gl2*) and *werewolf* (*wer*) mutants convert non-hair cells to root hair cells (Masucci et al., 1996; Lee and Schiefelbein, 1999). The *GL2* gene encodes a homeodomain leucine-zipper protein, and the *WER* gene encodes an R2R3-type MYB transcription factor that activates *GL2* expression preferentially in differentiating non-hair cells (Rerie et al., 1994; Di Cristina et al., 1996; Masucci et al., 1996; Lee and Schiefelbein, 1999). *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*) encode basic helix-loop-helix (bHLH) transcription factors that affect non-hair cell differentiation in a redundant manner, as evidenced by the conversion of non-hair cells to root-hair cells in the *gl3 egl3* double mutant (Bernhardt et al., 2003). Although, obvious increase in the number of root-hair cells was hardly observed in both *gl3* and *egl3* single mutants (Bernhardt et al., 2003). The *TRANSPARENT TESTA GLABRA1* (*TTG1*) gene is also involved in non-hair cell fate determination, as shown by the conversion of non-hair cells to root-hair cells in the *ttg1* mutant (Galway et al., 1994). The *TTG1* gene encodes a WD40-repeat protein (Walker et al., 1999). GL3 and EGL3 interact with WER (Bernhardt et al., 2003) and with TTG1 (Payne et al., 2000; Esch et al., 2003; Zhang et al., 2003) in yeast cells. A protein complex including WER, GL3/EGL3, and TTG1 acts upstream of the *GL2* gene in the root-hair regulatory pathway and promotes *GL2* gene expression (Galway et al., 1994; Rerie et al., 1994; Wada et al., 1997; Hung et al., 1998; Lee and Schiefelbein, 1999; Bernhardt et al., 2003, 2005). The cells expressing *GL2* differentiate into non-hair cells (**Figure 1**). In contrast, the root-hair cell differentiation is controlled by *CAPRICE* (*CPC*) as shown by a few root-hair phenotype of the *cpc* mutant (Wada et al., 1997). The *CPC* gene encodes R3-type MYB protein (Wada et al., 1997). The TTG1-GL3/EGL3-WER protein complex also up-regulates *CPC* gene expression in non-hair cells (Koshino-Kimura et al., 2005). The CPC protein moves from non-hair cells to neighboring cells and disturbs the formation of the TTG1-GL3/ETC3-WER transcriptional complex by competitively binding with WER (Wada et al., 2002; Koshino-Kimura et al., 2005; Kurata et al., 2005; Tominaga et al., 2007). The formation of the TTG1-GL3/EGL3-CPC protein complex represses expression of *GL2*, thereby inhibiting non-hair cell differentiation (Wada et al., 2002; Kurata et al., 2005; **Figure 1**).

THE *CPC* FAMILY PROMOTES ROOT-HAIR CELL DIFFERENTIATION

CAPRICE encodes a small protein with an R3 MYB motif and strongly promotes root-hair cell differentiation in *Arabidopsis* (Wada et al., 1997). In addition, we presented a model in which

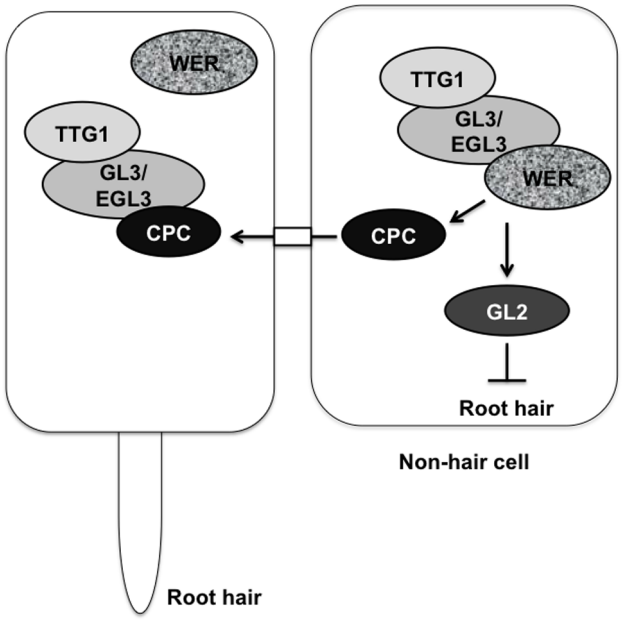

FIGURE 1 | Regulation of root-hair cell and non-hair cell fate determination by transcription factors. The TTG1-GL3/EGL3-WER complex promotes *GL2* and *CPC* expression. The cell expressing *GL2* differentiates into a non-hair cell. The CPC protein moves from the non-hair cell to a neighboring cell and competes with WER for binding to GL3/EGL3. The TTG1-GL3/EGL3-CPC complex cannot promote *GL2* expression, resulting in the differentiation of a root-hair cell.

CPC was proposed to have evolved from WER (Tominaga et al., 2007). Chimeric constructs made from the R3 MYB regions of CPC and WER and used in reciprocal complementation tests revealed that the CPC R3 could not functionally substitute for WER R3 in the differentiation of non-hair cells (Tominaga et al., 2007). In contrast, WER R3 can substitute for CPC R3 (Tominaga et al., 2007). Our results suggest that CPC evolved from WER after truncation of the activation domain and loss of DNA binding ability (Tominaga et al., 2007). *Arabidopsis* has six additional *CPC*-like MYB sequences in its genome, including *TRY*, *ETC1*, *ETC2*, *ETC3/CPL3*, *TCL1*, and *TCL2/CPL4* (Hulskamp et al., 1994; Schellmann et al., 2002; Esch et al., 2004; Kirik et al., 2004a,b; Simon et al., 2007; Wang et al., 2007, 2008, 2010; Tominaga et al., 2008; Wester et al., 2009; Gan et al., 2011; Tominaga-Wada and Nukumizu, 2012). These seven CPC-like MYB transcription factors act as positive regulators of root-hair cell differentiation and as negative regulators of trichome differentiation in a partially redundant manner (Tominaga-Wada et al., 2011; Tominaga-Wada and Nukumizu, 2012). The *try* mutant forms trichome clusters on leaves indicating that TRY functions in trichome differentiation (Hulskamp et al., 1994; Schellmann et al., 2002). *ETC1* and *ETC2* have redundant and enhancer functions with *CPC* and *TRY* in root-hair and trichome differentiation (Esch et al., 2004; Kirik et al., 2004a,b). Therefore, these genes were named *ENHANCER OF TRY AND CPC* (*ETC*; Esch et al., 2004; Kirik et al., 2004a,b). *TCL1* and *TCL2* negatively regulate trichome formation on the inflorescence stems and pedicels (Wang et al., 2007; Gan et al., 2011). These findings suggest functional divergence among *CPC* family genes.

RECENT FINDINGS ON THE FUNCTIONS OF THE *CPC* FAMILY

We have identified the *CPL4* gene between At2g30430 and *ETC2* (At2g30420) independently of Gan et al. (Gan et al., 2011; Tominaga-Wada and Nukumizu, 2012). Between *CPL4* and *ETC2*, there were several chimeric transcripts generated through alternative splicing (Tominaga-Wada and Nukumizu, 2012). Our study proposed that inter-genic alterative splicing also characterizes the CPC-like MYB gene family (Tominaga-Wada and Nukumizu, 2012).

A lateral inhibition mechanism mediated by cell-to-cell movement of CPC was thought to cause cell fate specification (Lee and Schiefelbein, 2002; Kwak and Schiefelbein, 2007, 2008). However, it is unclear how *CPC*, which is preferentially expressed in non-hair cells, specifically acts in the root-hair cells rather than in non-hair cells. Recently, nuclear trapping of CPC in the root-hair cells by EGL3 was suggested to be involved in root-hair cell differentiation (Kang et al., 2013). CPC protein accumulates predominantly in the nuclei of root-hair cells in the early meristematic region, and this localization requires specific expression of *EGL3* in the root-hair cells (Kang et al., 2013). These results suggest that cell-to-cell movement of CPC occurs within the meristem of root epidermal cells and that EGL3 traps the CPC protein in the root-hair cells (Kang et al., 2013). CPC and TRY were reported to recruit AtMYC1 into the nucleus, suggesting mutual control of the intracellular localization of patterning proteins (Pesch et al., 2013). *AtMYC1*, a homologue of *GL3* and *EGL3*, encodes a bHLH transcription factor predominantly localized in the cytoplasm (Urao et al., 1996; Pesch et al., 2013). AtMYC1 regulates the distribution of GL1 protein between the nucleus and the cytoplasm. On the other hand, AtMYC1 is recruited into the nucleus by TRY and CPC, subsequent to significant accumulation of TRY and CPC in the nucleus (Pesch et al., 2013). These results and genetic analyses imply that AtMYC1 represses the activity of TRY and CPC (Pesch et al., 2013).

Tissue-specific transcript profiling also indicated that there were some redundancies between *CPC* and *TRY* at the transcriptional level (Simon et al., 2013). We have extended the characterization of CPC-like MYB genes to include the identification of inter-genic alterative splicing and precise expression patterns using tissue-specific transcript profiling (Tominaga-Wada and Nukumizu, 2012; Simon et al., 2013). Recent findings have also revealed that in addition to the formation of the transcription complex, each type of transcription factor can regulate the inter- and intra-cellular localization of the other types to regulate root hair and trichome formation (Kang et al., 2013; Pesch et al., 2013).

A *CPC*-LIKE MYB IN TOMATO

Recently, we identified the tomato *SlTRY* gene as an ortholog of an *Arabidopsis CPC*-like MYB gene (Tominaga-Wada et al., 2013b). The *CPC*::*SlTRY* construct in *cpc-2* transgenic plants increased the number of root-hairs compared with that of the *cpc-2* mutant plants (**Figure 2**; Tominaga-Wada et al., 2013b). These results

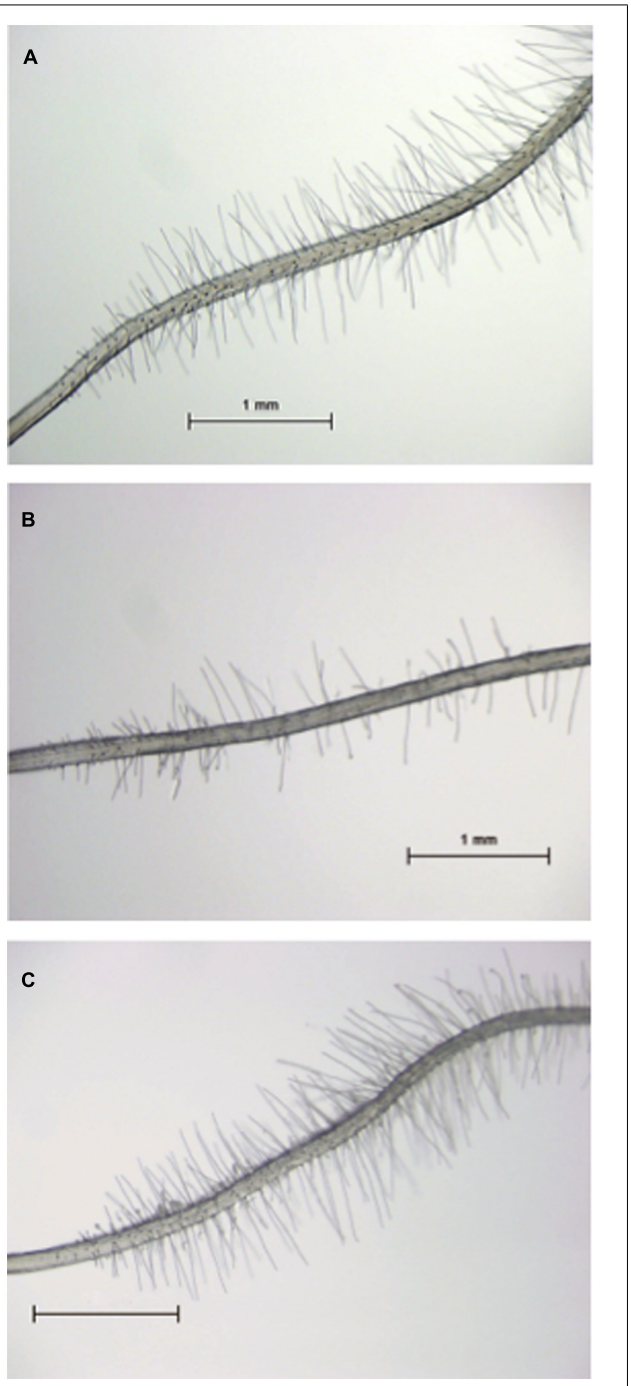

FIGURE 2 | Root-hair phenotype of *CPC*::*SlTRY* transgenic plants. **(A)** Root-hair phenotype of a 5-day-old *Arabidopsis* wild type (Col-0) seedling. **(B)** Root-hair phenotype of a 5-day-old *Arabidopsis cpc-2* mutant seedling. **(C)** Root-hair phenotype of a 5-day-old *Arabidopsis cpc-2* mutant seedling transformed with *CPC*::*SlTRY*. Scale bars: 1 mm.

suggest that tomato and *Arabidopsis* use common transcription factors for root-hair differentiation. In addition to root-hair cell differentiation, the *Arabidopsis CPC* gene is known to regulate anthocyanin biosynthesis (Zhu et al., 2009). Anthocyanin accumulation was repressed in the *CPC*::*SlTRY* transgenic plants as was observed in the *35S*::*CPC* transgenic plants, suggesting that *SlTRY* also influences anthocyanin pigment synthesis (Tominaga-Wada et al., 2013a). Tomato and *Arabidopsis* partially use similar transcription factors for root hair cell differentiation, and a CPC-like R3 MYB may be a key common regulator of plant root-hair development. Further analysis of CPC-like gene function in tomato is on-going.

FUTURE PERSPECTIVES

The cell-to-cell movement of CPC from non-hair cells to root-hair cells is important for root-hair cell specification; however, the precise mechanism of CPC movement is unknown. How CPC is targeted, transported through plasmodesmata, and trapped in the nucleus of the root-hair cells to define cell fate is an intriguing problem.

Transcriptome analyses provide detailed characterizations of transcription factors involved in root epidermal cell differentiation. Further molecular characterization of individual genes and mutant phenotypes is necessary to fully assess the precise mechanism for root epidermal cell differentiation, including an analysis of redundancies in the epidermal cell regulatory pathway.

TRY and *GL3* homologous genes were identified from the tomato genome and named *SlTRY* and *SlGL3*, respectively (Tominaga-Wada et al., 2013b). *SlTRY* showed a similar function to *TRY*, including inhibition of trichome formation and enhancement of root-hair differentiation. On the other hand, *SlGL3* did not show any obvious effect on trichome or non-hair cell differentiation (Tominaga-Wada et al., 2013b). There may be other GL3 ortholog(s) in the unannotated tomato genomes, or tomato uses other pathways to regulate epidermal cell differentiation. Further studies to determine the functions of R3-MYB and bHLH in epidermal cell differentiation in tomato are required.

ACKNOWLEDGMENT

JSPS KAKENHI Grant numbers 24658032, 23570057, and 25114513 financially supported this work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 November 2013; accepted: 24 February 2014; published online: 13 March 2014.
*Citation: Tominaga-Wada R and Wada T (2014) Regulation of root hair cell differentiation by R3 MYB transcription factors in tomato and Arabidopsis. Front. Plant Sci. **5**:91. doi: 10.3389/fpls.2014.00091*
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 08 April 2014
doi: 10.3389/fpls.2014.00133

Regulation of cell fate determination by single-repeat R3 MYB transcription factors in Arabidopsis

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MYB transcription factors regulate multiple aspects of plant growth and development. Among the large family of MYB transcription factors, single-repeat R3 MYBs are characterized by their short sequence (<120 amino acids) consisting largely of the single MYB DNA-binding repeat. In the model plant Arabidopsis, R3 MYBs mediate lateral inhibition during epidermal patterning and are best characterized for their regulatory roles in trichome and root hair development. R3 MYBs act as negative regulators for trichome formation but as positive regulators for root hair development. In this article, we provide a comprehensive review on the role of R3 MYBs in the regulation of cell type specification in the model plant Arabidopsis.

Keywords: CPC, GL1, GL2, GL3, R3 MYB, root hair, trichome, TRY

INTRODUCTION

In plants, MYB transcription factors are encoded by a large family of genes (Stracke et al., 2001; Chen et al., 2006; Dubos et al., 2010; Katiyar et al., 2012). They play important roles in regulating plant growth and development and plant responses to environmental stimuli. There are several sub-families of MYB transcription factors, defined by the number of MYB DNA-binding domain repeats. These include 4R-MYB, 3R-MYB, R2R3-MYB, and 1R-MYB subfamilies that contain four, three, two and one MYB DNA-binding domain repeats, respectively (Dubos et al., 2010).

In the model plant Arabidopsis, there are a total of 64 1R-MYB or MYB-related proteins (Dubos et al., 2010). Among this subfamily, there are a unique set of 1R-MYBs that are characterized by their short sequence (<120 amino acids) consisting largely of the single R3 MYB repeat. These small proteins are referred as single-repeat R3 MYB transcription factors (R3 MYBs) and are subjects of this review article. We provide a comprehensive review about the function and action of R3 MYBs in the model plant Arabidopsis, particularly, in trichome and root hair development.

R3 MYBs IN ARABIDOPSIS

In the completely-sequenced model plant *Arabidopsis thaliana* (hereafter referred as Arabidopsis), a total of seven genes encoding R3 MYBs have been reported so far. These include TRIPTYCHON (TRY)(Schnittger et al., 1999; Schellmann et al., 2002), CAPRICE (CPC) (Wada et al., 1997, 2002), ENHANCER OF TRY AND CPC1 (ETC1) (Esch et al., 2004; Kirik et al., 2004a), ETC2 (Kirik et al., 2004b), ETC3/CAPRICE-LIKE MYB3 (CPL3) (Simon et al., 2007; Tominaga et al., 2008; Wang et al., 2008), TRICHOMELESS1 (TCL1) (Wang et al., 2007) and TCL2/CPL4 (Gan et al., 2011; Tominaga-Wada and Nukumizu, 2012). These seven R3 MYB proteins share approximately 52–82% similarity, and 37–68% identity to each other at the amino acid level (**Figure 1**). They consist of largely the single MYB DNA-binding repeat, with the total number of amino acid of each protein ranging from 77 to 112.

In order to search for any other potential R3 MYBs encoded by the Arabidopsis genome, the amino acid sequence of TRY was used as a template for searching protein sequence homologs encoded by the fully-sequenced Arabidopsis genome using the "Protein Homologs" tool of Phytozome (www.phytozome.net). In addition to CPC, ETC1, ETC2, ETC3, TCL1 and TCL2, the search identified eight other proteins. These included MYB3, MYB4, MYB5, MYB23, MYB82, GL1, TT2, and WER (**Figure S1**). However, R3 MYBs were clustered in one distinct branch of the phylogenetic tree (**Figure S2**). Furthermore, those eight MYBs are mostly R2R3-MYBs, are typically twice the size of R3 MYBs in term of number of amino acids, and share low similarity (17–34%) and identity (10–23%) with R3 MYBs at the amino acid level (**Figure S3**). Therefore, these MYBs are not considered to be R3 MYB proteins and no additional R3 MYB was identified.

It should be noted that an R3 MYB-related protein, designated as MYBL2, has been shown to be involved in the regulation of flavonoid biosynthesis (Dubos et al., 2008; Matsui et al., 2008). Amino acid alignment and phylogenetic analysis indicated that MYBL2 is more similar to other MYBs than R3 MYBs. For example, the number of amino acid of MYBL2 (195 aa) is almost twice that of R3 MYBs (**Figure S1**). In addition, the cell-cell movement motif WxM (further discussed in the next section) is not conserved in MYBL2 (**Figure S1**). Therefore, MYBL2 is not included in our further discussion of R3 MYBs in this review article.

It should also be noted that there are three transcript variants for *ETC3*, designated as *ETC3.1*, *ETC3.2* and *ETC3.3*, according to the current annotation by TAIR (http://www.arabidopsis.org)

Abbreviations: CPC, CAPRICE; CPL3, CAPRICE-LIKE MYB3; CPL4, CAPRICE-LIKE MYB4; EGL3, ENHANCER OF GLABRA3; ETC1, ENHANCER OF TRY AND CPC1; ETC2, ENHANCER OF TRY AND CPC2; ETC3, ENHANCER OF TRY AND CPC3; GL1, GLABRA1; GL2, GLABRA2; GL3, GLABRA3; TCL1, TRICHOMELESS1; TCL2, TRICHOMELESS2; TRY, TRIPTYCHON; TTG1, TRANSPARENT TESTA GLABRA1.

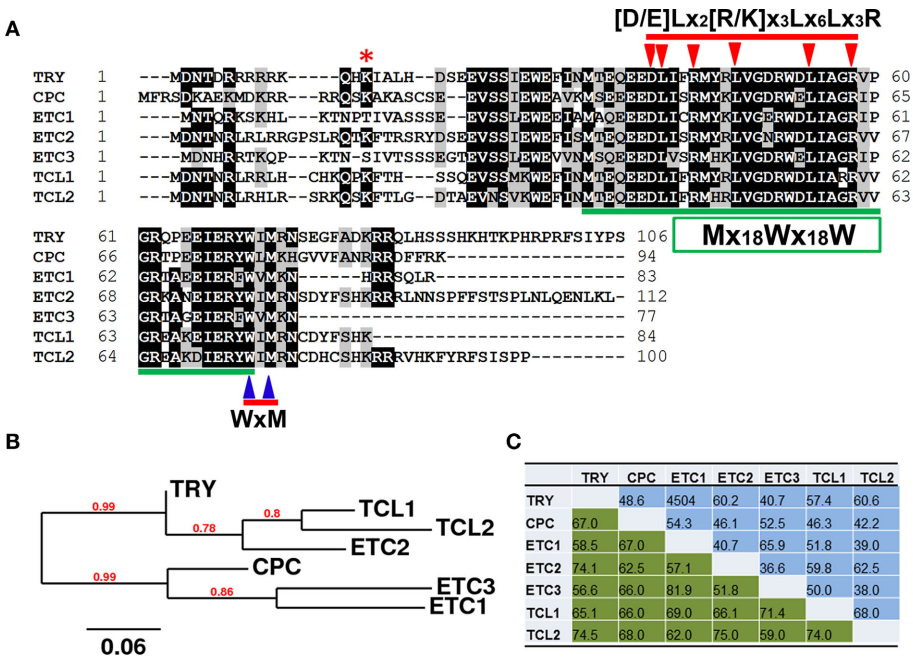

FIGURE 1 | Single-repeat R3 MYB transcription factors in *Arabidopsis thaliana*. (A) Amino acid sequence alignment of R3 MYBs. Identical and similar amino acids are shaded in black and gray, respectively. The amino acids of [D/E]Lx$_2$[R/K]x$_3$Lx$_6$Lx$_3$R signature that is required for interaction between MYBs and R/B-like bHLH transcription factors are indicated by arrowheads on the top of amino acids. The amino acids of WxM signature that is crucial for cell-to-cell movement are indicated by arrowheads on the bottom of amino acids. The pattern of primary structure of MYB repeat, Mx$_{18}$Wx$_{18}$W, is indicated in a box and amino acids are underlined. The Lysine19 (K19) of ETC2 that was proposed to control its stability is indicated by an asterisk. **(B)** Phylogenetic analysis of R3 MYBs. The full-length amino acid sequences of each R3 MYB were used for the analysis. **(C)** Similarity and identity of amino acid sequences of R3 MYB proteins.

and Phytozome. Proteins encoded by these three *ETC3* transcript variants are identical except that compared with ETC.1, ETC3.2 has deletion of three amino acids and ETC3.3 has deletion of two amino acids at a site prior to the conserved bHLH binding sequence signature and cell-to-cell movement motif (described in the next section) (**Figure S4**). For simplicity, only ETC3.1 was included in our analysis.

Finally, several chimeric transcripts can be formed between *ETC2* and *TCL2/CPL4*, two tandem repeat genes in Chromosome II (*ETC2*: At2g30420; *TCL2/CPL4*: At2g30424), resulting from alternative splicing (Tominaga-Wada and Nukumizu, 2012).

In summary, the genome of model plant Arabidopsis encodes a total of seven R3 MYBs, and these seven R3 MYBs are the subjects of this review article.

R3 MYBs STRUCTURAL FEATURES

In addition to the small size (77–112 aa) and the constitution of a single MYB repeat, there are a number of structural characteristics of R3 MYBs (**Figure 1**). Firstly, R3 MYBs contain a sequence signature of [D/E]Lx$_2$[R/K]x$_3$Lx$_6$Lx$_3$R that has been shown to be required for the interaction between R3 MYBs and R/B-like bHLH transcription factors (Zimmermann et al., 2004). It should be noted that this bHLH interaction signature is also conserved in many other MYB proteins including those showed sequence homology with R3 MYBs (**Figure S1**). Secondly, R3 MYBs contain a sequence motif WxM that has been shown to be required for its cell-to-cell movement (Kurata et al., 2005). This cell-to-cell movement motif is not conserved in any other MYBs that showed sequence homology with R3 MYBs (**Figure S1**). Finally, the primary structure of MYB repeat in R3 MYBs follow a pattern of Mx$_{18}$Wx$_{18}$W, instead of [F/I]x$_{18}$Wx$_{18}$W that is typically found in the R3 MYB repeat of R2R3-MYB proteins and MYBL2 (**Figure S1**). Therefore, a combination of these three structural features together with the small size and the constitution of a single MYB repeat define a small family of R3 MYB transcription factors.

Due to its constitution of a single MYB repeat, R3 MYBs lack the activation domain that is typically present in most transcription factors. Therefore, from the domain structural perspective, it is believed that R3 MYBs rely on the interaction with other transcription factors to execute their transcriptional activity. As discussed in details below, this indeed represents a major mechanism of action of R3 MYBs.

FUNCTION OF R3 MYBs IN TRICHOME DEVELOPMENT

Trichomes are hair cells produced by the outward growth of epidermal cells (Marks, 1997; Hülskamp and Schnittger, 1998). They distribute on the surface of aerial organs including leaves, stems and flower organs of most land plants. Trichomes can act as barriers to protect plants from biotic (e.g., insect herbivores) and abiotic stresses, UV light irradiation and excessive transpiration (Mauricio and Rausher, 1997; Eisner et al.,

1998; Werker, 2000; Wagner et al., 2004). Some trichomes or trichome-produced compounds are of great commercial value. For example, single-celled seed trichomes of *Gossypium hirsutum*, cotton fibers, are the most important natural fiber for the textile industry (Kim and Triplett, 2001). Multi-celled glandular trichomes of *Artemisia annua* accumulates artemisinin, a drug that is widely used for the treatment of malaria (Liu et al., 2006; Lommen et al., 2006). Therefore, study of trichome development may lead to the improvement of agronomical and economical traits in plants such as resistance to insect herbivores, improvement of cotton fiber yield and quality, and increase in yield of oil or other plant products contained in glandular trichomes. Furthermore, trichome has become an excellent system to study cell type specification (Schiefelbein, 2003; Pesch and Hülskamp, 2004; Serna, 2005; Schellmann et al., 2007). In this review article, we specifically focus on discussing the role of R3 MYBs in single-celled trichome development in the model plant Arabidopsis.

The genetic control of trichome development has been studied extensively in the last 20 years. It has been generally recognized that trichome initiation is promoted by an activator complex consisting of a WD40-repeat protein, TRANSPARENT TESTA GLABRA1 (TTG1) (Galway et al., 1994; Walker et al., 1999), an R2R3 MYB-type transcription factor, GLABRA1 (GL1) (Oppenheimer et al., 1991), and a bHLH transcription factor, GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3)(Payne et al., 2000; Zhang et al., 2003). Yeast two hybrid experiments showed that both GL1 and TTG1 bind GL3 but at different regions of the GL3 protein and that TTG1 and GL1 do not interact (Payne et al., 2000). This TTG1-GL3/EGL3-GL1 activator complex induces the expression of a homeodomain protein, GLABRA2 (GL2) (Rerie et al., 1994; Masucci et al., 1996), which is required for trichome initiation. The trichome initiation involves feedback loop controls: in addition to inducing *GL2* expression, the TTG1-GL3/EGL3-GL1 activator complex induces the expression of R3 MYB genes. R3 MYBs can move from a trichome precursor cell to its neighboring cell and compete with GL1 for binding GL3 or EGL3 thus disrupting the functionality of the activator complex, resulting in the inhibition of trichome initiation (Hülskamp et al., 1994; Schellmann et al., 2002; Esch et al., 2003; Schiefelbein, 2003; Pesch and Hülskamp, 2004; Ishida et al., 2008; Pesch and Hülskamp, 2009).

According to this model, R3 MYBs act as negative regulators of trichome initiation. Consistent with this view, overexpression of each of seven R3 MYBs resulted in glabrous phenotypes and loss-of-function mutation in R3 MYBs resulted in more trichome formation except *etc1* single mutant (Wada et al., 1997; Schnittger et al., 1999; Schellmann et al., 2002; Wada et al., 2002; Esch et al., 2004; Kirik et al., 2004a,b; Simon et al., 2007; Wang et al., 2007; Tominaga et al., 2008; Wang et al., 2008; Wester et al., 2009; Gan et al., 2011; Tominaga-Wada and Nukumizu, 2012). However, differences in severity and pattern of trichome formation have been observed in the single mutants of R3 MYB genes. The trichome phenotypes of R3 MYB single mutants can be categorized into three groups: (i) increased trichome clustering on leaf (*try*). (ii) increased trichome density on leaf (*cpc*, *etc2*, and *etc3*), and (iii) normal trichome density on leaves but increased trichomes on inflorescence stems and pedicels (*tcl1*, *tcl2*). It should be noted that the phenotype of increased trichome number on leaves of *etc2* and *etc3* single mutants is generally weak, which have resulted in inconsistent results among different studies. For example, the trichome phenotype of *etc2* single mutant was not detected in a few studies (Simon et al., 2007; Tominaga et al., 2008; Wang et al., 2008) but was detected in the study by Kirik et al. (2004b). Analysis of Arabidopsis natural variation population supported that *ETC2* regulates trichome formation on leaves (Hilscher et al., 2009). As mentioned above, among all single mutants of R3 MYB genes, only *etc1* single mutant did not display any detectable trichome phenotypes (Kirik et al., 2004a). Taken together, analysis of single mutants of R3 MYB genes suggested that among seven R3 MYBs, TRY is the predominant member controlling trichome clustering, TCL1 and TCL2 are the predominant members controlling trichome development on inflorescence stem and pedicels, and CPC, ETC2, and ETC3 mainly regulate trichome development on leaves. **Table 1** summarizes the trichome and root hair phenotypes of R3 MYB single mutants and overexpression lines.

Analysis of double, triple, quadruple and higher order mutants revealed redundancy among R3 MYB genes in each of these three categories. For example, *try cpc* double mutants have more trichome clusters than *try* single mutant and have more trichomes on leaves than *cpc* single mutant (Schellmann et al., 2002; Kirik et al., 2004a; Wang et al., 2008). Analysis of *tcl1 cpc etc1 etc3* quadruple mutants revealed that *TCL1* is also involved in the regulation of trichome density on leaves (Wang et al., 2008). Analysis of *tcl1 cpc* double mutants revealed that CPC is also involved in the regulation of trichome formation on upper inflorescence stem and pedicles (Wang et al., 2007). *try cpc etc1 tcl1* quadruple mutants form trichomes in almost all aerial parts of the plant (Wang et al., 2008) (**Figure 2**). Although *etc1* single mutant did not display any detectable trichome phenotypes, analysis of double, triple and quadruple mutants suggested that *etc1* mutation can enhance trichome phenotypes in each of those three categories (Kirik et al., 2004a; Wang et al., 2008; Wester et al., 2009).

Taken together, all seven R3 MYBs negatively regulate trichome formation in a largely redundant manner. Specificities have also been observed, which will be further discussed in a later section.

FUNCTION OF R3 MYBs IN ROOT HAIR DEVELOPMENT

Root hair is a tubular outgrowth of a trichoblast, a hair-forming cell on the epidermis of a plant root (Gilroy and Jones, 2000). Root hairs are formed in the region of maturation zone of the root. They are lateral extensions of a single cell and rarely branched. Root hairs help plant collect water and mineral nutrients (Gilroy and Jones, 2000). Similar to the trichome system, root hair has become an excellent system to study cell type specification (Ishida et al., 2008; Schiefelbein et al., 2009; Tominaga-Wada et al., 2011; Grebe, 2012; Ryu et al., 2013). Root hair development follows a position-dependent pattern. Epidermal cells in contact with two underlying cortical cells differentiate into hair cells (H cells; trichoblasts) whereas cells that contact only a single cortical cell differentiate into mature hairless cells (N cells; atrichoblasts) (Schiefelbein, 2000; Dolan and Costa, 2001).

Table 1 | Summary of R3 MYB transcription factors in Arabidopsis.

	Full name	Locus identifier	Phenotype of single mutant		Phenotype of overexpressor		References
			Trichome	Root hair	Trichome	Root hair	
TRY	TRIPTYCHON	At5g53200	Trichome clusters on leaf	Reduced	Reduced	Increased	Schnittger et al., 1999; Schellmann et al., 2002
CPC	CAPRICE	At2g46410	Increased on leaf	Strongly reduced	Reduced	Increased	Wada et al., 1997, 2002
ETC1	ENHANCER OF TRY AND CPC1	At1g01380	Wild type-like	Wild type-like	Reduced	Increased	Esch et al., 2004; Kirik et al., 2004a
ETC2	ENHANCER OF TRY AND CPC2	At2g30420	Slightly increased on leaf	Wild type-like	Reduced	Increased	Kirik et al., 2004b
ETC3/ CPL3	ENHANCER OF TRY AND CPC3/CAPRICE-LIKE MYB3	At4g01060	Slightly increased on leaf	Slightly reduced	Reduced	Increased	Simon et al., 2007; Tominaga et al., 2008; Wang et al., 2008; Wester et al., 2009
TCL1	TRICHOMELESS1	At2g30432	Increased on upper inflorescence stem and pedicel	Wild type-like	Reduced	Wild type-like	Wang et al., 2007
TCL2/ CPL4	TRICHOMELESS2/CAPRICE-LIKE MYB4	At2g30424	Increased on upper inflorescence stem and pedicel	Wild type-like	Reduced	Wild type-like	Gan et al., 2011; Tominaga-Wada and Nukumizu, 2012

FIGURE 2 | R3 MYB transcription factors function redundantly to control trichome formation in Arabidopsis. In Col wild type plant (left), trichomes were observed on leaves, lower part of stems and flower organs. In *try cpc etc1 tcl1* quadruple mutant (right), trichomes were observed in almost all aerial parts of the plants.

The genetic control of root hair formation is remarkably similar to that of trichome initiation with all same components in the activator complex except that GL1 is replaced by another R2R3-MYB transcription factor, WEREWOLF (WER) (Lee and Schiefelbein, 1999). Recently, it was found that WER positively regulates the expression of *MYB23*, an R2R3 MYB, during root epidermis development (Kang et al., 2009). Similar to WER, MYB23 specifies non-hair cell type and can substitute for the function of WER. Unlike WER, MYB23 regulates its own expression, providing a positive feedback loop to reinforce cell fate decisions and root epidermal patterning (Kang et al., 2009). The TTG1-GL3/EGL3-WER activator complex induces the expression of *GL2* and *R3 MYBs*. R3 MYBs can move from an N cell to a neighboring H cell to compete with WER for binding GL3 or EGL3, thus limiting the activity of the TTG1-GL3/EGL3-WER activator complex (Ishida et al., 2008; Schiefelbein et al., 2009; Tominaga-Wada et al., 2011; Grebe, 2012; Ryu et al., 2013). Opposing to that in the trichome initiation system, positive regulators of trichome initiation function as negative regulators of root hair formation and vice versa. Therefore, R3 MYBs are positive regulators of root hair formation.

According to this model, overexpression of R3 MYBs is expected to result in increased root hair formation whereas loss-of-function mutation results in decreased root hair formation. This is indeed the general trend. However, differences have been observed. TCL1 and TCL2 appeared to have diverged the most among R3 MYB subfamily because overexpression of TRY, CPC, ETC1, ETC2, and ETC3 rendered more root hair formation (Tominaga et al., 2008) whereas overexpression of TCL1 and TCL2 had no effect on root hair formation (Wang et al., 2007; Gan et al., 2011). Among all single mutants of R3 MYBs, only *try* and *cpc* single mutants have been consistently shown to have

decreased root hair formation and *cpc* single mutant had stronger root hair phenotype than *try* mutant (Wada et al., 1997, 2002; Schellmann et al., 2002). Although *etc3* single mutant was shown to display wild-type root hair phenotypes in two studies (Simon et al., 2007; Wang et al., 2008), it was shown to produce less root hairs in two other studies (Tominaga et al., 2008; Wester et al., 2009), indicating that the root hair phenotype of *etc3* mutant is generally weak. Other single mutants including *etc1*, *etc2*, *tcl1*, and *tcl2* displayed little or no root hair phenotypes. These results suggested that CPC is the predominant member of R3 MYB family controlling root hair formation.

Analysis of double, triple and quadruple mutants revealed that other R3 MYB genes may function redundantly with CPC to positively regulate root hair formation. Analysis of *etc1 cpc* double mutant and *etc1 try cpc* triple mutant revealed that *ETC1* functioned redundantly with *TRY* and *CPC* to control root hair formation (Kirik et al., 2004a). Analysis of *cpc etc1 etc3 tcl1* quadruple mutants indicated that *TCL1* positively regulate root hair formation (Wang et al., 2008). Analysis of *etc3 cpc* double mutant revealed that *ETC3* functioned redundantly with *CPC* to control root hair formation (Tominaga et al., 2008). Taken together, five of the seven R3 MYB genes have been shown to be involved in the regulation of root hair formation. So far, a role of ETC2 and TCL2 in root hair formation has not been reported.

OTHER FUNCTIONS OF R3 MYBs

In addition to regulating trichome and root hair development, R3 MYBs have been shown to be involved in the regulation of other processes including flowering, anthocyanin accumulation and stomatal formation. This is consistent with the observation that the expression of R3 MYB genes is not restricted to trichomes and root hairs. For example, *TRY* is also expressed in inflorescence and siliques (Schellmann et al., 2002).

etc3/cpl3 mutant was shown to flower earlier with fewer leaves than the wild-type (Tominaga et al., 2008; Tominaga-Wada et al., 2013b) although the early flowering phenotype was not reproduced by another study (Wester et al., 2009). Subsequently, it was shown that mutations in *TRY* or *CPC* delayed flowering of *etc3*/*cpl3* mutant and a mutation in *ETC1* did not further delay flowering but reduced plant size (Tominaga-Wada et al., 2013b). Mutations in ETC3/CPL3 were also shown to affect endoreduplication (Tominaga et al., 2008).

CPC was found to be a positive regulator of stomatal formation in the hypocotyl (Serna, 2008). CPC was localized in the nucleus and peripheral cytoplasm of fully differentiated epidermal cells in hypocotyl. *CPC* expression in differentiating stomaless-forming cells was shown to be positively regulated by TOO MANY MOUTHS (TMM), a leucine-rich repeat-containing receptor-like protein expressed in proliferative post-protodermal cells (Nadeau and Sack, 2002). Furthermore, *CPC* acts redundantly with *TRY* to promote stomata formation.

Because GL3, EGL3, and TTG1 of the activator complex are also involved in the regulation of seed coat mucilage and anthocyanin production, in addition to the regulation of trichome and root hair formation (Zhang et al., 2003), and that TTG1-GL3/EGL3-GLl and TTG1-GL3/EGL3-WER complexes activate the expression of R3 MYB genes, it raises a question of whether R3 MYBs also regulate anthocyanin and seed coat mucilage production. Molecular and genetic studies suggested that R3 MYBs regulate anthocyanin biosynthesis but not seed coat mucilage production.

CPC was shown to be a negative regulator of anthocyanin biosynthesis (Zhu et al., 2009). Overexpression of *CPC* repressed a total of 85 genes at the whole genome level. Of these 85 genes, seven are later anthocyanin biosynthesis genes. As discussed above, the action of R3 MYBs in the regulation of trichome and root hair development involves the TTG1-GL3/EGL3-GL1 and TTG1-GL3/EGL3-WER activator complex, respectively. The regulation of anthocyanin biosynthesis involves a similar TTG1-bHLH-MYB activator complex (Walker et al., 1999; Zhang et al., 2003; Zimmermann et al., 2004; Gonzalez et al., 2008). Unlike the trichome and root hair activator complexes, the MYB function is executed by PAP1 and PAP2. GL3/EGL3 interacts with both TTG1 and PAP1/PAP2, acting as positive regulators of anthocyanin biosynthesis (Zhang et al., 2003; Zimmermann et al., 2004). Transient expression analysis indicated that CPC competes with PAP1/PAP2 for binding with GL3/EGL3. Similarly, a tomato ortholog of TRY was also shown to negatively regulate anthocyanin accumulation (Nukumizu et al., 2013). Among all R3 MYBs, only CPC appears to act in the negative feedback on anthocyanin accumulation in response to nitrogen starvation conditions (Nemie-Feyissa et al., 2014).

For seed coat mucilage, in addition to TTG1, it requires bHLH transcription factor EGL3 and TT8, and MYB transcription factor MYB61 (Zhang et al., 2003). Analysis of single, double and higher order R3 MYB mutants did not reveal defects in seed mucilage production (Zhu et al., 2009; Wang et al., 2010), suggesting that R3 MYBs do not regulate seed mucilage biosynthesis.

REDUNDANCY AND SPECIFICITY OF R3 MYBs

As discussed above, R3 MYBs can function redundantly to regulate trichome and root hair development. However, although R3 MYBs are small in size (77–112 aa) and are similar (52–82%) to each other at the amino acid level, some members exhibit distinct function than others in trichome and root hair development. For example, among all R3 MYBs, TRY is characteristic of its regulatory role in trichome clustering, CPC is characteristic of its role in root hair development, and TCL1 and TCL2 are characteristic of their roles in trichome development on the inflorescence stem and pedicels. What determines the functional specificity of each R3 MYB? In general, there are a number of attributes of functional specificity of any given genes including the expression level, spatiotemporal expression of the gene and the biochemical property of the protein (e.g., transcriptional activity, protein stability and protein subcellular localization). The expression level and pattern of a gene are largely determined by the promoter activity whereas the biochemical properties of the protein are determined by the amino acid constitution of the protein. R3 MYBs display differences in both categories.

PROMOTER ACTIVITY

At the transcript level, differences in tissue/organ expression of R3 MYB genes have been observed. Among all seven R3 MYB genes, *ETC2* and *TCL1* were not expressed in the root (Kirik et al.,

2004b; Wang et al., 2007). The transcript of *TCL2/CPL4* was also not detected or at very low level in the root as determined by RT-PCR (Gan et al., 2011; Tominaga-Wada and Nukumizu, 2012). These results implied that these three R3 MYB genes do not play a major role in regulating root hair formation. This view is consistent with the observation that none of *etc2*, *tcl1*, and *tcl2* single mutants displayed root hair phenotypes. Therefore, the differences in tissue/organ expression patterns of R3 MYBs determine the first level of specificities. Nonetheless, all of these three genes tested were able to partially rescue the root hair phenotype of *cpc* mutant when their expression was driven by *CPC* promoter (Simon et al., 2007). These results reinforce the concept that the normal functions of these genes are restricted by their promoter activities.

Results from promoter-swap experiments supported that transcriptional regulation is important for the functional diversity of R3 MYB genes (Wester et al., 2009). When *ETC3* was expressed under the promoter of *ETC3*, *TRY* or *CPC*, it could equally rescue the trichome phenotype of *etc3* mutant (Wester et al., 2009), suggesting that the promoters of *TRY*, *CPC*, and *ETC3* are interchangeable with respect to *etc3* single mutant rescue. However, when these constructs (*pETC3:ETC3*, *pTRY:ETC3*, and *pCPC:ETC3*) were used to complement triple mutants containing *etc3*, qualitative differences were observed. *pETC3:ETC3* construct rescued the *cpc try etc3* mutant to the same extent as the *try cpc* mutant whereas *pTRY:ETC3* and *pCPC:ETC3* displayed an over-rescued phenotype resembling that of *try*. Differences were also observed between *pTRY:ETC3* and *pCPC:ETC3* in terms of trichome cluster phenotypes. These results suggest that the regulation of expression of R3 MYB genes is important for their functioning.

PROTEIN PROPERTIES

TRY and *CPC* displayed indistinguishable expression patterns in leaves (Schellmann et al., 2002), yet the trichome phenotypes of *try* and *cpc* single mutants are very different. These suggested that the amino acid sequences of R3 MYBs play important roles in determining its functionality. The importance of protein properties in determining its functionality has been supported by genetic complementation studies, in particularly promoter swapping assays. For example, when *TCL1* was expressed under the control of *TRY* or *CPC* promoter in *try* and *cpc* mutant background, respectively, *TCL1* was only able to partially rescue the trichome and root hair phenotypes of *try* and *cpc* mutants (Wang et al., 2007), indicating that TCL1 does not function equivalently with TRY or CPC. On the other hand, the expression of *TRY* under the control of *TRY* or *CPC* promoter could completely rescue the trichome clustering phenotype of *try* mutant whereas the expression of *CPC* under *TRY* promoter could not (Pesch and Hülskamp, 2011), suggesting that the specific role of *TRY* in regulating trichome cluster formation is not based on its transcriptional regulation but on specific protein properties. Furthermore, it has been suggested that TRY protein has specific properties relevant in the context of both cluster formation and trichome density (Pesch and Hülskamp, 2011).

Differences in R3 MYB protein properties have also been observed in other studies. For example, by using promoter-swap experiments, Simon et al. (2007) compared the ability of several R3 MYBs to rescue the root hair phenotype of *cpc* mutant under *CPC* promoter. It was found that ETC1 possessed the best ability, followed by ETC3, TRY, and ETC2, revealing differences in protein properties.

Taken together, the functional specificity of each R3 MYB was determined both by the promoter activity of each gene and the biochemical property of each protein. Which aspect of protein properties do R3 MYBs differ? The precise answer for this question is unclear. Recent studies, however, have revealed some possibilities.

Binding strength to GL3/EGL3

During trichome patterning, two important actions of R3 MYBs at the protein level are the competition with GL1 for bindings GL3 and the movement from trichome cell to its neighboring cells. The transcriptional activity and their binding strength with bHLH transcription factors (GL3/EGL3) of R3 MYBs have been studied in the yeast system and the bi-molecular fluorescence complementation (BiFC) system (Wester et al., 2009), and in the Arabidopsis mesophyll protoplast transient expression system (Wang et al., 2008). Although these assays could not distinguish differences in protein stability, these studies indicate that differences in biochemical property do exist among members of R3 MYBs. It was found that R3 MYBs including TRY, CPC, ETC1, ETC2, and ETC3 differ in their binding strength to GL3 (Wester et al., 2009). Furthermore, by using the yeast three-hybrid system, it was found that R3 MYBs' capacity to compete with GL1 for binding to GL3 also differs with CPC being the most potent inhibitor followed by ETC1, TRY, ETC3, and ETC2 (Wester et al., 2009). Such differences in competing GL1 for binding GL3 may contribute to the functional specificities of R3 MYBs.

Cell-to-cell movement

The cell-to-cell movement motif is conserved in all R3 MYB proteins, and the cell-to-cell movement has been experimentally demonstrated for CPC and ETC3 (Kurata et al., 2005; Wester et al., 2009). It has been shown that CPC is able to move readily within the root epidermis when its expression level is high (Kang et al., 2013). CPC is capable of moving from the stele tissue in the center of the root to the outermost epidermal layer, where it can induce the hair cell fate (Kang et al., 2013). The accumulation and localization of CPC in the nuclei of H-position cells require EGL3. During trichome patterning, it was found that ETC3 protein was localized in the nucleus as well as in the cytoplasm in trichome initials whereas ETC3 protein was restricted to the nucleus in surrounding cells (Wester et al., 2009), indicating that ETC3 protein likely moves from the trichome initial into the neighboring epidermal cells. The cell-cell movement of ETC3 was further confirmed using the particle bombardment in single leaf epidermal cells (Wester et al., 2009). Mathematical analysis suggests that the mobility of the inhibitors depends on their affinity for GL3 and predicted that ETC3 moves faster than CPC (Wester et al., 2009). This prediction was experimentally validated in particle bombardment experiments (Wester et al., 2009). Therefore, the difference in cell-to-cell movement ability may also contribute to the function specificities of R3 MYB proteins.

Protein subcellular localization

At the protein level, all R3 MYBs are localized in the nucleus as expected for transcription factors but several R3MYB proteins such as TCL1 (Wang et al., 2007), CPC (Serna, 2008) and ETC3 (Wester et al., 2009) are also found to be localized to a site around plasma membrane. Recently, it has been found that subcellular localization of R3 MYBs and GL1 affects their functionality (Pesch et al., 2013). AtMYC1, a homolog of GL3 and EGL3, can regulate the intracellular localization of GL1, TRY and CPC. AtMYC1 can relocate GL1 from the nucleus into the cytoplasm and can be recruited into the nucleus by TRY and CPC. It was suggested that AtMYC1 represses the activity of TRY and CPC (Pesch et al., 2013). It remains unclear to what extent, differences in protein subcellular location of R3 MYBs may affect their functionalities.

Protein stability

It is known that the degradation of GL3 and EGL3 proteins is ubiquitin/26S proteasome-dependent (Patra et al., 2013). However, no study has been conducted to directly compare the protein stability of R3 MYBs though it was suggested that a mutation at Lysine19 (K19) of ETC2 may affect its protein stability (Hilscher et al., 2009). This amino acid is also conserved in TRY, CPC, TCL1, and TCL2, but not in ETC1 and ETC3 (**Figure 1**). It is unclear whether such a difference may contribute to the function specificities of R3 MYB proteins

REGULATION OF EXPRESSION OF R3 MYB GENES

As discussed above, the trichome and root hair initiation involves feedback loop controls. In addition to inducing *GL2* expression, the TTG1-GL3/EGL3-GL1 (in trichome initiation) and TTG1-GL3/EGLs-WER (in root hair formation) activator complexes also induce the expression of R3 MYB genes. Consistent with these modes, *CPC* has been identified as a direct target gene for WER (Koshino-Kimura et al., 2005; Ryu et al., 2005). WER protein binds three sites in the *CPC* promoter region, designated *WBSI*, *WBSII/CPCMBSI* and *CPCMBSII*, and regulates its transcription. Furthermore, both GL1 and GL3 have been shown to be recruited to the promoter region of *CPC* and *ETC1* (Morohashi et al., 2007; Zhao et al., 2008).

Recent studies suggested the expression of R3 MYB gene may also be controlled by other mechanisms. By using an Arabidopsis protoplast transient expression system, it was found that co-transfection of GL1 or WER, with GL3 or EGL3, is required and sufficient to activate *TRY*, *CPC*, *ETC1*, and *ETC3*, but not *TCL1*, *TCL2* or *ETC2* (Wang et al., 2008; Gan et al., 2011). Furthermore, *MIR156*-regulated SPLs (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE), which are known to play important roles in regulating phase transition and flowering, have been shown to be able to directly activate the expression of *TCL1* and *TRY* through binding to their promoters and that this activation is independent of GL1 (Yu et al., 2010). Reduced expression of *TCL2* in *35S:MIR156* transgenic plants suggested that *MIR156*-targeted SPLs may also regulate the expression of *TCL2* (Gan et al., 2011).

Finally, the proposed regulatory feedback loop between the activators and the inhibitors can also lead to an auto-repression of the inhibitors. Specifically, binding of R3 MYB to GL3 can displace GL1 thereby inactivating the activator complex by forming a different protein complex through the replacement of GL1 with R3 MYBs. Consequently, this creates a shortcut of the regulatory feedback and results in the repression of R3 MYB own expression.

MODES OF ACTION OF R3 MYBs

As discussed above, the mode of action of R3 MYBs in trichome and root hair development is very similar. The action of R3 MYBs in root hair patterning has been discussed in several recent review articles (Ishida et al., 2008; Schiefelbein et al., 2009; Tominaga-Wada et al., 2011; Grebe, 2012; Ryu et al., 2013; Schiefelbein et al., 2014). To avoid redundancy, here we only focus on describing the action of R3 MYBs in trichome development. Genetic and molecular studies suggested that R3 MYBs move from a trichome precursor cell to its neighboring cell to compete with GL1 for binding GL3 or EGL3, thus limiting the activity of the TTG1-GL3/EGL3-GL1 activator complex and resulting in decreased expression of *GL2*; this results in the inhibition of trichome formation (**Figure 3**). During trichome patterning, regulatory feedback loops include several important events: (1) the activation of the inhibitors by the activators, (2) the movement of inhibitors between cells, (3) the repression of the activators by the inhibitors, and (4) the auto-repression of the inhibitors. These events created differences between cells and ultimately result in a pattern of trichome and non-trichome cells. This model of action of R3 MYBs is supported both by the binding between R3 MYBs and GL3 and the cell-to-cell movement of MYBs. For example, all seven R3 MYBs have been shown to interact with GL3 in Arabidopsis protoplasts (Wang et al., 2008; Gan et al., 2011). CPC and ETC3 have been shown to be able to move from cell to cell (Kurata et al., 2005; Wester et al., 2009). Although the cell-to-cell movement of other R3 MYBs has not been experimentally demonstrated, the cell-to-cell movement motif is conserved in all seven R3 MYBs (**Figure 1**).

Recent studies revealed that R3 MYBs also use other distinctive mechanisms to regulate trichome development. Both TCL1 and TCL2 can directly suppress the transcription of *GL1* (Wang et al., 2007; Gan et al., 2011), a member of the TTG1-GL3/EGL3-GL1 activator complex. Chromatin immunoprecipitation assay indicated that TCL1 or its protein complex can directly bind the cis-acting elements that are required for the proper expression of *GL1* (Wang et al., 2007). Overexpression of TCL1 resulted in suppression of *GL1* and loss-of-function of TCL1 resulted in elevated expression of *GL1* (Wang et al., 2007). Thus, some R3 MYBs, such as TCL1 and TCL2, have dual roles in disrupting the functionality of the activator complex: competing with GL1 for binding GL3 and suppressing the expression of *GL1*. The suppression of *GL1* expression by R3 MYBs provides an additional regulation loop to control the activity of activator complex. However, it is unknown if this represent a general mechanism for all members of R3 MYBs. It has been shown that overexpression of *CPC* could also drastically suppress the expression of *GL1* (Wang et al., 2007), suggesting other members of R3 MYB family could also function in a similar manner as TCL1 to directly suppress the transcription of *GL1*. However, it remains elusive whether such a suppression mechanism may also operate similarly during root

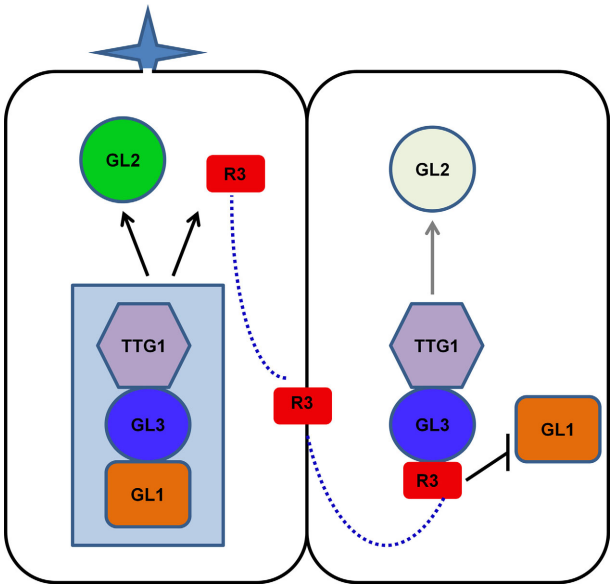

FIGURE 3 | Model of action of R3 MYB transcription factors in controlling trichome formation. TTG1, GL3/EGL3, and GL1 form an activator complex to regulate the transcription of *GL2* and R3 MYB genes. R3 MYBs can move from a trichome precursor cell (left) to its neighboring cell (right) to compete with GL1 for binding GL3 or EGL3, thus limiting the activity of the TTG1-GL3/EGL3-GL1 activator complex and resulting in decreased expression of *GL2*. In addition, some R3 MYBs, such as TCL1 and TCL2, can suppress the transcription of *GL1*. These events create differences between cells and ultimately result in a pattern of trichome and non-trichome cells. For simplicity, EGL3 is not shown in the model. R3, R3 MYBs.

hair patterning because overexpression of *CPC* was not able to change either the expression pattern or expression level of *WER* in roots (Song et al., 2011).

Cell fate in both trichome and root hair development is determined by competition between positive regulators and negative regulators. For both systems, models explaining pattern formation are similar. These models are largely based on a feedback loop in which the positive regulators activate the negative regulators and the negative regulators inhibit the activators, with the negative regulators being able to move between cells. Although the same machinery is central to the spatial regulation of root hair and trichome patterning, the context is different. Trichome formation can occur without a recognizable reference to other structures except for other trichomes whereas root hair patterning is position-dependent with root hairs normally form only in epidermal cells overlying a cleft between two underlying cortex cells. Such a position-dependent pattern is dependent on a leucine-rich repeat receptor-like kinase SCRAMBLED (SCM) which inhibits *WER* expression in the H cell position (Kwak et al., 2005; Kwak and Schiefelbein, 2007).

Since both *GL2* and R3 MYB genes are activated by the same TTG1-GL3/EGL3-GL1 activator complex but having opposite roles in trichome formation, one might wonder the relationship between GL2 and R3 MYBs in regulating trichome formation. One genetic approach answering this question is to analyze double mutants and higher order mutants between *gl2* and R3 MYB mutants. By doing that, it was found that R3 MYBs could still negatively regulate trichome formation in a redundant manner in *gl2* mutant background (Wang et al., 2010). These studies suggested that R3 MYBs could control trichome formation in a GL2-independent manner. The implication of these studies is that trichome formation may require the expression of other genes in addition to *GL2*. These studies called for additional models for the regulation of trichome development.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

R3 MYBs are best characterized for their regulatory roles in trichome and root hair development. In the last two decades, great progress has been made to elucidate the molecular mechanism of action of R3 MYBs in cell type specification. However, several questions remained to be answered. Firstly, the regulation of expression of R3 MYB genes needs further exploration. Not all members of R3 MYB genes are regulated by the activator complex. Only the expression of *TRY*, *CPC*, *ETC1*, and *ETC3* were found to be regulated by the TTG1-GL3/EGL3-GL3 activator complex. The expression of *TCL1*, *TCL2*, and *TRY* can be regulated by *MIR156*-mediated SPLs. Little is known about how the expression of *ETC2* is regulated. When tested in protoplasts, none of the SPLs tested could activate *Gal4:GUS* reporter gene when recruited to the *Gal4* promoter by a Gal4 DNA binding domain, suggesting that SPLs may need co-activator(s) to activate the transcription of R3 MYB genes (Gan et al., 2011). Secondly, the molecular mechanism of action of R3 MYBs in regulating trichome formation needs to be investigated further. It has been previously thought that R3 MYBs inhibit trichome formation via their competition with GL1 for binding GL3/EGL3, thus limiting the activity of the TTG1-GL3/EGL3-GL1 activator complex and resulting in the down regulation of *GL2*. Now it is known some R3 MYBs, such as TCL1 and TCL2, also directly suppress the expression of *GL1*, which also results in the down regulation of *GL2* that is required for trichome formation. Adding another layer of complexity, recent results suggested R3 MYBs may regulate trichome formation in a GL2-independent manner (Wang et al., 2010). Therefore, the relationship between GL2 and R3 MYB genes in the regulation of trichome formation deserves further investigation. A comprehensive systems approach integrating genetic, genomic and computational analyses would be essential for dissecting complex regulatory networks (Bruex et al., 2012). Thirdly, the movement of R3 MYBs and components of activator complex deserves further investigation. Mathematical model suggested that the movement of both R3 MYBs and GL3 are important for root epidermal patterning (Savage et al., 2008). However, discrepancies in the movement of components of activator complex have been observed. In roots, it was reported that GL3 protein moves from the hair cells to the non-hair cells (Bernhardt et al., 2005). However, in a particle bombardment experiment using GFP fusion proteins, it was found that TRY and CPC, but not GL1 or GL3, can move between cells (Digiuni et al., 2008). In a similar particle bombardment experiment, it was shown that none of TTG1, GL3, GL1 or GL2 moves between adjacent epidermal cells whereas CPC moves to neighboring cells (Zhao et al., 2008). Furthermore, it is unclear whether the cell-to-cell movement of

R3 MYB proteins is directional. If so, what controls the directional movement of R3 MYB proteins? A recent study has shed lights on the mechanism of possible directional cell-to-cell movement of R3 MYB proteins (Kang et al., 2013). Specifically, CPC is preferentially expressed in non-hair cells but acts in the root-hair cells. This directional cell-to-cell movement of CPC involves EGL3 which traps CPC protein in the root hair cells (Kang et al., 2013). Fourthly, trichome and root hair patterning involve both negative and positive feedback within and between cells. Much is known about the negative feedback mechanism in which R3 MYBs disrupt the functionality of TTG1-bHLH-MYB activator complex but less is known about the positive feedback. In root hair patterning, MYB23 functions in the positive feedback loop which can substitute the function of WER and bind to its own promoter (Kang et al., 2009). Such a positive feedback can reinforce cell fate decision and ensure robust establishment of the cell type pattern. Finally, R3 MYBs are wildly distributed in plant kingdom. It would be of great interest to examine if they regulate trichome formation in other plant species in a similar manner as that in Arabidopsis. Emerging evidence supports that this is likely the case (e.g., Tominaga-Wada et al., 2013a). Trichomes in many plant species (e.g., cotton fiber) are of great economic values. Manipulation of R3 MYBs expression can potentially contribute to the increase of productivity and quality in economic crops.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant no. 31170262), the Program for Introducing Talents to Universities (B07017) and a startup fund from Northeast Normal University to Shucai Wang, and by the Laboratory Directed Research and Development Program of Oak Ridge National Laboratory to Jin-Gui Chen. Oak Ridge National Laboratory is managed by UT-Battelle, LLC, for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725. This manuscript has been authored by UT-Battelle, LLC, under Contract No. DE-AC05-00OR22725 with the U.S. Department of Energy. The United States Government retains and the publisher, by accepting the article for publication, acknowledges that the United States Government retains a non-exclusive, paid-up, irrevocable, world-wide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00133/abstract

Figure S1 | Amino acid sequence alignment of R3 MYBs with other MYBs. Identical and similar amino acids are shaded in black and gray, respectively. The amino acids of [D/E]Lx$_2$[R/K]x$_3$Lx$_6$Lx$_3$R signature that is required for interaction between MYBs and R/B-like bHLH transcription factors are indicated by red arrowheads on the top of amino acids. The amino acids of WxM motif that is crucial for cell-to-cell movement are indicated by blue arrowhead on the top of amino acids. The pattern of primary structure of MYB repeat, Mx$_{18}$Wx$_{18}$W, is indicated by arrows on the bottom of amino acids.

Figure S2 | Phylogenetic analysis of R3 MYBs with other MYBs. The entire amino acid sequences of single repeat R3 MYB transcription factors were used for phylogenetic analysis using tool on www.phylogeny.fr with default settings.

Figure S3 | Amino acid similarity and identity of R3 MYBs and other MYBs.

Figure S4 | Amino acid sequence alignment of ETC3 variants. Identical and similar amino acids are shaded in black and gray, respectively. The amino acids of [D/E]Lx$_2$[R/K]x$_3$Lx$_6$Lx$_3$R signature that is required for interaction between MYBs and R/B-like bHLH transcription factors are indicated by arrowheads on the top of amino acids. The amino acids of WxM motif that is crucial for cell-to-cell movement are indicated by arrowheads on the bottom of amino acids.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 19 February 2014; paper pending published: 10 March 2014; accepted: 20 March 2014; published online: 08 April 2014.

*Citation: Wang S and Chen J-G (2014) Regulation of cell fate determination by single-repeat R3 MYB transcription factors in Arabidopsis. Front. Plant Sci. **5**:133. doi: 10.3389/fpls.2014.00133*

This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 10 June 2014
doi: 10.3389/fpls.2014.00262

Control of trichome formation in *Arabidopsis* by poplar single-repeat R3 MYB transcription factors

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In *Arabidopsis*, trichome formation is regulated by the interplay of R3 MYBs and several others transcription factors including the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1), the R2R3 MYB transcription factor GLABRA1 (GL1), the bHLH transcription factor GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3), and the homeodomain protein GLABRA2 (GL2). R3 MYBs including TRICHOMELESS1 (TCL1), TCL2, TRYPTICHON (TRY), CAPRICE (CPC), ENHANCER OF TRY AND CPC1 (ETC1), ETC2 and ETC3 negatively regulate trichome formation by competing with GL1 for binding GL3 or EGL3, thus blocking the formation of TTG1–GL3/EGL3–GL1, an activator complex required for the activation of the trichome positive regulator gene *GL2*. However, it is largely unknown if R3 MYBs in other plant species especially woody plants have similar functions. By BLASTing the *Populus trichocarpa* protein database using the entire amino acid sequence of TCL1, an *Arabidopsis* R3 MYB transcription factor, we identified a total of eight R3 MYB transcription factor genes in poplar, namely *P. trichocarpa TRICHOMELESS1* through *8* (*PtrTCL1–PtrTCL8*). The amino acid signature required for interacting with bHLH transcription factors and the amino acids required for cell-to-cell movement of R3 MYBs are not fully conserved in all PtrTCLs. When tested in *Arabidopsis* protoplasts, however, all PtrTCLs interacted with GL3. Expressing each of the eight *PtrTCL* genes in *Arabidopsis* resulted in either glabrous phenotypes or plants with reduced trichome numbers, and expression levels of *GL2* in all transgenic plants tested were greatly reduced. Expression of *PtrTCL1* under the control of *TCL1* native promoter almost completely complemented the mutant phenotype of *tcl*. In contrast, expression of *PtrTCL1* under the control of *TRY* native promoter in the *try* mutant, or under the control of *CPC* native promoter in the *cpc* mutant resulted in glabrous phenotypes, suggesting that PtrTCL1 functions similarly to TCL1, but not TRY and CPC.

Keywords: trichome formation, R3 MYBs, transcription factors, *Arabidopsis*, *Populus trichocarpa*

INTRODUCTION

Single-repeat R3 MYB transcription factors (R3 MYBs) are small proteins that typically contain ∼100 amino acids, largely consist of a single MYB DNA-binding repeat, and are best characterized for their regulatory roles in trichome and root hair development (Wang and Chen, 2014). R3 MYBs are widely distributed in the plant kingdom, and they are encoded by a small subset of MYB transcription factor genes (Dubos et al., 2010). In *Arabidopsis*, there are a total of seven genes encoding R3 MYBs, including *TRYPTICHON* (*TRY*; Schnittger et al., 1999), *CAPRICE* (*CPC*; Wada et al., 1997), *TRICHOMELESS1* (*TCL1*; Wang et al., 2007), *TCL2*/*CAPRICE-LIKE MYB4* (*CPL4*; Gan et al., 2011; Tominaga-Wada and Nukumizu, 2012), *ENHANCER OF TRY AND CPC1* (*ETC1*; Esch et al., 2004; Kirik et al., 2004a), *ETC2* (Kirik et al., 2004b), and *ETC3*/*CPL3* (Simon et al., 2007; Tominaga et al., 2008). All seven R3 MYBs contain the residues [D/E]L×2[R/K]×3L×6L×3R, a conserved amino acid signature required for interaction of MYBs with R/B-like bHLH transcription factors (Zimmermann et al., 2004), and W×M, a sequence motif that has been shown to be required for cell-to-cell movement of CPC (Kurata et al., 2005).

Trichome formation in *Arabidopsis* is controlled by the interplay of R3 MYBs and several other transcription factors including the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1; Walker et al., 1999), the R2R3 MYB-type transcription factor GLABRA1 (GL1; Oppenheimer et al., 1991), the bHLH transcription factor GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3; Payne et al., 2000; Zhang et al., 2003), and the homeodomain protein GLABRA2 (GL2; Rerie et al., 1994). TTG1, GL1, and GL3 or EGL3 form an activator complex to induce the expression of *GL2*, a positive regulatory gene of trichome formation (Rerie et al., 1994). The same TTG1–GL3/EGL3–GL1 activator complex can also induce the expression of R3 MYB genes. R3 MYBs can move from a trichome precursor cell to its neighboring cells, and compete with GL1 for binding GL3 or EGL3, thus limiting the formation of the activator complex (Hülskamp et al., 1994; Schellmann et al., 2002; Esch et al., 2003; Schiefelbein, 2003; Pesch and Hülskamp, 2004; Ishida et al., 2008), resulting in inhibition of trichome initiation.

In addition to competing with GL1 for binding GL3, some of the R3 MYBs including TCL1 and TCL2 also directly suppress the expression of *GL1* (Wang et al., 2007; Gan et al., 2011). Not all the R3 MYB genes in *Arabidopsis* are activated by the TTG1–GL3/EGL3–GL1 activator complex (Wang et al., 2008), and *microRNA156* (*MIR156*)-targeted SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) 9 has been shown to activate *TCL1*, *TCL2*, and *TRY* (Yu et al., 2010; Gan et al., 2011). These results indicate that R3 MYBs may use different mechanisms to regulate trichome formation in *Arabidopsis*.

Although single mutants of *Arabidopsis* R3 MYB genes have different phenotypes (Wada et al., 1997, 2002; Schnittger et al., 1999; Schellmann et al., 2002; Wang et al., 2007), over-expression of any of the R3 MYB genes in *Arabidopsis* resulted in glabrous phenotypes. Analysis of double, triple, and higher mutants also revealed that all the seven R3 MYBs function in a highly redundant manner to control trichome formation (Esch et al., 2004; Kirik et al., 2004a,b; Wang et al., 2007, 2008; Tominaga et al., 2008; Wester et al., 2009; Gan et al., 2011).

Functional homologs of some of these transcription factors that regulate trichome formation have been identified in other plants. For example, GaHOX1 from cotton has been identified as a functional homolog of GL2 (Guan et al., 2008) and GaMYB2 as a functional homolog of GL1 (Wang et al., 2004; Guan et al., 2011, 2014). MYB like genes from *Mimulus guttatus* and peach regulate trichome formation (Scoville et al., 2011; Vendramin et al., 2014), and expression of a tomato R3 MYB gene in *Arabidopsis* resulted in glabrous phenotypes (Tominaga-Wada et al., 2013). On the other hand, expression of *Arabidopsis GL3* in *Brassica napus* induced ectopic trichome formation (Gruber et al., 2006). These results suggest that trichome formation in other plant species may be controlled by similar mechanisms as in *Arabidopsis*. However, trichome regulators in plants other than *Arabidopsis* remain largely unidentified.

Populus trichocarpa is the first tree whose genome has been fully sequenced (Tuskan et al., 2006), and it is also a good model plant for studies in areas such as wood development, ecological interactions, and other aspects of perennial plants that cannot be studied in the annual model plant *Arabidopsis* (Groover, 2005; Whitham et al., 2006; Jansson and Douglas, 2007). By using the entire amino acid sequence of TCL1 to BLAST search the *P. trichocarpa* protein database, we found there are a total of eight genes in poplar encoding R3 MYB transcription factors, namely *P. trichocarpa TRICHOMELESS1* through *8* (*PtrTCL1–PtrTCL8*). In the study described here, we examined if R3 MYBs from poplar can functionally substitute for *Arabidopsis* R3 MYBs to regulate trichome formation in *Arabidopsis* plants.

MATERIALS AND METHODS

IDENTIFICATION OF POPLAR HOMOLOGS OF *Arabidopsis* R3 MYB TRANSCRIPTION FACTOR TCL1

To identify poplar homologs of *Arabidopsis* R3 MYB transcription factors, the entire amino acid sequence of *Arabidopsis* R3 MYB transcription factor TCL1 was used in BLAST searches of the *P. trichocarpa* proteome (www.phytozome.net). The entire amino acid sequences of identified poplar R3 MYB transcription factors were then used in BLAST searches until no more poplar R3 MYBs were identifiable. Full-length amino acid sequences of *Arabidopsis* and poplar R3 MYBs were subjected to phylogenetic analysis using "One Click" mode of Phylogeny (www.phylogeny.fr) with default settings.

PLANT MATERIALS AND GROWTH CONDITIONS

Poplar xylem tissue from *P. trichocarpa* was collected as described previously (Geraldes et al., 2011; Liu et al., 2013; Wang et al., 2014), and used for RNA isolation and poplar R3 MYB gene cloning. The *tcl1* and *try* mutants, and the *35S:HA-TCL1* transgenic plant were in the Columbia-0 (Col-0) background (Esch et al., 2003; Wang et al., 2007). The *cpc* mutant was in the Ws background (Wada et al., 1997).

Unless specified otherwise, *Arabidopsis* ecotype Col-0 was used for plant transformation. Seedlings used for RNA isolation were obtained by growing sterilized seeds on 1/2 Murashige and Skoog (MS) basal medium with vitamins (Plantmedia) and 1% (w/v) sucrose. Seedlings used for phenotypic analysis were obtained either by growing seeds on 1/2 MS medium or by directly sowing seeds into soil. All plants were grown in growth rooms at 22°C with 14/10 h photoperiod, and light density of approximately 120 μmol m^{-2} s^{-1}.

RNA ISOLATION AND RT-PCR

Total RNA from poplar samples was isolated using PureLink Plant RNA Reagent (Invitrogen), and cleaned with RNeasy Plant Mini Kit (Qiagen) as described previously (Geraldes et al., 2011; Wang et al., 2014). Total RNA from *Arabidopsis* seedlings was isolated using EasyPure^{TM} Plant RNA Kit (Transgene) according to the manufacturer's instructions. All RNA samples were treated with RNase-Free DNase set (Qiagen) to eliminate possible DNA contamination.

cDNA was synthesized using 2 μg total RNA by Oligo(dT)-primed reverse transcription using Omniscript RT Kit (Qiagen). Some of the primers used for cloning or examining the expression of corresponding genes have been described previously (Wang et al., 2007, 2008, 2010; Gan et al., 2011), and poplar R3 MYB gene-specific primers are shown in **Table 1**.

CONSTRUCTS

To generate HA (human influenza hemagglutinin)- or GD (Gal4 DNA binding domain)-tagged constructs for poplar R3 MYB genes, the full-length, open-reading frames (ORF) of corresponding poplar R3 MYB genes were amplified by RT-PCR using RNA isolated from poplar xylem samples, and the PCR products were then cloned in-frame with an N-terminal HA or GD tag into the *pUC19* vector under the control of the double *35S* enhancer promoter of *CaMV* (Wang et al., 2005).

The *35S:PtrTCL1-GFP* construct was cloned by fusing *PtrTCL1* in frame with GFP (Green fluorescent protein) and then cloned into the *pUC19* vector under the control of the *35S* promoter. The *TCL1p:HA-PtrTCL1*, *TRYp:HA-PtrTCL1*, and *CPCp:HA-PtrTCL1* constructs were cloned by replacing the *35S* promoter in *35S:HA-PtrTCL1* with *TCL1*, *TRY*, and *CPC* promoters, respectively (Wada et al., 1997; Esch et al., 2003; Wang et al., 2007).

Table 1 | Primers using for poplar R3 MYB gene cloning and expression assays.

Primers	Sequences
PtrTCL1-Nde1F	5′-CAACATATGGATAGACGTCGCAGG-3′
PtrTCL1-Sac1R	5′-CAAGAGCTCTTAAGAGGTATTAGGAATTAC-3′
PtrTCL2-Nde1F	5′-CAACATATGGATAGACGTCGCAAG-3′
PtrTCL2-Sac1R	5′-CAAGAGCTCTTAGGAATGACATCTC-3′
PtrTCL3-Nde1F	5′-CAACATATGGAGAGTATGAACCGC-3′
PtrTCL3-Sac1R	5′-CAAGAGCTCTCAACTAGAAGTCCTAG-3′
PtrTCL4-Nde1F	5′-CAACATATGTCCCTTCAATTTCAC-3′
PtrTCL4-Sac1R	5′-CAAGAGCTCTTACTGACTTGTAGAGTATC-3′
PtrTCL5-Nde1F	5′-CAACATATGGCTGACTTGGATCAC-3′
PtrTCL5-Sac1R	5′-CAAGAGCTCTTACTGACTTGTAGAGTATC-3′
PtrTCL6-Nde1F	5′-CAACATATGGCTGACTCTGAACATTC-3′
PtrTCL6-Sac1R	5′-CAAGAGCTCTCATTCACTTGTAGAGTATC-3′
PtrTCL7-Nde1F	5′-CAACATATGGCTGACACTGAACATTC-3′
PtrTCL7-Sac1R	5′-CAAGAGCTCTCATTCACTCGTAGAGC-3′
PtrTCL8-Nde1F	5′-CAACATATGGCTTGCTCGGGTCAC-3′
PtrTCL8-Sac1R	5′-CAAGAGCTCTCATCTTTCCTTTGATGATC-3′

For plant transformation, corresponding constructs in the *pUC19* vector were digested with *EcoRI* and subcloned into the binary vector *pPZP211* or *pPZP221* (Hajdukiewicz et al., 1994).

PLANT TRANSFORMATION AND TRANSGENIC PLANT SELECTION

About five-week-old plants with several mature flowers on the main inflorescence were used for plant transformation. Plants were transformed by using the floral dip method via *Agrobacterium tumefaciens* GV3101 (Clough and Bent, 1998). T1 seeds were germinated on plates containing antibiotics to select transgenic plants. For each construct, more than 70 transgenic lines were obtained. Phenotypes of transgenic plants were examined in the T1 generation and at least five transgenic lines with similar phenotypes were collected. The phenotypes observed were confirmed in the following two to three generations. Expression of corresponding genes in related lines was confirmed by RT-PCR. Homozygous T3 or T4 seeds were used for further experiments, and data from one representative line for each construct are presented.

PLASMID DNA ISOLATION, PROTOPLAST TRANSFECTION, AND GUS ACTIVITY ASSAY

All reporter and effector plasmids were prepared using the GoldHi EndoFree Plasmid Maxi Kit (Kangwei) according to the manufacturer's instructions. The procedures for protoplast isolation, transfection, and GUS activity assay have been described previously (Tiwari et al., 2003; Wang et al., 2005, 2008; Wang and Chen, 2008). Briefly, protoplasts were isolated from rosette leaves collected from ∼4-week-old *Arabidopsis* plants. Effector and reporter plasmids were co-transfected into protoplasts and incubated at room temperature for 20–22 h under darkness. GUS activities were measured using a Synergy^{TM} HT microplate reader (BioTEK).

MICROSCOPY

Trichomes were analyzed and photographed using a Motic K microscope equipped with a Canon digital camera. Localization of PtrTCL1-GFP proteins in transgenic plants expressing *PtrTCL1–GFP* under the control of the *35S* promoter was examined under an Olympus FV1000 confocal microscope. Protoplast cells isolated from the *PtrTCL1–GFP* transgenic plants were stained with DAPI and then examined under an Olympus FV1000 microscope.

RESULTS

IDENTIFICATION OF R3 MYB TRANSCRIPTION FACTORS IN POPLAR

A total of eight poplar R3 MYB transcription factors were identified, and collectively named as PtrTCL1 to PtrTCL8. Corresponding gene names for the PtrTCLs identified are as follows: *PtrTCL1*, *Potri.002G168900*; *PtrTCL2*, *Potri.014G096300*; *PtrTCL3*, *Potri.015G022000*; *PtrTCL4*, *Potri.007G122800*; *PtrTCL5*, *Potri.017G03700*; *PtrTCL6*, *Potri.011G026300*; *PtrTCL7*, *Potri.004G021300*; and *PtrTCL8*, *Potri.004G015100*.

Similar to their homologs in *Arabidopsis*, nearly the entire protein of poplar R3 MYBs is made up of the single MYB domain (**Figure 1A**). The amino acid signature [D/E]L×2[R/K]×3L×6L×3R, that is required for the interaction with R/B-like bHLH transcription factors (Zimmermann et al., 2004), is fully conserved in all seven R3 MYB transcription factors in *Arabidopsis* (Wang et al., 2007, 2008; Gan et al., 2011), but is found in only three poplar R3 MYBs including PtrTCL1, PtrTCL2, and PtrTCL3 (**Figure 1A**). In the other four poplar R3 MYBs, D/E, the first amino acid in the amino acid signature is replaced by T/N (**Figure 1A**). Similarly, the amino acid motif W×M, that has been shown to be required for the cell-to-cell movement of CPC (Kurata et al., 2005), is conserved in all seven *Arabidopsis* R3 MYBs (Wang et al., 2007, 2008; Gan et al., 2011), but is found in only PtrTCL1, PtrTCL2, and PtrTCL3 (**Figure 1A**). In the other four poplar R3 MYBs, the M in the motif is replaced by S/T (**Figure 1A**).

Phylogenetic analysis using full-length protein sequences of poplar R3 MYBs and TCL1 showed that the clade of PtrTCL1–PtrTCL3 is most closely related to the clade of TCL1, TCL2, ETC2 and TRY (**Figure 1B**). Together, PtrTCL1–PtrTCL3 and seven *Arabidopsis* R3 MYBs formed one subgroup, and PtrTCL4–PtrTCL8 formed another subgroup.

PtrTCLs INTERACT WITH GL3

We have previously demonstrated that TRY, CPC, ETC1, ETC2, TCL1, TCL2, and ETC3 interact with GL3 in plant cells (Wang et al., 2008; Gan et al., 2011), supporting the proposal that R3 MYBs control trichome formation in *Arabidopsis* by competing with GL1 for binding GL3, and thus eliminating the formation of TTG1–GL3–GL1 activator complex. Considering that only three of the identified poplar R3 MYBs have the fully conserved amino acid signature that is required for the interaction with R/B-like bHLH transcription factors, we tested if PtrTCLs interact with GL3 in plant cells.

Arabidopsis protoplast transfection assays were used to test the interaction between PtrTCLs and GL3. Plasmids of the reporter gene *Gal4-GUS*, together with the effector genes *GL3* and GD

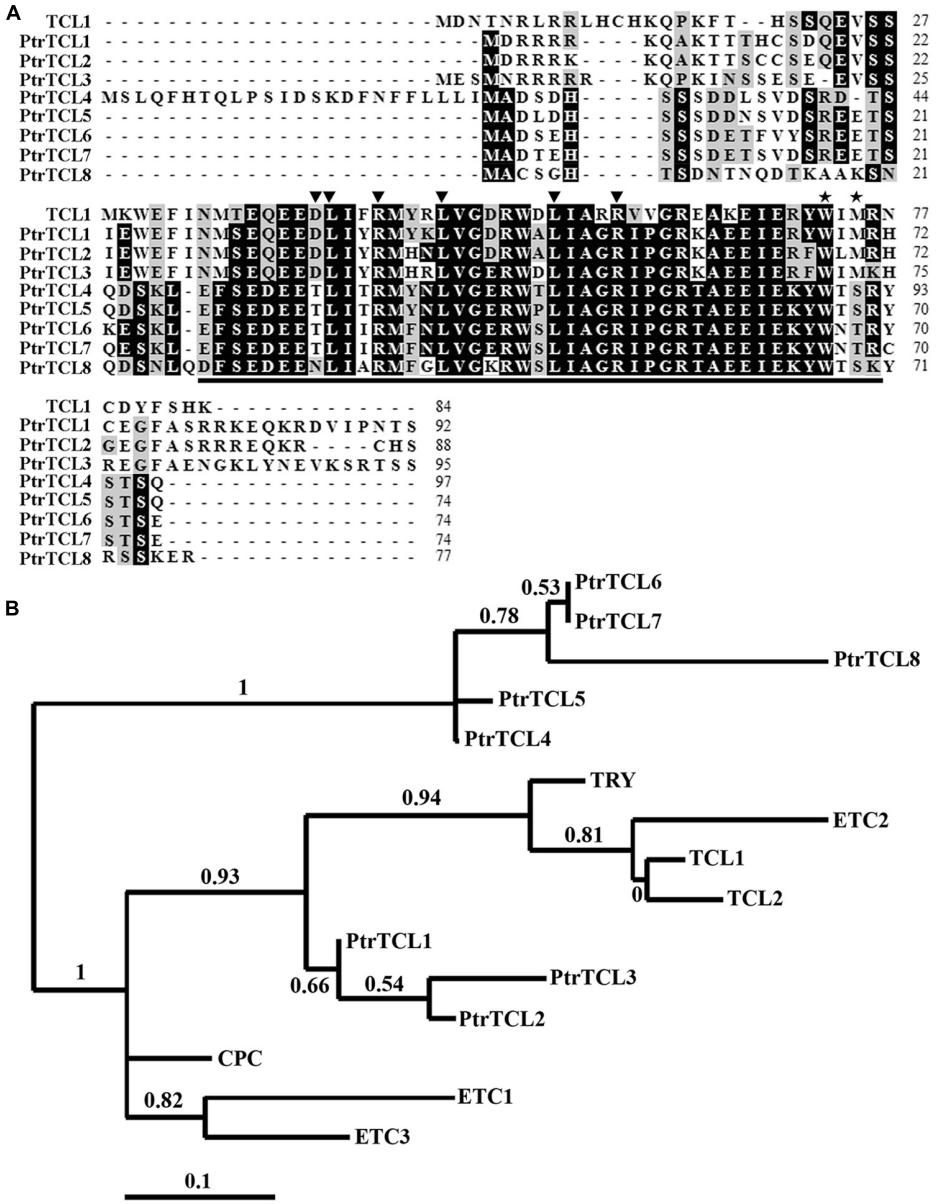

FIGURE 1 | R3 MYB transcription factors in poplar. (A) Sequence alignment of TCL1 with eight poplar R3 MYB proteins. Identical amino acids are shaded in black and similar amino acids in gray. The R3 MYB domain is indicated by underline, the amino acid signature [D/E]L×2[R/K]×3L×6L×3R that is required for interacting with R/B-like BHLH transcription factors is indicated by arrowheads, and the amino acids within the MYB domain that are crucial for cell-to-cell movement of CPC are indicated by asterisks. **(B)** Phylogenetic analysis of seven *Arabidopsis* and the eight poplar R3 MYB transcription factors. The entire amino acid sequences were used to generate the phylogenetic tree by using "One Click" mode of Phylogeny (www.phylogeny.fr) with default settings. Branch support values are indicated above or below branches, bar indicates branch length.

fused PtrTCLs (*GD–PtrTCLs*; **Figure 2**), were co-transfected into *Arabidopsis* protoplasts. *GD* and *GD–TCL1* were used as negative and positive controls, respectively. As expected, neither GD nor GD–TCL1 activated the reporter gene in the absence of GL3. In the presence of GL3, GD–TCL1 but not GD activated the reporter gene. Similarly, none of the eight poplar R3 MYBs activated the reporter gene in the absence of GL3; however, all of them activated the reporter gene in the presence of GL3 (**Figure 2**).

PtrTCLs NEGATIVELY REGULATE TRICHOME FORMATION WHEN EXPRESSED IN *Arabidopsis*

Having shown that all the eight poplar R3 MYBs interact with GL3 in plant cells, we further analyzed if PtrTCLs regulate trichome formation by generating transgenic *Arabidopsis* plants expressing HA-tagged PtrTCLs under the control of *35S* promoter (*35S:HA-PtrTCLs*). As shown in **Figure 3A**, expression of *PtrTCL1*, *PtrTCL2*, *PtrTCL3*, *PtrTCL5*, *PtrTCL7* and *PtrTCL8* in *Arabidopsis* resulted in glabrous phenotypes, similar to the phenotype observed in

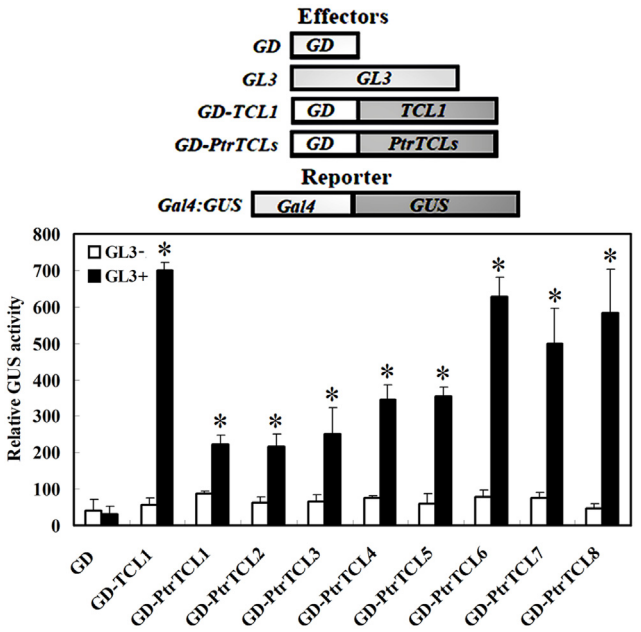

FIGURE 2 | Poplar R3 MYBs interact with GL3 in plant cells. Reporter gene and effector gene plasmids were co-transfected into protoplasts isolated from *Arabidopsis* rosette leaves. Protoplasts were incubated in darkness for 20–22 h after transfection, and then GUS activity was measured. Data represent the mean±SD of three replicates. Reporter and effector constructs are diagrammed at the top of the figure. *significantly different from the absence of GL3 (GL3-; $P < 0.001$).

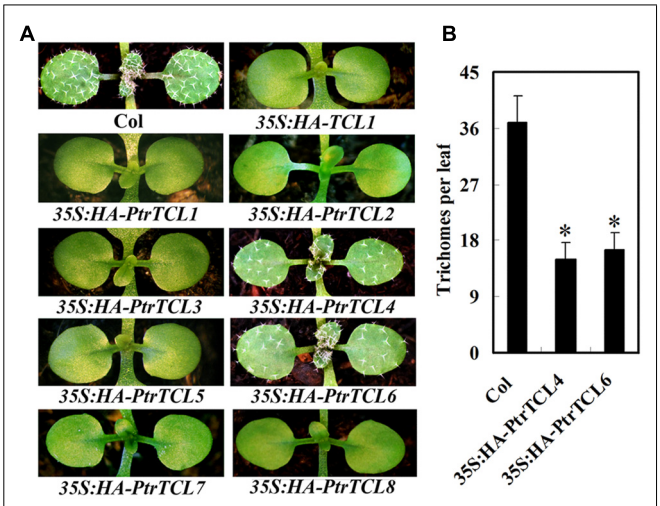

FIGURE 3 | Poplar R3 MYBs negatively regulate trichome formation in *Arabidopsis*. **(A)** Trichome phenotypes in *Arabidopsis* plants expressing poplar R3 MYB genes. All transgenic lines are in Col background. Pictures were taken from 2-week-old, soil-grown seedlings. **(B)** Trichome density on the first two rosette leaves of *Arabidopsis* plants expressing *PtrTCL4* and *PtrTCL6*. Data represent the mean±SD of 18 plants. *significantly different from Col wild-type plants ($P < 0.001$).

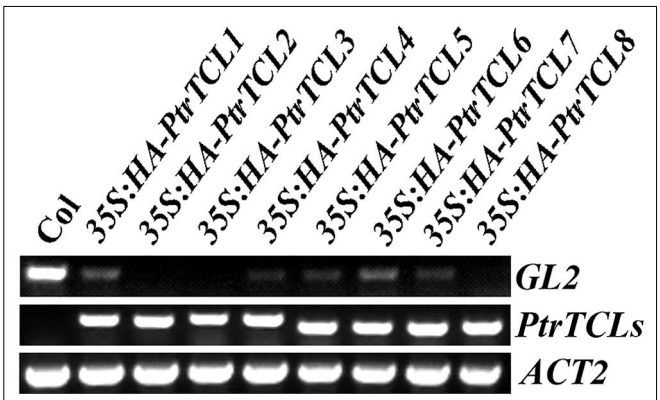

FIGURE 4 | Expression of *GL2* and poplar R3 MYB genes in transgenic *Arabidopsis* plants. RNA was isolated from Col wild-type and transgenic *Arabidopsis* seedlings, RT-PCR was used to examine the expression of *GL2* and polar R3 MYB genes. The expression of *ACTIN2* (*ACT2*) was used as a control.

plants over-expressing *TCL1* (Wang et al., 2007). In contrast, transgenic plants expressing *PtrTCL4* and *PtrTCL6* had greatly reduced number of trichomes on rosette leaves, but none of the transgenic plants obtained showed glabrous phenotypes (**Figure 3**). RT-PCR analysis showed that poplar R3 MYB genes were highly expressed in their corresponding transgenic lines (**Figure 4**), indicating that the phenotypes observed in *PtrTCL4* and *PtrTCL6* transgenic plants were not due to relative lower expression levels of the corresponding genes.

GL2 is one of the target genes of the activator complex TTG1–GL3/EGL3–GL1, and it positively regulates trichome formation in *Arabidopsis*. Because binding of PtrTCLs to GL3 indicated that expression of *PtrTCLs* in *Arabidopsis* resulted in the inhibition of the formation of TTG1–GL3/EGL3–GL1 activator complex, we examined the expression of *GL2* in *Arabidopsis* transgenic plants expressing poplar R3 MYB genes. As shown in **Figure 4**, expression of *GL2* was dramatically reduced in transgenic plants expressing any of the poplar R3 MYB genes.

SUBCELLULAR LOCALIZATION OF PtrTCL1

Among the eight poplar R3 MYB transcription factors, PtrTCL1 has highest amino acid similarity with TCL1, and phylogenetic analysis also showed that the clade of PtrTCL1–PtrTCL3 is most closely related to TCL1 (**Figure 1**). We wanted to further explore the functions of poplar R3 MYB transcription factors in the regulation of trichome formation in *Arabidopsis* by taking PtrTCL1 as an example. We first examined the subcellular localization of the PtrTCL1 protein by generating transgenic plants expressing *PtrTCL1–GFP* under the control of the *35S* promoter. As shown in **Figure 5A**, expression of *PtrTCL1–GFP* in *Arabidopsis* resulted in a glabrous phenotype, a phenotype similar to transgenic plants expressing *PtrTCL1* (**Figure 3**). This indicated that the PtrTCL1–GFP fusion protein is likely functional. By examining the transgenic plants obtained, we found that GFP florescence was mainly observed in the nucleus of epidermal cells (**Figure 5B**); examination of DAPI stained protoplast cells isolated from the *PtrTCL1–GFP* transgenic plants confirmed that the GFP was mainly observed in the nucleus (**Figure 5C**), indicating that PtrTCL1 is a nuclear localized protein.

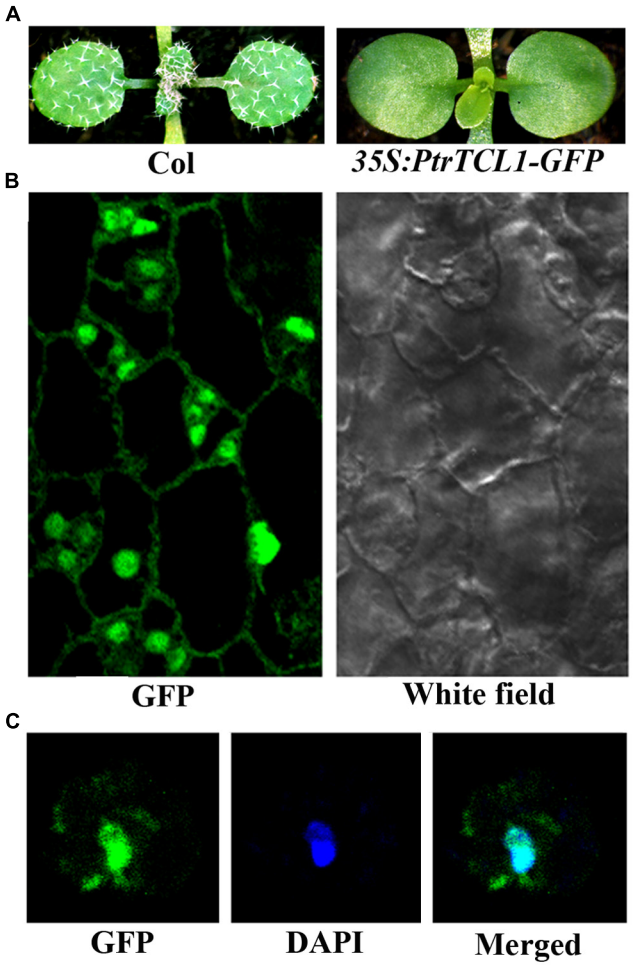

FIGURE 5 | Subcellular localization of PtrTCL1. (A) Expression of *PtrTCL1-GFP* under the control of *35S* promoter in *Arabidopsis* resulted in a glabrous phenotype. Pictures were taken from 2-week-old, soil-grown seedlings. **(B)** GFP fluorescence in the epidermal cells of the rosette leaves in a 2-week-old transgenic plant expressing *PtrTCL1-GFP* under the control of the *35S* promoter. Left panel: GFP channel, right panel: white field image. **(C)** GFP fluorescence in a protoplast cell isolated from transgenic plant expressing *PtrTCL1-GFP* under the control of the *35S* promoter. Left panel: GFP channel; Middle panel: DAPI channel; right panel: merged.

PtrTCL1 ALMOST COMPLETELY RESCUED *tcl1* MUTANT TRICHOME PHENOTYPES

Previously, we identified *TCL1* as a major R3 MYB transcription factor that regulates trichome formation on the inflorescences and pedicels, and we showed that knock-out of *TCL1* resulted in ectopic trichome formation on the inflorescence stems and pedicels. In addition, we showed that over-expression of *TCL1* in *Arabidopsis* resulted in a glabrous phenotype (Wang et al., 2007). Here, we showed that PtrTCL1, that is in the most closely related clade to TCL1 (**Figure 1**), resulted in a glabrous phenotype when over-expressed in *Arabidopsis* (**Figure 3**). Therefore, we wanted to further examine if PtrTCL1 is the functional equivalent of TCL1 by testing if *PtrTCL1* could rescue the *tcl1* mutant phenotype when expressed under the control of *TCL1* native promoter.

Transgenic plants were generated in a *tcl1* background to express *PtrTCL1* under the control of the *TCL1* native promoter (*TCL1p:HA-PtrTCL1/tcl1*). Previously, we showed that expression of *TCL1–GFP* under the control of the *TCL1* promoter fully rescued the *tcl1* trichome phenotype (Wang et al., 2007), indicating that the *TCL1* promoter used is functional in *Arabidopsis*. As shown in **Figure 6**, expression of *PtrTCL1* under the control of *TCL1* native promoter almost completely rescued *tcl1* mutant phenotype, i.e., *tcl1* mutant forms ectopic trichomes in both stem internodes above the first flower and pedicels, while *TCL1p:HA-PtrTCL1/tcl1* plants do not have trichomes on stem internodes after the first flowers, but have a few trichomes on first pedicel only. These results indicate that PtrTCL1 is likely the functional equivalent of TCL1 in controlling trichome formation on the inflorescence stems and pedicels.

In addition to *tcl1*, single mutants *try* and *cpc* showed trichome phenotypes; *try* mutants have trichome clusters, and *cpc* mutants have increased numbers of trichome on leaves (Wada et al., 1997; Schnittger et al., 1999), so we also examined if PtrTCL1 is functionally equivalent to *TRY* or *CPC*. Transgenic plants were generated to express *PtrTCL1* under the control of *TRY* native promoter (*TRYp:HA-PtrTCL1/try*) in the *try* background, and under the control of *CPC* native promoter (*CPCp:HA-PtrTCL1/cpc*) in the *cpc* background. As shown in **Figure 7**, expression of *PtrTCL1* under the control of *TRY* native promoter in a *try* mutant background, or under the control of *CPC* native promoter in a *cpc* mutant background resulted in glabrous phenotypes.

DISCUSSION

In this study we identified poplar homologs of *Arabidopsis* R3 MYB transcription factors, and analyzed their function in trichome formation in *Arabidopsis*. We showed that expression of any of the eight poplar R3 MYB genes under the control of the *35S* promoter in *Arabidopsis* resulted in either a glabrous phenotype or a great reduction in trichome numbers (**Figure 3**). These results suggest that poplar R3 MYBs act as negative regulators for trichome formation and may have overlapping functions, similar to their *Arabidopsis* R3 MYB homologs.

Among the eight poplar R3 MYB transcription factors, PtrTCL1 is in the most closely related clade to TCL1 (**Figure 1B**), is localized in the nucleus (**Figure 5**), and expression of *PtrTCL1* under the control of the *TCL1* promoter in the *tcl1* mutant background (*TCL1p:HA-PtrTCL1/tcl1*) almost fully restored the trichome phenotype of the *tcl1* mutant (**Figure 6**). Expression of *PtrTCL1* under the control of the *TRY* native promoter in the *try* background (*TRYp:HA-PtrTCL1/try*), or under the control of the *CPC* native promoter in the *cpc* background (*CPCp:HA-PtrTCL1/cpc*) resulted in glabrous phenotypes (**Figure 7**), suggesting that PtrTCL1 may be the functional equivalent of TCL1, rather than TRY and CPC. It should be noted that ETC2 and TCL2 are also in the clade that is sister to the clade of PtrTCL1–PtrTCL3; however, because no mutant available for *TCL2* and *etc2* mutant does not have any trichome phenotype, we could not test whether PtrTCL1 might be functionally equivalent to TCL2 or ETC2.

In *Arabidopsis*, R3 MYBs inhibit trichome formation by competing with GL1 for binding of GL3 or EGL3, thus inhibiting

FIGURE 6 | Rescue of *tcl1* phenotype by *PtrTCL1*. Trichome formation on inflorescences in Col, *tcl1* and *TCL1p:HA-PtrTCL1/tcl1-1* plants. Pictures were taken from 5-week-old, soil-grown plants.

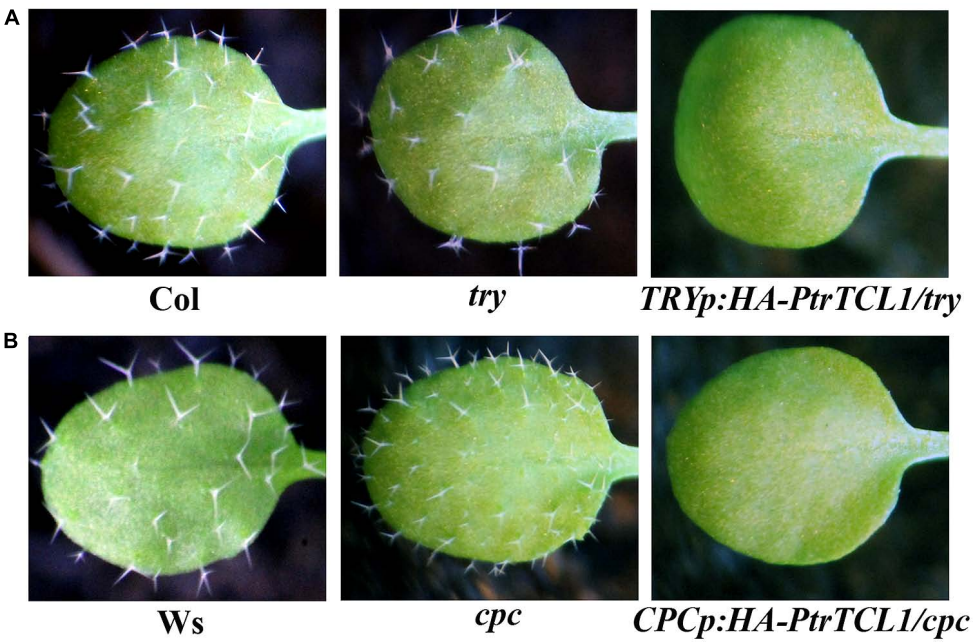

FIGURE 7 | Expression of *PtrTCL1* in *try* and *cpc* resulted in glabrous phenotypes. (A) Expression of *PtrTCL1* under the control of *TRY* native promoter in the *try* mutant background resulted in glabrous phenotype. **(B)** Expression of *PtrTCL1* under the control of *CPC* native promoter in the *cpc* mutant background resulted in glabrous phenotype. Pictures were taken from 2-week-old, soil-grown seedlings.

the formation of the TTG1–GL3/EGL3–GL1 activator complex (Schellmann et al., 2002; Esch et al., 2003; Schiefelbein, 2003; Pesch and Hülskamp, 2004; Ishida et al., 2008). In accordance with these results, all seven *Arabidopsis* R3 MYBs have the conserved [D/E]L×2[R/K]×3L×6L×3R amino acid signature that is required for interaction with R/B-like bHLH transcription factors (Zimmermann et al., 2004), and protoplast transfection assays showed that they all interact with GL3 in plant cells (Wang et al., 2008; Gan et al., 2011). Sequence alignment results showed that only three of the poplar R3 MYBs, namely PtrTCL1, PtrTCL2, and PtrTCL3, have the fully conserved amino acid signature required for interacting with bHLH transcription factors (**Figure 1A**). When tested in protoplasts, however, all the eight poplar R3 MYBs interacted with GL3 (**Figure 2**), and the interaction with GL3 may be stronger for those R3 MYBs without the fully conserved amino acid signature as judged by the GUS activities (**Figure 2**). Our results indicate that either the conserved amino acid signature is not required for the interaction of poplar R3 MYBs with GL3, or a single-amino-acid substitution (D/E > T/N) in poplar R3 MYBs does not affect their interaction with GL3. It is also possible that previous designations of conserved residues may be biased due to a smaller set of proteins analyzed. Furthermore, interaction of poplar R3 MYBs with GL3 in plant cells suggests that poplar R3 MYBs can block the formation of the activator complex required for trichome formation. In accordance with this, RT-PCR results showed that expression of *GL2* is dramatically reduced in the transgenic plants expressing poplar R3 MYB genes (**Figure 4**). Since our previous results showed that R3 MYBs in *Arabidopsis* may regulate

trichome formation in a GL2 independent manner (Wang et al., 2010), we cannot rule out the possibility that poplar R3 MYBs may also regulate trichome formation through other mechanisms.

ACKNOWLEDGMENTS

The authors thank Drs. Tom Guilfoyle and Gretchen Hagen (University of Missouri-Columbia) for providing vectors for protoplast transfection assays, and Dr. Tom Guilfoyle for critical reading of the manuscript. This work was supported by the National Natural Science Foundation of China (grant no. 31170262), the Programme for Introducing Talents to Universities (B07017), and the startup funds from Northeast Normal University (www.nenu.edu.cn) to Shucai Wang.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 March 2014; accepted: 21 May 2014; published online: 10 June 2014.
*Citation: Zhou L, Zheng K, Wang X, Tian H, Wang X and Wang S (2014) Control of trichome formation in Arabidopsis by poplar single-repeat R3 MYB transcription factors. Front. Plant Sci. **5**:262. doi: 10.3389/fpls.2014.00262*
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 23 June 2014
doi: 10.3389/fpls.2014.00297

Signaling to stomatal initiation and cell division

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Stomata are two-celled valves that control epidermal pores whose opening and spacing optimizes shoot-atmosphere gas exchange. *Arabidopsis* stomatal formation involves at least one asymmetric division and one symmetric division. Stomatal formation and patterning are regulated by the frequency and placement of asymmetric divisions. This model system has already led to significant advances in developmental biology, such as the regulation of cell fate, division, differentiation, and patterning. Over the last 30 years, stomatal development has been found to be controlled by numerous intrinsic genetic and environmental factors. This mini review focuses on the signaling involved in stomatal initiation and in divisions in the cell lineage.

Keywords: stomata, development, signaling, cell fate, cell division

INTRODUCTION

Stomata formation in *Arabidopsis thaliana* involves at least one asymmetric division as well as a single symmetric division. The meristemoid mother cell (MMC) undergoes an asymmetric entry division that produces a small triangular meristemoid and a larger sister cell. The large cell, termed a stomatal lineage ground cell (SLGC), can either differentiate into a pavement cell (ubiquitous epidermal cell) or undergo asymmetric spacing divisions that produce a satellite meristemoid. Meristemoids can undergo one to three rounds of amplifying divisions before they finally differentiate into an oval-shaped guard mother cell (GMC). GMCs divide symmetrically once thus generating a pair of guard cells (GCs; **Figure 1**; Nadeau and Sack, 2002b; Bergmann and Sack, 2007).

INTERCELLULAR SIGNALING TO STOMATAL INITIATION AND PATTERNING

Stomata are distributed and spaced throughout the plant shoot epidermis. Communication between stomata and their with neighboring epidermal cells ensures that stomata are spaced at least one cell apart ("one cell spacing" rule). The *TOO MANY MOUTHS* (*TMM*) gene was the first stomatal gene identified in *Arabidopsis* (Yang and Sack, 1995). TMM as well as three ERECTA-family (ERf) members are leucine-rich repeat (LRR) receptor-like protein and kinases. Mutations in *TMM* lead to excessive clustered stomata in leaves (Nadeau and Sack, 2002a). The loss of function of three *Arabidopsis* ERf genes, *ER*, *ERECTA-LIKE 1* (*ERL1*), and *ERECTA-LIKE 2* (*ERL2*), induces stomatal clustering (Shpak et al., 2005).

Genetic and biochemical evidence indicate that ERfs act specifically with respect to ligands and developmental stage during stomatal development. The *EPIDERMAL PATTERNING FACTOR* (*EPF*) and *EPF-LIKE* (*EPFL*) genes encode secreted cysteine-rich peptides (Torii, 2012). EPF1 and EPF2 were the first two peptides identified that are used as intercellular signals in stomatal patterning (Hara et al., 2007, 2009; Hunt and Gray, 2009). *EPF1* is expressed in late meristemoids and in GMCs in the stomatal lineage (Hara et al., 2007). *EPF2* expression is restricted to MMCs and early meristemoids, a stage earlier in stomatal development than that of *EPF1*. The loss of *EPF2* function induces excessive divisions and increased stomatal density (Hara et al., 2009; Hunt and Gray, 2009). Overexpression of *EPF2* represses *TMM* expression and blocks stomatal formation, consistent with EPF2 restricting the formation of stomatal precursors (Hunt and Gray, 2009). The loss of *EPF1* function leads to the formation stomata in contact, whereas the overexpression of *EPF1* results in few or no stomata, consistent with EPF1 regulating stomatal patterning (Hara et al., 2007). Bioactive EPF peptides have been shown to directly bind ERf receptors using biosensor chips. The *in vivo* specificities of EPFs and ERf have also been defined (Lee et al., 2012). The EPF2–ER pair prevents cells next to MMCs or Ms from acquiring a stomatal cell fate. The EPF1–ERL1 pair ensures the one-celled spacing between stomata. ER and ERL1 form homodimers or heterodimers, but TMM only forms heterodimeric receptor complexes with ERf members. Thus, stomatal lineage cell determination and patterning are precisely controlled by diverse ligand–receptor pairs (Torii, 2012).

STOMAGEN/EPFL9 is also a member of the EPF/EPFL-family of peptides that is secreted from mesophyll cells of immature leaves (Hunt et al., 2010; Kondo et al., 2010; Sugano et al., 2010). The loss *STOMAGEN/EPFL9* function using gene silencing via RNA interference resulted in a reduction in stomatal density (Hunt et al., 2010; Sugano et al., 2010). In contrast to role of *EPF1* and *EPF2*, the ectopic overexpression of *STOMAGEN* or the application of synthetic STOMAGEN peptides induce the formation of clusters containing numerous stomata in contact (Kondo et al., 2010; Sugano et al., 2010).

While the *TMM* gene was named based upon the loss of function phenotype of excessive stomata in leaves, stomata are absent from stems and hypocotyls (Yang and Sack, 1995; Geisler et al., 1998; Bhave et al., 2009). The loss-of-function of *CHALLAH*

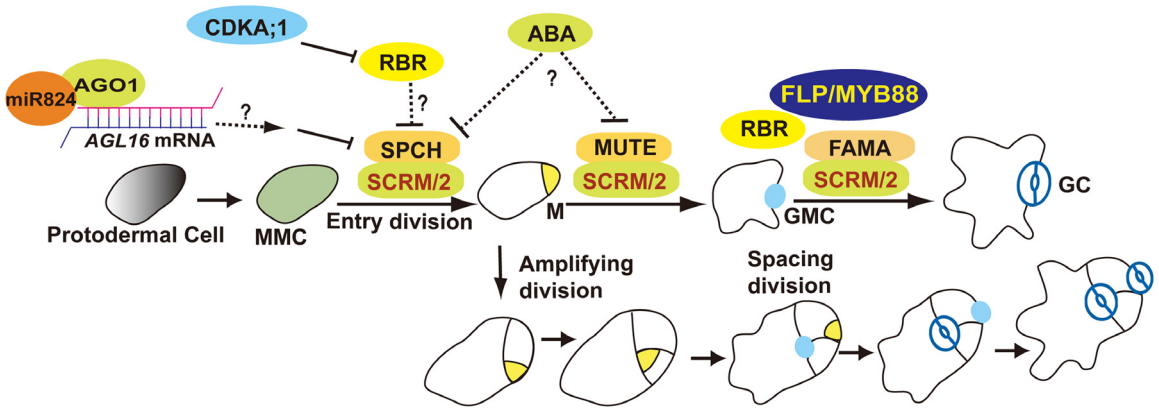

FIGURE 1 | Overview of stomatal development. The stomatal lineage initiates from meristemoid mother cells (MMCs). MMC undergoes asymmetric entry divisions and produces a meristemoid (M) as well as a larger sister cell (termed a stomatal lineage ground cell, SLGC). Meristemoids can undergo asymmetric amplifying divisions before differentiating into a guard mother cell (GMC). SLGCs can differentiate into a pavement cells or reacquire a MMC fate and initiate asymmetric spacing divisions that produce "satellite meristemoids." Stomatal complexes form after at least one unequal division of a stem cell, and then by a single equal division of a GMC. SPCH, MUTE, FAMA, and FLP/MYB88 are transcription factors that regulate key fate transitions during stomatal development. SCRM and SCRM2 heterodimerize with SPCH, MUTE, and FAMA to promote stomatal–lineage transitions. AGO1 is involved in an AGL16-mediated microRNA post-transcriptional regulatory pathway that blocks asymmetric spacing divisions in SLGCs. ABA might be involved in stomatal initiation and differentiation by repressing SPCH and MUTE. RBR activity is predominantly regulated by CDKA;1. RBR participates in the regulation of asymmetric as well as symmetric divisions.

(*CHAL*), which encodes the EPFL6 peptide from the EPF/EPFL family, has been shown to restore stomata to *tmm-1* stems and hypocotyls (Abrash and Bergmann, 2010). Two CHAL paralogs, *CHAL-LIKE1/EPFL5* and *CHAL-LIKE2/EPFL4*, are also involved in stomatal development (Abrash et al., 2011). Higher order mutations in *CHAL* family (*CHALf*) produce stomatal clusters in the hypocotyls of *tmm-1* mutants. A model has been proposed in which TMM dampens CHALf signaling while it promotes EPF1/2-ERf-mediated stomatal formation. When *TMM* function is lost, CHALf ligands inhibit stomatal initiation and differentiation via ERf receptors (Abrash et al., 2011; **Figure 2**). In contrast to the specificity of EPF1/2 to the stomatal lineage, STOMAGEN and CHALf peptides are secreted from internal tissues, indicating that underlying cells are also involved in optimizing the stomatal formation and patterning (Abrash and Bergmann, 2010; Kondo et al., 2010; Sugano et al., 2010).

INTRACELLULAR SIGNAL TRANSDUCTION PATHWAY

The signals received at the cell periphery, such as by TMM–ERf receptors, must in turn be transduced to act on nuclear or cytoplasmic targets. Mitogen-activated protein kinase (MAPK) cascades are candidates for intracellular signaling pathways that connect extrinsic signals to stomatal development. Loss-of-function mutations in the *MAPKK kinase YODA* (*YDA*) gene lead to the massive overproliferation of stomata in the epidermis. Normally, the two daughter cells of an asymmetric division exhibit distinct cell fates, those of a meristemoid and SLGC, and they eventually form a stoma and a pavement cell, respectively. But in a *yda* mutant, both progeny develop into stomata (Bergmann et al., 2004). MPK3 and MPK6 function downstream of YDA-MKKs during stomatal development (Wang et al., 2007). The functions of the MAPK cascade in stomatal development have been systematically examined using the targeted expression of constitutively active (CA) and dominant-negative (DN) kinase variants in the stomatal lineage. Together these results reveal that the MAPK signaling pathway functions during each stage of stomatal development from initiation to differentiation (Lampard et al., 2009).

The CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) protein acts as an E3 ubiquitin ligase that transduces light signals perceived by photoreceptors. Loss-of-function mutants of *cop1* display stomatal clusters but this phenotype is suppressed by the expression of CA-YDA, consistent with the MAPK signaling pathway mediating light signals that regulate stomatal production. This signaling pathway is parallel to that of TMM, but upstream of the basic-helix–loop–helix transcription factor SPEECHLESS (SPCH; Kang et al., 2009; **Figure 2**).

SPCH, MUTE, and FAMA are key regulators that direct three successive stages of stomatal development (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). SPCH is required for epidermal cells to acquire an MMC fate and to undergo asymmetric entry divisions. A strong allele *spch-1* is completely devoid of stomatal lineage cells. Overexpression of *SPCH* increases the number of asymmetric divisions and leads to extra stomata. *SPCH* overexpression can restore stomatal formation to *tmm* hypocotyls, consistent with SPCH acting downstream of TMM (Ohashi-Ito and Bergmann, 2006). The functions of SPCH during stomatal initiation required INDUCER OF CBF EXPRESSION 1 (ICE1)/SCREAM (SCRM), that regulates freezing tolerance, as well as SCRM2 (Kanaoka et al., 2008; **Figure 1**).

Disruption of brassinosteroid (BR) biosynthesis, perception, or signaling caused opposite effects on stomatal production in cotyledons and hypocotyls (Fuentes et al., 2012; Gudesblat et al.,

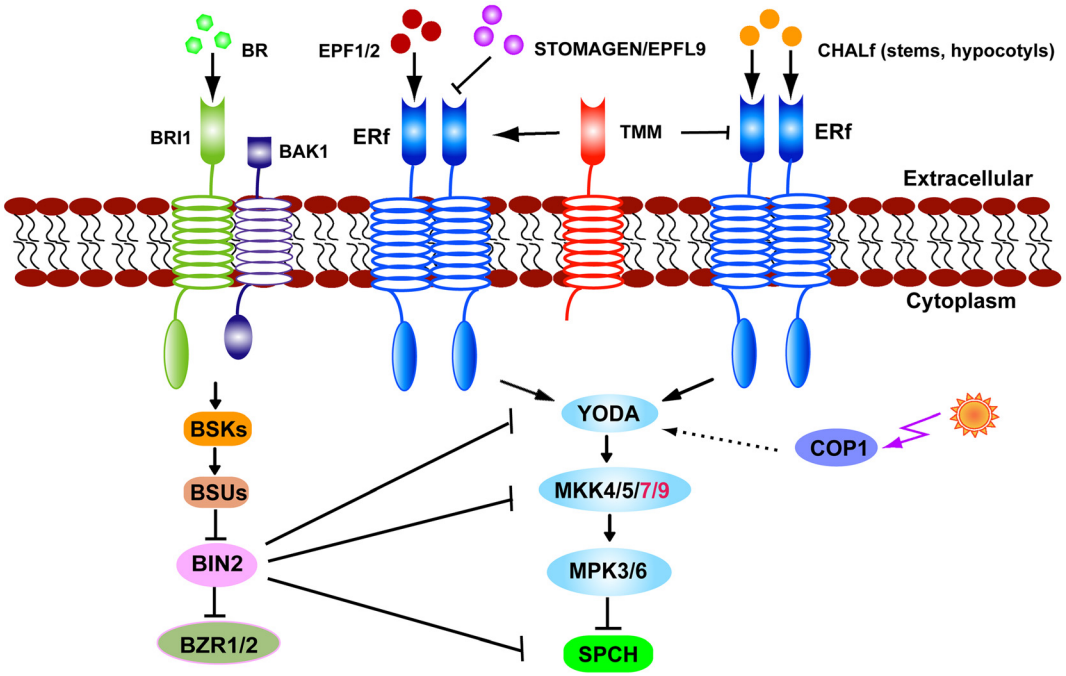

FIGURE 2 | The stomatal signaling pathway. EPF1, EPF2, and STOMAGEN/EPFL9 signals are received by TMM–ERf leading to transduction and the YODA–MKK–MPK cascade. Light regulates stomatal production via COP1 which is upstream of YODA. In stems or hypocotyls TMM dampens CHALf peptide binding with the ERf receptor. BR signals received by BRI1–BAK1 inhibit BIN2 activity. BIN2 suppresses SPCH by directly phosphorylating or indirectly thorough YODA or MKK4/5.

2012a; Kim et al., 2012; Khan et al., 2013). The serine/threonine glycogen synthase kinase 3 (GSK3)/SHAGGY-like BRASSINOSTEROID INSENSITIVE 2 (BIN2) phosphorylates YDA *in vitro* as well as the substrates of YDA, MKK4, and MKK5 (Kim et al., 2012; Khan et al., 2013). SPCH activity is inhibited after its being phosphorylated by MPK3 or MPK6 (Lampard et al., 2008). Thus the lowered MPK3/6 activity in BR mutants assumed the formation of excessive stomata in cotyledons (Kim et al., 2012). By contrast, BRs promote stomatal production in hypocotyls where BIN2 might directly phosphorylates SPCH residues that overlap with those targeted by the MAPKs, as well as BIN2-specific residues outside the MPK target domain (Gudesblat et al., 2012b). Either MPK- or BIN2-mediated phosphorylation leads to the degradation of SPCH protein and blocks entry into the stomatal cell lineage (**Figure 2**).

Since a similar organ-dependent stomatal phenotype is present in the *tmm* mutants (Geisler et al., 1998), a model about BR organ-specific effects was proposed (Casson and Hetherington, 2012; Serna, 2013). In cotyledons, BIN2 phosphorylates YDA or MKKs at low BR levels, and switches off the degradation of SPCH by MPKs, resulting in the production of extra stomata. In hypocotyls, the presence of CHALf signaling might lead to a reduction in MAPK activity. Consequently, the BIN2-mediated direct phosphorylation of SPCH is the predominant pathway in hypocotyls (Serna, 2013). Interestingly, BRs can induce new meristemoids to form in *tmm* hypocotyls, a phenotype similar to the presence of increased meristemoids in *tmm chal* hypocotyls (Fuentes et al., 2012; Gudesblat et al., 2012b), indicating that organ-specific functions of *CHALf* might be responsible for the opposite effects of BRs on stomatal production (**Figure 2**).

Detailed analyses of stomatal development in sterol biosynthesis *fk* mutants suggest that sterols (BR-independent) are required for stomatal cell fate determination and maintenance. Physically asymmetric divisions progress normally in *fk* mutants, but their cell-fate asymmetry is disrupted (Qian et al., 2013).

Additional plant hormones, such as gibberellins (GAs) can contribute to organ-specific effects of BRs. Stomatal formation in hypocotyls, but not in cotyledons, can be induced by GA or ethylene, and this effect is pronounced when both hormones are present. Conversely, no stomata form in hypocotyls of the GA-deficient mutant *ga1-3* (Saibo et al., 2003).

Abscisic acid (ABA) not only induces stomatal closure, but also prevents stomatal initiation, since stomatal numbers increase in the ABA-deficient *aba2-2* mutant. Time-course analysis reveals that meristemoid formation is prolonged in *aba2-2*. By contrast, in the ABA-over-accumulating mutant *cyp707a1 a3*, meristemoid formation is restricted. Compared to the wild-type, *SPCH* and *MUTE* transcripts are abundant in the *aba2-2* mutant but reduced in the *cyp707a a3* mutant (Tanaka et al., 2013). Interestingly, new *tmm* alleles display differential sensitivity to ABA in seedling growth and seed germination, but not in stomatal development (Yan et al., 2014).

Auxin widely regulates plant development by coordinating the placement and patterning of organs and cells. Dynamic changes of auxin activity during stomatal development were monitored using

auxin input (35S::DII-VENUS) and output (DR5::VENUS) markers by time-lapse imaging. The disruption of auxin efflux induced a delayed switching from meristemoids to GMCs, indicating that auxin depletion is essential for M-GMC differentiation. The disruption of auxin efflux also causes excessive stomata to arise in clusters, indicating that auxin is also involved in stomatal stem cell fate determination (Le et al., 2014).

MicroRNAs (miRNAs) play important roles in regulating gene expression in multicellular plants and animals. The miR824 regulates the asymmetric division of SLGCs by repressing the *AGAMOUS-LIKE16 (AGL16*) gene in the stomatal lineage (Kutter et al., 2007). The components of the miRNA pathway *HYPONASTIC LEAVES1* (*HYL1*), *ARGONAUTE1* (*AGO1*), and the *HUA ENHANCER1* (*HEN1*) genes participate in stomatal production (Jover-Gil et al., 2012). Time-lapse analysis revealed that *AGO1* acts as a negative regulator in restricting the asymmetric spacing divisions in SLGCs. *AGO1* may act by negatively regulating *SPCH* transcript levels downstream of *TMM* (Yang et al., 2014a; **Figure 1**).

REGULATION OF CELL DIVISION IN STOMATAL DEVELOPMENT

Arabidopsis stomata are generated after at least one asymmetric and one symmetric division. Thus division polarity is important for the regulation of cell fate determination, proliferation, and patterning during stomatal development. The BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) and POLAR proteins are novel proteins regulating stomatal divisions (Dong et al., 2009; Pillitteri et al., 2011). The localization and levels of BASL and SPCH have been tracked in developing leaves which led to a "polarity switching" model that predicts the sitting of the BASL protein during successive divisions (Robinson et al., 2011). The regulation of asymmetric divisions during stomatal development has been comprehensively discussed in recent reviews (Lau and Bergmann, 2012; Pillitteri and Torii, 2012; Wengier and Bergmann, 2012). Here we emphasize recent work on the control of terminal divisions in stomatal development.

The loss of *FAMA* function induces cell overproliferation, resulting in the stacking of narrow epidermal cells that lack GC fate (Ohashi-Ito and Bergmann, 2006). The R2R3 MYB transcription factors *FOUR LIPS (FLP)* and *MYB88* function at the same stage as *FAMA*. *flp myb88* double mutants resemble *fama* mutants in that they harbor extra divisions, although the latter lack GCs (Lai et al., 2005). *CDKB1;1* is expressed specifically in the stomatal lineage cells. Reducing CDKB1 activity, either by overexpressing a DN form *CDKB1;1.N161*, or via the loss-of-function of both the *CDKB1;1* and *CDKB1;2* genes (*cdkb1;1 1;2*) blocks the symmetric division of GMCs, resulting in the formation of single GCs (SGCs; Boudolf et al., 2004; Xie et al., 2010). GCs usually harbor 2C DNA levels, but SGCs in *CDKB1;1.N161* have a 4C DNA content, consistent with an arrest during the cell cycle transition before G2-to-M. FLP can directly bind to a *cis*-regulatory element within the *CDKB1;1* promoter and can negatively regulate *CDKB1;1* transcript levels.

Chromatin immunoprecipitation microarray (ChIP-chip) analysis also reveals that many core cell cycle genes are putative transcriptional targets of FLP/MYB88, including *CDKA;1, CDC6, CYCD4;1* (Xie et al., 2010). The loss of *CDKA* function in *cdka;1* homozygous mutants, also results in SGCs forming in the epidermis. But SGCs in *cdka;1* mutants contain a 2C levels of DNA, indicating that *CDKA;1* acts at the G1-to-S transition of the cell cycle. Moreover, *CDKA;1*, like *CDKB1;1*, is also a direct target of FLP/MYB88 through binding to *cis*-regulatory elements in these promoters (Yang et al., 2014b). CDKA;1 activity is generally more important for the G1-to-S transition, while CDKB1's are required for the G2-to-M progression, but the overexpression of *CDKA;1* can partially rescue GMC divisions in a *cdkb1;1 1;2* double mutant, suggesting that elevating CDKA;1 activity can at least partially substitute for CDKB1 activity (Weimer et al., 2012; Yang et al., 2014b). The combined loss of FLP/MYB88 and CDKB1 function, such as in the *flp-1 myb88 cdkb1;1 1;2* quadruple mutant, induces SGCs to undergo endoreduplication, that can lead to mean DNA levels of 6C in SGCs. Thus FLP/MYB88 can also conditionally restrict the G1/S transition (Lee et al., 2013).

Since CDK activation depends on its association with cyclin partners, the co-expression of *CDKB1;1* and *CYCLIN A2;3* (*CYCA2;3*) enhanced the kinase activity of CDKB1;1 and triggered ectopic cell divisions (Boudolf et al., 2009). Defective GMC divisions are present in *cyca2* mutants, while the *cdkb1;1 cyca2;234* quadruple mutant displays more SGCs than the *cyca2;234* triple mutant, suggesting that *CYCA2s* and *CDKB1s* synergistically promote GMC division (Vanneste et al., 2011). The overexpression of *CYCA2;3* at the stage when *FAMA* is expressed induced a differential increase in *CDKB1;1* expression in some subdivided GCs. Strikingly, ectopic *TMM* expression was present in some of these subdivided cells, indicating a cell fate reversion from a GC to a precursor cell fate (Yang et al., 2014b).

FAMA overlaps in function with FLP/MYB88 in limiting GMC division, but likely acts in a parallel or different pathway (Ohashi-Ito and Bergmann, 2006). However, the FAMA protein, like that of FLP/MYB88, also bind to *CDKB1;1* promoter (Hachez et al., 2011). Recently, a functional redundancy between FLP/MYBB and FAMA in maintaining the GC fate and integrity has been found (Lee et al., 2014). While a *FAMA* transgene driven by its native promoter, i.e., *proFAMA:cFAMA-GFP*, rescued the *fama* mutant phenotype of tumor-like clusters, over time this transformation generated a gain-of-function phenotype, that of the asymmetric division of GCs themselves. This subdivision produces two cells with unequal size and fate with the smaller daughter cell often developing into a stoma, leading to a "stoma-in-stoma" (SIS) phenotype. Notably, the levels of trimethylation on lysine27 histone3 (H3K27me3) of stomatal stem cell genes was disrupted, i.e., on *SPCH*, *MUTE*, and *FAMA*. The constitutive expression of the Polycomb-group gene *CURLY LEAF* was found to suppress this SIS phenotype. Moreover, a FLP transgene also induced a SIS phenotype (Lee et al., 2014). RETINOBLASTOMA-RELATED (RBR), is a homolog of the human tumor suppressor *Retinoblastoma* gene, which is involved in H3K27me3-mediated gene silencing (Gutzat et al., 2012). Down-regulation of *RBR* by RNAi induced GC subdivisions as well as the resetting of GC fate to that of stomatal lineage stem cells (Borghi et al., 2010; Lee et al., 2014). Both FLP and FAMA directly bind to RBR, suggesting that FAMA and/or

FLP/MYB88 might interact with RBR in repressing stomatal stem cell genes.

Suppression of RBR in the leaf epidermis also leads to the formation of small cells, consistent with RBR also regulating asymmetric divisions in the epidermis (Desvoyes et al., 2006; Borghi et al., 2010). The RBR protein is phosphorylated predominantly by CDK kinases, such as CDKA;1 and CDKB1s (Nowack et al., 2012). Consistent with the role of CDK in stomatal cell fate determination, the loss-of-function in a *cdkb1;1 1;2* double mutant, in a *35Spro:CDKB1;1.N161* line, as well as in *cdka;1* mutants all lead to a reduced stomatal production (Boudolf et al., 2004; Xie et al., 2010; Yang et al., 2014a). The expression of *CDKB1;1* under control of the *CDKA;1* native promoter partially rescued stomatal formation in *cdka;1* mutants. A mechanism in which asymmetric and symmetric divisions are controlled by the CDK activity levels is presented (Weimer et al., 2012).

CHALLENGES AND PERSPECTIVES

A key breakthrough in understanding stomatal development was the finding of a set of transcription factors required for successive stages of development that include lineage initiation, differentiation, and proliferation. Recent work demonstrates that the stability of the SPCH protein is regulated by multiple kinases. A remaining challenge is to define how SPCH proteins are selectively phosphorylated by different kinases *in planta* depending on different cells, organs, and growth conditions.

SPCH is proposed to be transcriptionally regulated by CDKA;1 via the regulation of RBR activity (Weimer et al., 2012). It is also possible that FAMA or FLP maintain GC fate by interacting with RBR to suppress *SPCH* expression (Lee et al., 2014). Future studies in different organs and cell types in response to signaling should help elucidate the precise spatial control mechanism of stomatal cell fate determination and maintenance.

ACKNOWLEDGMENTS

We apologize to colleagues whose work has not been mentioned because of space limitations. This work is supported by grants from the National Natural Science Foundation of China (31271463, 31071198, and 31000119) and the Hundred Talents Program (KSCX2-YW-N-073), Chinese Academy of Sciences. We thank Fred Sack and the anonymous reviewers for the discussion and suggestion on this review.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 May 2014; paper pending published: 19 May 2014; accepted: 06 June 2014; published online: 23 June 2014.

*Citation: Le J, Zou J, Yang K and Wang M (2014) Signaling to stomatal initiation and cell division. Front. Plant Sci. **5**:297. doi: 10.3389/fpls.2014.00297*

This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

OPINION ARTICLE
published: 11 October 2013
doi: 10.3389/fpls.2013.00401

Antagonistic regulation of the meristemoid-to-guard mother-cell-transition

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Keywords: SPCH, MUTE, meristemoid self-renewal, guard mother cell fate, MAPK

The building of a multicellular organism from a single cell is the result of coordinated acquisition of different cell identities in an ordered spatial arrangement. But, how do cells learn about their identity? Stomatal development in Arabidopsis provides perhaps one of the most tractable contexts in which to examine concepts of cell fate determination, as a variety of lineage tracing techniques, including long-term confocal time lapse imaging (Peterson and Torii, 2012), can be deployed in different genetic contexts and environmental conditions to trace the history of only a few cell types. In this essay, I focus on the choice between meristemoid cell self-renewal, in which one of daughters of a dividing meristemoid retains the properties of the parent cell, and its transition through guard mother cell (GMC) fate to produce stomata.

In Arabidopsis, stomatal development, which starts at the tip of the leaf and proceeds basipetally, takes place through a series of stereotyped yet flexible cell division pattern (Lau and Bergmann, 2012; Pillitteri and Torii, 2012). The first sign of stomatal development is an unequal cell division from a protodermal cell called the meristemoid mother cell (MMC). This cell division produces a small triangular meristemoid and a larger neighboring cell. Meristemoids are self-renewing cells that divide (amplifying divisions) in an inward spiral and always yield a larger cell and a smaller meristemoid that maintains its self-renewal activity. The meristemoid eventually loses its self-renewal character and adopts a rounded shape giving rise to the GMC. The GMC undergoes an equal and symmetric cell division that generates the paired guard cells. Some of the larger cells that result from the unequal divisions of MMCs or meristemoids and that come into contact with the stoma (or its precursor) can adopt MMC fate initiating the cell division pattern that culminates with stomatal formation. Others differentiate into pavement cells. This lineage is responsible for generating the majority of the epidermal cells in the leaves (Geisler et al., 2000).

All stomatal lineages originate by an unequal division, but the number of subsequent cell divisions is not fixed, and it varies from zero to three. For example, the study of cell divisions through time using serial imprints revealed that, in the leaves (abaxial side) of C24 ecotype, 44% of complexes develop their stomata prematurely, that is, after the second, or even the first, unequal cell division (Berger and Altmann, 2000). Similar studies in Columbia background (abaxial epidermis of leaves and cotyledons) showed that 66% of their stomata develop also after the first or second unequal cell division (Geisler et al., 2000). Clonal analysis in the leaves (adaxial side) of Landsberg *erecta* ecotype also showed some flexibility with at least 13% of the complexes developing stomata prematurely (Serna et al., 2002). The differences in the degree of flexibility of stomatal development among different studies could be due to the genetic background, the face analyzed, the growth conditions and/or the methodology used to trace stomatal lineages. Anyway, what is certain is that, a greater or lesser extent, the number of amplifying divisions that take place before GMC formation is flexible. More recently, long-term confocal time lapse imaging in Arabidopsis leaves underlined also this variability in the number of amplifying divisions (Robinson et al., 2011). But, what are the molecular bases of this plasticity?

Time-lapse confocal imaging in live leaves showed that the basic helix-loop-helix (bHLH) factor SPEECHLESS (SPCH) locates in MMCs and meristemoids, and disappears in GMCs. This finding strongly suggests a role for SPCH, which drives the cell division that initiates the stomatal-cell lineage (MacAlister et al., 2007; Pillitteri et al., 2007), in the maintenance of meristemoid self-renewal activity in the leaves (Robinson et al., 2011). Agree with this interpretation, and suggesting that the proposed role of *SPCH* in leaves extends to other plant organs, meristemoids of the pedicel epidermis of the weak *spch-2* mutant undergo significantly fewer amplifying divisions compared with wild type plants (MacAlister et al., 2007). In addition to SPCH, MAPKs also provide flexibility to stomatal development through repression of the meristemoid-guard mother cell switch (Lampard et al., 2009): constitutive activation, beginning in meristemoids, of either the mitogen-activated protein kinases (MAPK) kinase kinase YODA (YDA) or the MAPK kinases MKK4, MKK5, MKK7 or MKK9 prevents stomatal formation giving rise to an epidermis consisting of pavement cells and arrested meristemoids. Because both MKK4 and MKK5 function upstream of the MAP kinases MPK3 and MPK6 during stomatal development (Wang et al., 2007), it is expected that constitutive activation, beginning in meristemoids, of MPK3 or MPK6 also blocks GMC formation. If this is true, and given that MPK3 and MPK6 phosphorylate SPCH protein *in vitro* (Lampard et al., 2008), these phosphorylation events may activate SPCH function in meristemoids, promoting their self-renewal behavior and so repressing their transition to guard-mother cell (**Figure 1**). This scenario contrasts with that in which

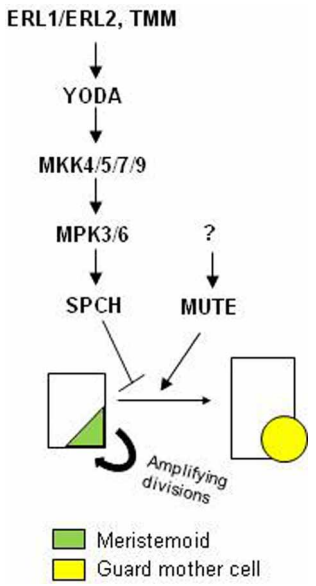

FIGURE 1 | Model for the regulation of the self-renewal behavior of the meristemoids and their transition to guard mother cell (GMC) fate. Predominance of SPCH, which is activated by YDA MAPK module, inhibits the meristemoid-to-GMC transition by promoting the self-renewal behavior of the meristemoids. In contrast, predominance of MUTE represses the amplifying divisions of the meristemoids and enhances their transition through the GMC fate. GMC symmetrically divides to produce paired-guard cells (not shown). The components upstream of MUTE remain unknown. SCRM1 and SCRM2 function in this transition through direct interaction with SPCH and MUTE (not shown).

SPCH hyper-phosphorylation by both MPK3 and MPK5 suppresses its activity preventing the first unequal cell division of stomatal development (Lampard et al., 2008).

The *ERECTA*-family leucine-rich repeat receptor-like kinases members *ERECTA-LIKE1* (*ERL1*) and *ERECTA-LIKE2* (*ERL2*) maintain the division activity of the meristemoids and prevent them from differentiating into GMCs (Shpak et al., 2005): *erl1*, *erl2* and *erl1 erl2* mutants show a reduction in the number of larger cells that surround the stoma (or its precursor), which arise from amplifying divisions. Like *ERL1* and *ERL2*, both the subtilisin-related extracellular protease *STOMATAL DENSITY AND DISTRIBUTION1* (*SDD1*) and the plasma membrane-anchored leucine-rich repeat receptor-like protein *TOO MANY MOUTHS* (*TMM*) have been also involved in the maintaining of the self-renewal activity of the meristemoids, with *sdd1-1* and *tmm* mutants undergoing also less amplifying divisions than wild-type plants (Berger and Altmann, 2000; Geisler et al., 2000; Bhave et al., 2009). Because TMM associates with ERL1 *in vivo* (Lee et al., 2012), the formation of heterodimeric complexes between TMM and ER1, and most probably between TMM and ERL2, might be required for the initiation of this signaling cascade. Moreover, the fact that constitutive activation of YDA suppresses the defects of *tmm* places this extracellular signaling upstream of YDA (Bergmann et al., 2004). It is known that these receptors interact and are activated by peptide ligands of the EPIDERMAL PATTERNING FACTOR (EPF)/EPIDERMAL PATTERNING FACTOR-LIKE (EPFL)-family (Torii, 2012). However, at the moment, no peptide ligand has been directly involved in the self-renewal activity of the meristemoids.

MUTE also encodes a bHLH protein, which, in addition, exhibits high homology with SPCH (Pillitteri et al., 2007). The loss-of-function *mute* mutant lacks stomata but develops meristemoids that arrest after excessive amplifying cell divisions that take place in an inward-spiral pattern (MacAlister et al., 2007; Pillitteri et al., 2007). This suggests that *MUTE* represses self-renewal activity of the meristemoids and induces GMC formation. Both *MUTE* promoter activity and the MUTE protein localization are restricted to a subset of meristemoids (MacAlister et al., 2007; Pillitteri et al., 2007), which presumably will undergo GMC transition. Interestingly, MUTE does not seem to be a substrate of either MPK3 or MPK6 (Lampard et al., 2008). Even more, MUTE is phosphorylated *in vitro* by the MAP kinase MPK4. However, both loss-of-function and gain-of-function of the MAPK kinase kinase MEKK1, which acts upstream of MPK4 (Asai et al., 2002), do not induce any effect on stomatal development (Wang et al., 2007). In addition, the expression, beginning in meristemoids (or MMCs), of constitutively activate MAPK kinase kinase MKK1 or MKK2, which phosphorylate MPK4 (Popescu et al., 2009), also confers no apparent effect on the control of stomatal development, including meristemoid self-renewal activity (Lampard et al., 2009). Then, if MPK4 phosphorylates MUTE in meristemoids, this phosphorylation event should not be correlated with the MUTE function in the control of the meristemoid-to-guard mother-cell-transition. If this is true, both loss- and gain-of-function of MPK4 plants should exhibit no effect on stomatal development.

Together, these finding suggest that at least two parallel pathways control the switch from meristemoid to GMC (**Figure 1**):(1) a pathway that controls SPCH function, and (2) an unknown pathway that regulates the activity of MUTE. Predominance of MUTE pathway may repress meristemoid self-renewal behavior, accelerating stomata formation. In contrast, predominance of SPCH pathway may induce the opposite effect, promoting self-renewal behavior of the meristemoids, and delaying GMC formation. In addition, RT-PCR analysis showed that SPCH promotes its own transcription and the transcription of *MUTE* (MacAlister et al., 2007; Pillitteri et al., 2007). Genetic and biochemical data also suggest that both SCRM1 and SCRM2 function in this transition through direct interaction with SPCH and MUTE (Kanaoka et al., 2008).

Stomatal number, and so gas exchange with the atmosphere, depends on the number of MMCs formed and on how long it takes for GMCs to divide. Since stomata premature formation reduces the distance among neighboring stomata, and vice versa, a coordinated control of self-renewal of meristemoids and their transition to GMC fate is fundamental to avoid maladaptive responses. Meristemoids may perceive signals, adjusting the levels of SPCH vs. MUTE activity, in order to mount physiologically appropriate developmental responses. But, how could these signals trigger the regulation of SPCH and/or MUTE activity? Agree with the general view that MAPK modules are integrating point of multiple signals, several studies have highlighted that YDA-MAPK module is a point of integration for communicating brassinosteroids and light signaling to stomatal development (Kang et al., 2009; Kim et al., 2012; Khan et al., 2013). Brassinosteroids, through

the glycogen synthase kinase 3-like kinase BRASSINOSTEROID INSENSITIVE 2, can also regulate SPCH activity directly (Gudesblat et al., 2012), which, most probably, takes place in situations that trigger a reduction of YDA-MAPK levels (Serna, 2013). ABA also controls stomatal development (Tanaka et al., 2013), and YDA-MAPK module may also serve as a transmitter of the ABA-dependent cascade. Certainly, MPK6 is a target of ABSCISIC ACID-INSENSITIVE 1 (Leung et al., 2006), which represses stomatal formation (Tanaka et al., 2013). Considering that YDA-MAPK module controls the meristemoid to GMC transition, it is likely that these factors also regulate this switch. MUTE may also perceive, directly or indirectly through their yet unknown upstream regulators, systemic signals to optimize the ratio of stomata vs. pavement cells to the state of the plant and the environment.

Interestingly, analysis of static pictures showed that the number of meristemoids and GMCs is not constant throughout development (Geisler and Sack, 2002). Instead, there are periods when, for example, both meristemoids and GMCs increase and decrease, respectively (Geisler and Sack, 2002). This suggests that, in this particular period, meristemoids receive signals that accentuate the predominance of SPCH pathway, thus prolonging their self-renewal capacity. There are also periods when meristemoids drop, while GMCs increase (Geisler and Sack, 2002), suggesting, in this period, a predominance of MUTE pathway in these meristemoids, even though they had divided a variable number of times. Agree with this interpretation, data from serial imprints showed that in some temporal windows the majority of meristemoids become mature stomata, even though they do it prematurely (Geisler and Sack, 2002). Meristemoids also pause divisions when subject to mild osmotic stress, and quickly recover them once this stress is alleviated (Skirycz et al., 2011). Mild osmotic stress may block MUTE activity, which in absence, or under low levels, of SPCH activity, would pause meristemoid progression. Selective activation of either SPCH or MUTE may resume meristemoid self-renewal capacity or its transition to GMC fate, respectively. Supporting this interpretation, arrested meristemoids of 13 days-old *iMUTEmute* plants transit to GMC fate upon late (13 days post-germination) induction of β-estradiol-dependent *MUTE* expression (Triviño et al., 2013).

The story gets more complicated when we consider stomatal development in the context of the entire developing leaf, instead of considering individual lineages. For example, if two meristemoids from two separate lineages arise adjacent to each other, they do not progress to become stomata, but instead one of them undergoes an additional cell division to prevent the physical contact with its neighbor, or even undergoes a transdifferentiation process (Geisler et al., 2000). This example highlights that cell-to-cell signaling guides meristemoid fate, most probably, by imposing to that dictated by environmental factors or phytohormones.

Detailed knowledge about the process that regulates self-renewal of meristemoids and their transit to GMC fate is crucial to enable better adapted and more productive plants to local site conditions. To deep into the understanding of the control of this step is necessary to identify the components acting upstream of MUTE. Challenges for the future also include verifying that MPK3 and MPK6 are downstream components of the four MAPK kinases, MKK4, MKK5, MKK7, and MKK9, in the meristemoid to GMC transition, and that MPK3 and MPK6 trigger SPCH regulation in this switch. Identifying the components upstream of TMM-ERL1/2 is also needed.

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Received: 29 August 2013; accepted: 20 September 2013; published online: 11 October 2013.

*Citation: Serna L (2013) Antagonistic regulation of the meristemoid-to-guard mother-cell-transition. Front. Plant Sci. **4**:401. doi: 10.3389/fpls.2013.00401*

This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 01 July 2014
doi: 10.3389/fpls.2014.00315

Regulation of xylem cell fate

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The vascular system is organized throughout the plant body for transporting water, nutrients, and signaling molecules. During vascular development, xylem, phloem, and procambial/cambial cells are produced in a spatiotemporally organized manner. Several key regulators for xylem cell patterning and differentiation have been discovered, including auxin, cytokinin, CLE peptides, microRNAs, HD-ZIPIIIs, VNDs, and moving transcription factors SHR and AHLs. Recent studies are identifying functional interactions among these factors that ultimately determine xylem cell fate. This review focuses on regulatory networks underlying xylem cell fate determination in root vascular development.

Keywords: xylem, hormone, transcription factor, differentiation, patterning

INTRODUCTION

Cell fate determination is a fundamental mechanism underlying complex morphogenesis in multicellular organisms. Vascular tissues consist of phloem, xylem, and procambial cells. In *Arabidopsis thaliana* roots, the pattern of the central xylem axis, two phloem poles, and their intervening procambium is maintained during development, suggesting a robust mechanism for determining the spatial fate of each vascular cell (Cano-Delgado et al., 2010). Therefore, root vascular development is considered as an excellent system for studying cell fate determination (Bonke et al., 2003; Kubo et al., 2005). Recent studies on root vascular development have uncovered novel machineries regulating xylem cell fates in roots, such as cell-to-cell communication mediated by ligand–receptor interaction and intercellular movement of transcription factors (Hirakawa et al., 2011; Miyashima et al., 2012). We summarize recent advances on xylem cell fate determination in roots and discuss the regulatory networks controlling xylem cell fate determination.

CYTOKININ IS A CENTRAL REGULATOR OF PROTOXYLEM VESSEL CELL FATE IN ROOTS

Root xylem vessels are classified into two types, protoxylem vessels and metaxylem vessels, which are equipped with a spiral-patterned and a pitted-patterned secondary cell-wall, respectively. The root vascular system is organized with precise cell patterning, in which five xylem vessel cells occupy the central xylem axis (**Figure 1**). In the xylem axis, two protoxylem vessels are always located on the outer side, and 2−4 metaxylem vessels are located on the inner side (**Figure 1**). It is widely recognized that root vascular cell identities are determined in the root apical meristem (RAM). The well-known vascular-specific marker genes *ALTERED PHLOEM DEVELOPMENT* (*APL*) and *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*) are expressed in phloem and protoxylem vessel cell files, respectively, in the RAM (Bonke et al., 2003; Mähönen et al., 2006).

The plant hormone cytokinin (CK) has been implicated in the specification of protoxylem vessels in roots (Mähönen et al., 2000; Fukuda, 2004). Application of the synthetic CK benzyladenine causes the loss of root protoxylem vessels in a dose-dependent manner (Yokoyama et al., 2007; Kondo et al., 2011; Ren et al., 2013). Conversely, reduction of CK content by expressing *CYTOKININ OXIDASE 2* (*CKX2*), which encodes a CK degradation enzyme, leads to the formation of extra protoxylem vessels (Mähönen et al., 2006). Thus, the number of protoxylem vessels depends on CK levels (**Figure 2**). CK signal transduction is initiated by the receptors *ARABIDOPSIS* HISTIDINE KINASE 2–4 (AHK2, AHK3, and CRE1/AHK4/WOL; Kieber and Schaller, 2014; **Figure 2A**). *AHK* mutants form extra protoxylem vessels adjacent to two original protoxylem vessels in the stele, due to the reduced CK sensitivity (Mähönen et al., 2006; Kondo et al., 2011; **Figure 2B**). The *wol* mutant displays a more severe phenotype, in which phloem cells are completely lost and only protoxylem vessels are formed in the stele (Scheres et al., 1995; Mähönen et al., 2006). The CK signal is transduced via phosphotransfer from AHKs to AHPs (**Figure 2A**). The *AHP* quintuple mutant (*ahp1 ahp2 ahp3 ahp4 ahp5*) exhibits the extra protoxylem phenotype similar to that of *ahk* mutants (Hutchison et al., 2006). These results indicate that CK negatively regulates protoxylem vessel formation via AHKs and AHPs. The atypical AHP, AHP6, lacks the histidine residue conserved among other AHPs. *AHP6* is expressed in future protoxylem vessel cell files in RAM, and loss-of-function mutants often cause a partial loss of protoxylem vessels (Mähönen et al., 2006). These results strongly suggest that AHP6 functions as a pseudo-phosphotransfer protein that interferes with phosphorelay from AHKs to AHPs by competing with other AHPs, leading to inactivation of CK signaling in protoxylem vessel formation (Mähönen et al., 2006; **Figure 2A**).

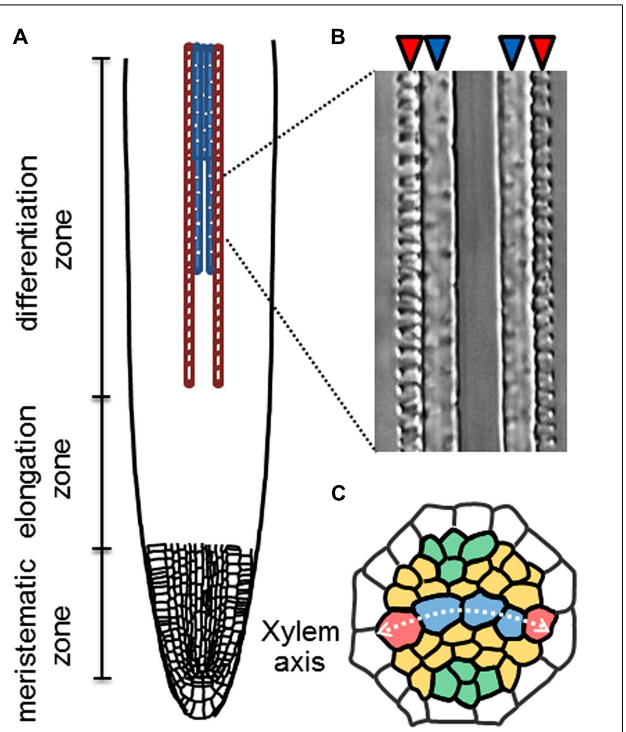

FIGURE 1 | Xylem cell specification in roots. (A) Schematic illustration of longitudinal pattern of protoxylem (red) and metaxylem (blue) vessel cells in a root. **(B)** A Nomarski differential interference image of protoxylem (red arrowheads) and metaxylem vessels (blue arrowheads) in an *Arabidopsis* root. **(C)** Schematic illustration of radial cell patterning in the root stele. Red; protoxylem vessels (PX), blue; metaxylem vessels (MX), orange; procambial cells and green; phloem cells.

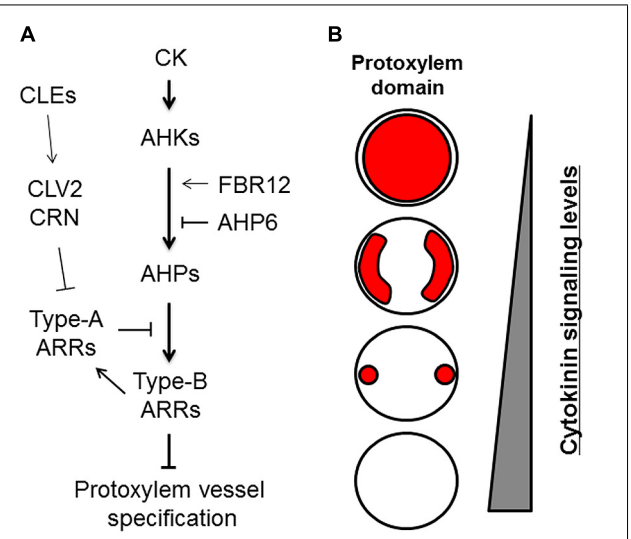

FIGURE 2 | Roles of cytokinin signaling in protoxylem vessel specification. (A) Cytokinin (CK) signaling and its modulators involved in protoxylem vessel specification. **(B)** Schematic illustration of protoxylem domains (red) formed differentially depending on cytokinin signaling levels in the stele of roots.

Typical AHPs activate transcription factors named type-B *ARABIDOPSIS* RESPONSE REGULATORs (ARRs), which are the final targets in CK signal transduction (Yokoyama et al., 2007; Argyros et al., 2008). Triple mutants of centrally acting type-B ARRs (*arr1 arr10 arr12*) develop ectopic protoxylem vessels similar to those of other CK-related mutants (Yokoyama et al., 2007; Ishida et al., 2008). Type-B ARRs directly up-regulate type-A *ARRs*, which negatively regulate CK signaling by interacting with AHPs and interfering with type-B ARR functions (To et al., 2007). Mutants for type-A *ARRs* exhibit elevated CK sensitivity (To et al., 2004) and have fewer protoxylem vessels in lateral roots but not in the primary root (Ren et al., 2009; Kondo et al., 2011). These studies indicate that the CK signaling cascade consisting of AHKs, AHPs, and ARRs has a central role in regulating protoxylem vessel cell specification (**Figure 2**).

MODULATORS OF CYTOKININ SIGNALING REGULATE PROTOXYLEM VESSEL FORMATION

Modulators of CK signaling are involved in the regulation of protoxylem vessel formation. There are 32 genes encoding CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE) peptides in *Arabidopsis* (Ito et al., 2006; Jun et al., 2008). Many CLE peptides, including CLE10, inhibit protoxylem vessel formation in wild-type plants (Kondo et al., 2011). By contrast, CLE10 does not inhibit protoxylem vessel formation in the type-B *arr10 arr12* double mutant (Kondo et al., 2011). Gene expression analysis shows that CLE10 down-regulates type-A *ARRs*. These results suggest that CLE10 activates CK signaling through the down-regulation of type-A *ARRs*, thereby suppressing protoxylem vessel formation (Kondo et al., 2011). Further genetic analysis suggests that CLAVATA2 (CLV2) may act as a receptor that mediates CLE10 signaling and regulates protoxylem vessel formation (Kondo et al., 2011).

A recent study reported that the loss-of-function mutant of *FUMONISIN B1-RESISTANT 12* (*FBR12*) produced extra protoxylem vessels due to reduced CK sensitivity (Ren et al., 2013). *FBR12* encodes a eukaryotic translation initiation factor (eIF5A) that is believed to play various roles via interactions with different proteins and RNAs (Thompson et al., 2003; Jao and Chen, 2006; Feng et al., 2007). FBR12 physically and genetically interacts with CRE1/AHK4/WOL and AHPs, which results in enhanced CK signaling (Ren et al., 2013). The modulation of CK signaling by various factors at different signaling steps enables fine spatiotemporal regulation of the protoxylem vessel domain (**Figure 2**).

SPATIAL REGULATION OF THE CYTOKININ ACCUMULATION DOMAIN IN ROOTS

Precise protoxylem vessel patterning requires spatial control of CK accumulation. Reporter-GUS analysis using hormone-response markers shows that domains with high auxin and high CK levels are localized in xylem axis and procambium in the stele, respectively (Bishopp et al., 2011a; **Figure 3**). The auxin distribution pattern is formed by auxin lateral transport through auxin efflux carriers PIN-FORMED 3 (PIN3) and PIN7 (Bishopp et al., 2011a). High auxin level in the xylem axis directly

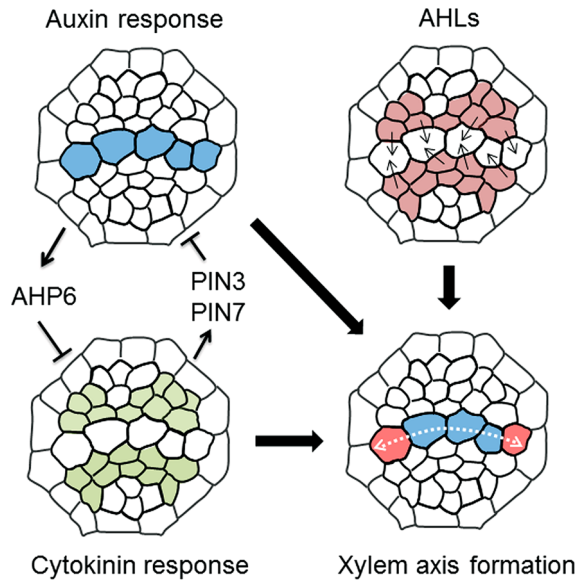

FIGURE 3 | A molecular mechanism determining xylem axis domain. Schematic illustration showing a machanism underlying the formation of xylem axis domain in the root stele. (1) Mutual inhibition of high auxin and cytokinin domain defines protoxylem vessel domain, in which auxin inhibits cytokinin signaling via AHP6 and cytokinin restricts the auxin accumulation via PIN3 and PIN7. (2) AHLs movement plays a role in the determination of not only protoxylem vessel but also metaxylem vessel domain, thereby establishing the boundary between xylem axis and procambium domain in root stele. (3) The combination of these regulations leads to the formation of spatially regulated xylem axis domain. The details are described in the main text.

up-regulates *AHP6* expression in the protoxylem vessel position via auxin-responsive elements in its promoter (Bishopp et al., 2011a). NPA treatment, which inhibits polar auxin transport, blocks *AHP6* expression in the protoxylem vessel position, resulting in the loss of protoxylem vessels (Bishopp et al., 2011a). AHP6 has a negative role in CK signaling (Mähönen et al., 2006). These results indicate that PIN-mediated polar auxin transport and auxin accumulation induces *AHP6* expression, which in turn attenuates CK signaling at the protoxylem vessel position (**Figure 3**).

Conversely, *PIN7* expression is regulated by CK (Bishopp et al., 2011a). The *PIN7* expression domain overlaps the high CK-response domain, and *PIN7* transcript levels are increased in response to CK treatment (Bishopp et al., 2011a). A recent study discusses a new technique for blocking symplastic connections by inducing the expression of mutated *CALLOSE SYNTHASE 3* (*CALS3*), which substantially increases callose deposition at plasmodesmata (Vaten et al., 2011). Using this technique to inhibit symplastic transport revealed that basipetal transport of CK via the phloem is required for controlling the *PIN7* expression domain (Bishopp et al., 2011b; Vaten et al., 2011). This result indicates that basipetal CK transport toward the RAM restricts the high auxin-response domain in the xylem axis by modulating auxin lateral transport (Bishopp et al., 2011b). Consequently, the fact that the *ahk3 cre1* double mutant forms ectopic protoxylem vessels adjacent to the original protoxylem vessels (Mähönen et al., 2006; Kondo et al., 2011) can be explained because the high auxin-response domain in the xylem axis expands due to reduced CK signaling in that mutant (Mähönen et al., 2006; Bishopp et al., 2011a,b; Kondo et al., 2011; **Figure 2B**). Therefore, this mutually inhibitory feedback loop between auxin and CK allows precise establishment and maintenance of the protoxylem vessel position (**Figure 3**).

FACTORS THAT REGULATE METAXYLEM VESSEL FORMATION

The protoxylem domain is determined by the balance between auxin and CK; however, the molecular mechanisms that determine the metaxylem vessel domain remain unclear. Recently, Ursache et al. (2014) isolated mutants defective in *TRP2*, which is involved in tryptophan biosynthesis and tryptophan-mediated auxin biosynthesis. These mutants have a defect in metaxylem vessel formation but not in protoxylem vessel formation, suggesting an involvement of auxin biosynthesis in metaxylem vessel formation.

The conserved CLE–WOX signaling pathway is involved in metaxylem vessel development in rice (*Oryza sativa*; Chu et al., 2013). A rice CLE peptide named FON2-LIKE CLE PROTEIN2 (FCP2) negatively controls the expression of *quiescent-center-specific-homeobox* (*QHB*), which is an ortholog of AtWOX5 and is expressed in the QC and metaxylem precursor cells (Kamiya et al., 2003; Chu et al., 2013). Negative regulation of QHB by application of exogenous FCP2 causes the loss of metaxylem identity, leading to aberrant cell division in the metaxylem vessel position (Chu et al., 2013). As mentioned previously, CLE peptides can inhibit protoxylem vessel formation in *Arabidopsis* (Kondo et al., 2011). Therefore, the role of CLE signaling in the regulation of root xylem development is not conserved between *Arabidopsis* and rice.

BOUNDARY FORMATION BETWEEN XYLEM AND PROCAMBIUM DOMAINS

A recent study reported that the boundary between the procambium and xylem axis is determined by moving transcription factors named AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEINs (AHLs; Zhou et al., 2013; **Figure 3**). In *ahl3* and *ahl4* single mutants, both ectopic protoxylem vessels and ectopic metaxylem vessels are formed in the procambial region adjacent to the xylem axis, suggesting an enlargement of the xylem axis domain (Zhou et al., 2013). This phenotype has some similarity with that of CK-defective mutants in terms of excess protoxylem vessel formation, but is distinctive in terms of extra metaxylem vessel formation adjacent to the original metaxylem. *AHL4* is expressed in the procambium and its protein product can move into the xylem axis domain (Zhou et al., 2013). This intercellular movement is required for correct boundary formation between the procambium and the xylem (Zhou et al., 2013). The *ahl3 ahl4* double mutant does not exhibit a more severe xylem phenotype compared with those of the single mutants, indicating that AHL3 and AHL4 function together (Zhou et al., 2013). AHL3 and AHL4 form a heterodimer, and have the potential to move from the procambium to the xylem (Zhou et al., 2013). High auxin and

high CK-response domains are altered in *ahl* mutants (Zhou et al., 2013), but the relationship between AHLs and hormonal regulation of xylem axis formation is unknown. Further analysis of the function of AHLs may provide new insights into the mechanisms underlying boundary formation between xylem axis and the procambium.

MOLECULAR SWITCHES FOR PROTOXYLEM AND METAXYLEM VESSEL CELL FATE

The GRAS-family transcription factor SHORT-ROOT (SHR), which is known to establish the identity of endodermis and cortex (Helariutta et al., 2000; Nakajima et al., 2001; Gallagher et al., 2004; Cui et al., 2007), also functions in the regulation of protoxylem and metaxylem specification (Carlsbecker et al., 2010). The *shr* mutant forms metaxylem vessels at the protoxylem position, indicating a switch of vessel types from protoxylem to metaxylem (Carlsbecker et al., 2010). SHR moves from the stele to the endodermis and induces expression of *miR165* and *miR166*, in co-operation with SCARECROW (SCR; Carlsbecker et al., 2010; **Figures 4A–C**). Then, *miR165* and *miR166* move from the endodermis to the stele, leading to their higher accumulation in the outer region than in the inner domain of the stele (Carlsbecker et al., 2010; **Figures 4D,E**). *miR165* and *miR166* destabilize the mRNAs of *class III homeodomain-leucine zipper* (*HD-ZIPIII*) family genes, which include *AtHB8*, *PHABULOSA* (*PHB*), *PHAVOLUTA*, *REVOLUTA*, and *CORONA/AtHB15* (Prigge et al., 2005; Carlsbecker et al., 2010). This results in higher expression of these genes in the inner domain of the stele (Carlsbecker et al., 2010; **Figure 4F**).

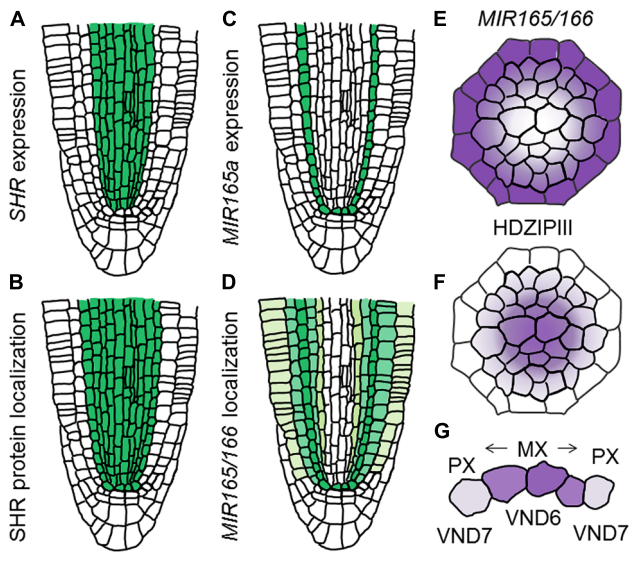

FIGURE 4 | Determination of xylem vessel types in the xylem axis. **(A–D)** Schematic illustration showing difference between gene expression and localization of gene products for *SHR* and *MIR165/166* in the RAM (based on the data reported in Carlsbecker et al., 2010). **(A)** Expression pattern of *SHR*. **(B)** Localization of SHR protein. **(C)** Expression pattern of *MIR165a*. **(D)** Localization of *MIR165/166*. **(E,F)** Schematic illustration showing localization patterns of *MIR165/166* **(E)** and HD-ZIPIII transcripts **(F)**. **(G)** Schematic illustration of xylem vessel type determination. Vessel types are determined by VND6 and VND7 as well as HD-ZIPIII gradients.

The *miR165/miR166*-insensitive mutant *phb-7d* exhibits ectopic metaxylem vessel formation at the protoxylem vessel position, similarly to that of the *shr* mutant (Carlsbecker et al., 2010). Conversely, quadruple mutants for HD-ZIPIIIs only produce protoxylem vessels in the xylem axis, which is confirmed by the loss of metaxylem vessel marker ACAULIS 5 (ACL5) and ectopic expression of protoxylem vessel marker AHP6 (Mähönen et al., 2006; Muniz et al., 2008; Carlsbecker et al., 2010). These results indicate that *HD-ZIPIII* genes ultimately determine the xylem vessel types; high expression induces metaxylem vessels, whereas low expression induces protoxylem vessels (Miyashima et al., 2011).

VASCULAR-RELATED NAC-DOMAIN 6 (VND6) and VND7, which belong to the NAM, ATAF1/2, and CUC2 (NAC) transcription factor family, are master regulators for xylem cell differentiation and determine the cell fate of the metaxylem and protoxylem vessels, respectively (Kubo et al., 2005). Both VND6 and VND7 directly up-regulate genes involved in programmed cell death and secondary cell-wall thickening (Ohashi-Ito et al., 2010; Yamaguchi et al., 2011). *VND6* is expressed in the central metaxylem vessels, whereas *VND7* is expressed primarily in the protoxylem vessel cell files (Kubo et al., 2005). Overexpression of *VND6* and *VND7* leads to ectopic formation of metaxylem and protoxylem vessel elements, respectively (Kubo et al., 2005; Yamaguchi et al., 2010a). Conversely, expression of *VND6* and *VND7* fused with the chimera repression domain SRDX under control of their own promoters causes a failure of central metaxylem and protoxylem vessel development, respectively (Kubo et al., 2005). However, loss-of-function mutants for *VND6* and *VND7* do not show any defect in root xylem development (Kubo et al., 2005), indicating that seven VND family members function differently but in some cases redundantly in the regulation of xylem cell differentiation. VND-interacting 2 (VNI2) was identified as an interacting protein with VND7 by a yeast two-hybrid screen (Yamaguchi et al., 2010b). VNI2 negatively regulates xylem vessel differentiation in opposition to VND7 (Yamaguchi et al., 2010b). Further analyses are required to elaborate the relationship between VNDs, VNIs, and HD-ZIPIIIs in the context of switching xylem vessel types (**Figure 4G**).

CONCLUDING REMARKS

Xylem cell fate is regulated by spatiotemporal actions of various signaling factors. Mutual inhibition between CK and auxin determines the precise xylem vessel domains, in particular protoxylem vessels. Some CLE peptides play a role in fine-tuning the CK signal. The movement of AHLs defines the boundary between the procambial domain and the xylem domain, thereby establishing the xylem axis in root stele. The opposite movement of SHR and *miR165/miR166* between outer endodermis and inner stele ultimately regulates the level of HD-ZIPIII proteins, resulting in the fate determination of different xylem vessel types. Finally, the master transcription factors VND6 and/or VND7 execute the program of metaxylem and protoxylem vessel differentiation, respectively. Collectively, in roots, xylem cell fates are controlled precisely by a regulatory network consisting of hormone signaling pathways and transcription factors in a hierarchical organization.

However, the basic root vascular pattern is determined during embryogenesis. Therefore, to understand the regulation of xylem cell fate, we should elucidate the mechanism underlying the onset of vascular cells in early embryos. Recent studies demonstrated that two bHLH transcription factors, LONESOME HIGHWAY (LHW) and TARGET OF MONOPTEROS 5 (TMO5), play crucial roles in the initiation of vascular cells (De Rybel et al., 2013; Ohashi-Ito et al., 2013). Further functional analysis of downstream targets of these transcription factors may provide novel insights into understanding the determination of xylem cell fates.

ACKNOWLEDGMENTS

This work was supported partly by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (NC-CARP project) to Hiroo Fukuda, from the Japan Society for the Promotion of Science (23227001 to Hiroo Fukuda, and JSPS Research Fellowships for Young Scientists to Takayuki Tamaki).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 April 2014; accepted: 13 June 2014; published online: 01 July 2014.
Citation: Kondo Y, Tamaki T and Fukuda H (2014) Regulation of xylem cell fate. Front. Plant Sci. 5:315. doi: 10.3389/fpls.2014.00315
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

OPINION ARTICLE
published: 01 May 2014
doi: 10.3389/fpls.2014.00179

Members of the *MYBMIXTA-like* transcription factors may orchestrate the initiation of fiber development in cotton seeds

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Keywords: Gossypium, ovule, protoplast, transient assay, phylogeny, transcription, HDzip, fiber initiation

MYBMIXTA-like (MML) transcription factors form the subgroup 9 of R2R3-MYBs (Stracke et al., 2001) whose first characterized member was *MIXTA* from *Antirrhinum majus*. Various *MML* genes have been shown to be important regulators of epidermal cell differentiation in different plant species, including specifying cell shape in petals, vegetative trichome initiation and branching and seed fiber initiation (Martin et al., 2002; Machado et al., 2009; Walford et al., 2011). Indeed, the conical cells of petals look very much like young fibers shortly after they protrude from the epidermal surface of the cotton seed and begin to elongate, so it is likely there is some commonality in cellular regulation between the different tissue types. In tetraploid cotton, *Gossypium hirsutum* L. (*Gh*), the silencing or over-expression of two *MML* genes (*GhMYB25* and *GhMYB25Like)* expressed predominantly in the ovule epidermis during fiber initiation affect the initiation or timing of expansion of fiber initials (Machado et al., 2009; Walford et al., 2011). Based on silencing phenotypes and gene expression data, *GhMYB25Like* may be one of the most upstream genes in a regulatory cascade currently known to involve *GhMYB25*, *GhMYB109* (an R2R3 MYB from subgroup 15, Pu et al., 2008) and other types of transcription factors, such as the homeodomain leucine zipper (HDzip) factor *GhHD-1*, that may act in a protein complex with GhMYB25 (Zhang et al., 2010; Walford et al., 2012). The recent release of the genome sequence from the diploid *G. raimondii* (*Gr*), an extant species most closely related to the D-genome progenitor of tetraploid cotton, may help draw a more complete picture about the evolution of the *MML* gene subgroup in cotton and their apparent expansion and recruitment to specialized functions in epidermal seed fiber development.

GENOMIC ORGANIZATION AND PHYLOGENY OF COTTON MMLs

The *Gr* genome contains over 200 R2R3 MYBs (Paterson et al., 2012), but 10 (*GrMML1-10*, **Figure 1A**) cluster with MIXTA, and all of these contain the signature protein motif AQWESARxxAExRLxRES previously indicated to be unique to subgroup 9 (Stracke et al., 2001). This number is considerably greater than the three *MML* genes found in Arabidopsis: *AtMYB16*, proposed to control the shape of petal epidermal cells (Baumann et al., 2007), *AtMYB17* a putative regulator of early inflorescence development and seed germination (Zhang et al., 2009) and *AtMYB106/NOK* a negative regulator of trichome branching (Jakoby et al., 2008). The *MML* factors are distinct from the *AtGL1*-like MYBs (*AtMYB0*, *AtMYB23*, *AtMYB66*) of subgroup 15, involved in trichome and root hair initiation and development, that are often suggested as the likely models for regulators of seed fiber development in cotton. Subgroup 15 in *Gr* appears to have only one member, Gorai.012G061800/GrMYB109 (Paterson et al., 2012) that is a homolog of GhMYB109 with a demonstrated role in fiber elongation rather than initiation (Pu et al., 2008). *Gr* has very close homologs of *GhMYB25* (Gorai.012G186500, *GrMML7*) and *GhMYB25Like* (Gorai.008G179600, *GrMML3*) as well as eight other novel *MML* genes, each with a pair of very close homologs in the A- and D-genomes of tetraploid *Gh* (**Figure 1A**, **Supplementary data 1**). The 10 MML proteins fall into 4 distinct clades supported by elevated bootstrap scores. One of these, Sg9-2, includes GrMML3/GhMYB25Like, GrMML7/GhMYB25, Gr/GhMML9 and 10, but no Arabidopsis or Antirrhinum MML MYBs (**Figure 1A**). In the *Gr* genome the 10 *MML* genes are distributed across 6 of the 13 diploid cotton chromosomes. However, on chromosomes Chr08 and Chr13 there are clusters of two (*GrMML3* and *4* and a fragment of another *MML* gene) and three genes (*GrMML8, 9,* and *10*), respectively, in tandem arrangements (Paterson et al., 2012; **Figure 1B**). This suggests that they may have evolved from gene duplications of ancestral *MML* genes and subsequently evolved new functions and in some cases different expression patterns.

MML GENE EXPRESSION DURING THE EARLY EVENTS OF FIBER INITIATION AND DEVELOPMENT

The 10 *MML* genes are nearly all expressed predominantly in early fiber development in whole ovules/seeds of tetraploid cotton

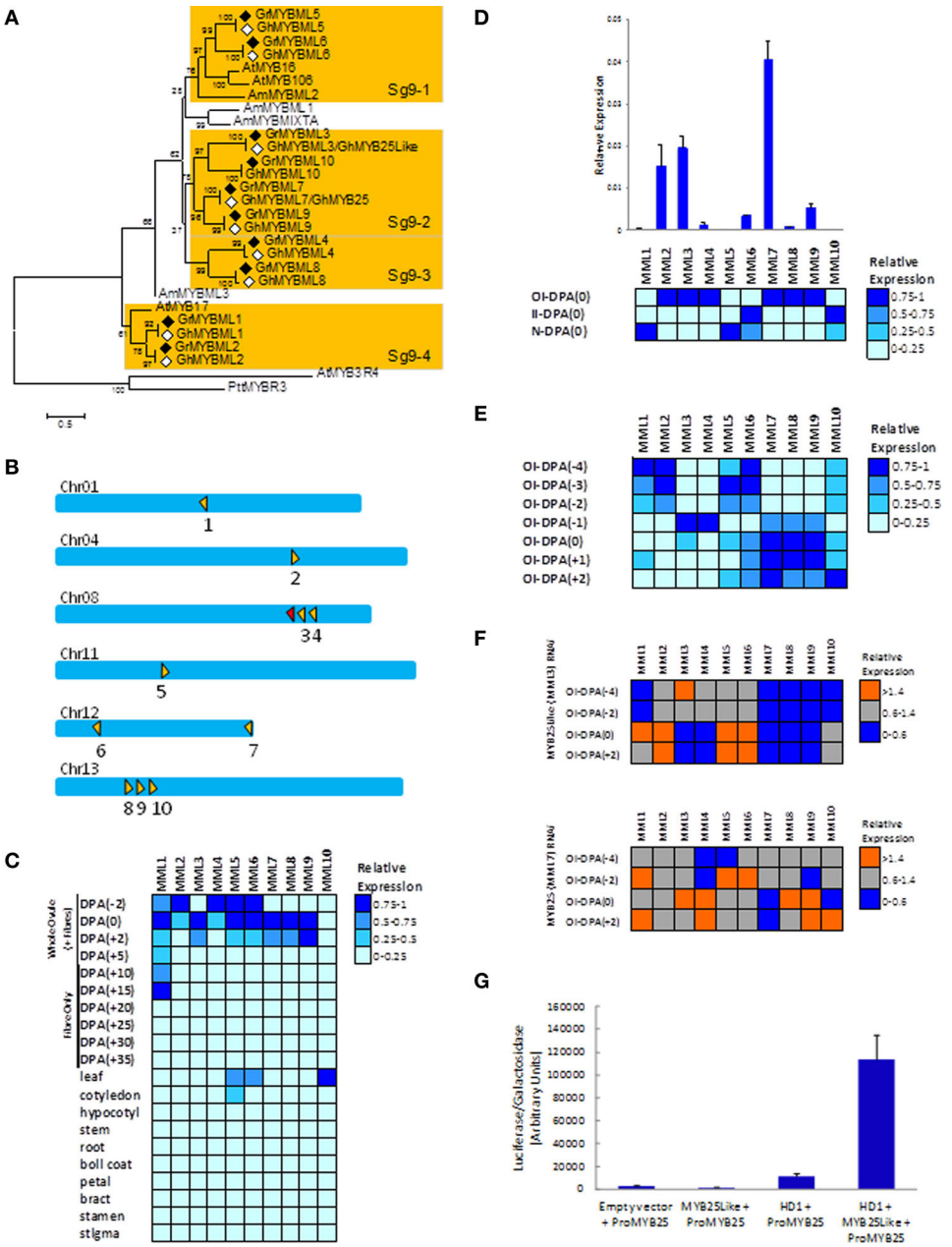

FIGURE 1 | Phylogenetic analysis, chromosomal location, gene expression of the cotton *MML* genes in wild type and transgenic *G. hirsutum*, and transactivation of the *GhMML3* promoter in cotton protoplasts. (A) Phylogenetic analysis of the *G. raimondii* (Gr, filled diamonds) MML proteins and their putative *G. hirsutum* (Gh, empty diamonds) D-genome homologs. Four clades (Sg9-1 to 4) are indicated by shading. The rooted Neighbour–Joining tree was obtained in MEGA 5.0 with Clustal W alignment using the full length amino acid sequences (details in **Supplementary data 1**). *Arabidopsis thaliana* (At) MYB3R4 and *Populus tremula* × *P. Tremuloides* (Ptt) MYBR3 are R1R2R3-MYBs used as outgroups and the other AtMYB and *Anthirrinum majus* (Am) sequences are landmarks of Subgroup 9. **(B)** Schematic of the chromosomal distribution of the 10 *GrMML* genes indicated by the filled triangles, while the unfilled triangle represents a fragment of a *MML* gene. The directions of the triangle indicate the coding strand of the transcripts and the numbers under the triangle the particular *GrMML* gene. Adjacent triangles are tandemly arranged genes. (Chromosomes are only approximately to scale). The heat maps visualize the transcript level differences between the *G. hirsutum* homologues of the *GrMML* genes in: **(C)** cotton fibers, ovules and selected other plant organs, **(D)** three dissected tissues (OI, II and N) from wild type (*G. hirsutum*) ovules collected the day of anthesis [DPA(0)], **(E)** dissected OI of wild type ovules collected from 4 days before anthesis [DPA(−4)] to 2 days after anthesis [DPA(+2)], **(F)** OI dissected from *G. hirsutum* ovules silenced by RNAi for *GhMYB25Like* (ie., *MYBML3*) and *GhMYB25* (i.e., *MYBML7*) and their respective controls (ie., null segregant plants) collected at DPA(−4), (−2), (0), and (+2) **(F)**. Heat maps were made using Expander software based on gene expression relative to the cotton ubiquitin gene and normalized for each *MML* gene and separate experiment (details in **Supplementary data 2**).Primers used detect both the A- and D-genome homoeologues of each *MML* gene. **(G)** Transactivation assay of the *GhMML3/GhMYB25* promoter-Luciferase reporter by GhMYB25Like and/or GhHD-1 in cotton cotyledon protoplasts (details in **Supplementary data 3**).

from −2 to +2 day post anthesis (DPA) (**Figure 1C**) with little expression in other cotton tissues. A few (*GhMML5, 6, 10*) are also expressed in leaves and/or cotyledons which are rich in either hair or glandular trichomes that are structurally related to seed fibers and share some common regulators like *GhHD-1* (Walford et al., 2012). *MML* gene expression levels were also investigated using hand-microdissected tissues (Bedon et al., 2013) from whole ovules and young seeds to focus just on the fiber initiation process occurring in the epidermis. This circumvents the transcript dilution or confounding with expression from the other layers of ovule tissues. Six of the *GhMML* genes were preferentially expressed at 0 DPA in outer-integuments (OI; *GhMML2, 3, 4, 7, 8,* and *9*), two in inner-integument (II; *GhMML6* and *10*) and two in the nucellus (N; *GhMML1* and *5*) (heat map in **Figure 1D**). Based on their differences in relative expression levels in the OI, three groups can be classified as having; high (*GhMML2, 3* and *7*), low to medium (*GhMML 4, 6, 8,* and *9*), and very low (*GhMML1, 5,* and *10*) expression (chart in **Figure 1D**). Further expression analysis in the OI was performed from −4 to +2 DPA which covered the stages of ovule epidermal cell differentiation (−4 to −1 DPA), fiber initiation (i.e., ballooning of fiber initials above the epidermis surface at 0 DPA), and early fiber elongation (+1 to +2 DPA) (**Figure 1E**). There are four different patterns with some *GhMML* genes having their highest expression from −4 to −2 DPA (*GhMML1, 2, 5, 6*); genes peaking at −1 DPA (*GhMML3* and *4*); genes peaking at 0 to +1 DPA (*GhMML7, 8, 9*); and one member with highest expression at +2 DPA (*GhMML10*) (**Figure 1E**). These expression patterns support the specialization of different sets of *MML* genes for specific aspects of epidermal cell differentiation, although they may still have some roles in other tissues.

MML REGULATORY CASCADES AND INTERACTIONS

To unravel the potential transcriptional networks among the different *GhMML* members their expression levels were assessed in dissected OI from transgenic tetraploid cotton silenced (through RNAi) for *GhMML3/GhMYB25Like* (Walford et al., 2011) or *GhMML7/GhMYB25* (Machado et al., 2009) compared to the transcript levels from the corresponding null segregant plants as controls (**Figure 1F**, **Supplementary data 2**). In the *GhMYB25Like* silenced plants **Figure 1F** (upper panel), the transcript level of *GhMYB25Like/GhMML3* was decreased at 0 and +2 DPA, as previously reported (Walford et al., 2011). Transcripts of *GhMML7/GhMYB25, GhMML8,* and *9* were completely abolished at 0 and +2 DPA, and *GhMML4* was decreased to a lesser extent, indicating that they may all be downstream of *GhMYB25-like*. A significant increase in *GhMML2* and *6* transcripts were observed at 0 and +2 DPA (**Figure 1F**), suggesting that they may be repressed by GhMYB25-like. A slight decrease in *GhMML1* at −4 DPA was also seen (see chart representation in **Supplementary data 2**). In the *GhMYB25* silenced plants (**Figure 1F**-lower panel), the transcript levels of *GhMML7/GhMYB25* was decreased at 0 and +2 DPA as previously reported by Machado et al. (2009). Transcript levels of *GhMML3/GhMYB25Like, GhMML4* and *8*, and to a lesser extent *GhMML9*, were increased at 0 DPA (**Supplementary data 2**), suggesting there may be some feedback regulation of *GhMYB25Like* by GhMYB25. The direct requirement of GhMYB25Like/GhMML3 for expression of *GhMML7/GhMYB25* was assessed using a transient assay in cotton protoplasts (**Figure 1G**, **Supplementary data 3**). Transactivation of the *GhMYB25* promoter, fused to the *luciferase* reporter, did occur in the presence of GhMYB25Like, but only when co-expressed with the HDzip factor GhHD-1, recently shown to be involved in fiber initiation (Walford et al., 2012), so the two may be involved in a complex to activate *GhMYB25*.

CONCLUSION

The MYBMIXTA-like subgroup appears to have expanded in cotton compared to non-fiber plants, probably following a cotton specific triplication as reported in Paterson et al. (2012), thus contributing to the evolution of seed fiber. The spatio-temporal expression study of the different *GhMML* genes reported here distinguishes them according to the plant organs and/or ovule tissues in which they are expressed and the timing of their expression during the early stages of seed fiber development. The newly described *GhMML2* is one of the more highly expressed *MML* genes in the OI along with *GhMML7/GhMYB25* and *GhMML3/GhMYB25Like* which have already been shown to be involved in seed fiber initiation (Machado et al., 2009; Walford et al., 2011), but *GhMML2* shows a different pattern of expression with highest transcript accumulation before the fiber initiation step. *GhMML2,* together with *GhMML6,* transcripts are increased rather than decreased at 0 and +2 DPA in *GhMYB25Like* RNAi plants, suggesting a molecular function different to *GhMML7, 8,* and *9* that are down-regulated in this transgenic background. The differences in *GhMML* expression in OI in the two *MMLs* silenced plants highlights a possible hierarchical network between MMLs; with MML3/GhMYB25-like activating the transcription of *MML7/GhMYB25*, *MML8,* and *MML9* and perhaps being involved in the repression of *MML2* and *MML6* at 0 and +2 DPA as the fibers begin to expand and elongate. MML7/GhMYB25 might be involved in a feedback loop to transcriptionally activate *MML3*, *4*, *8,* and *9* at 0 DPA. We were able to show that MML3/GhMYB25Like is able to transactivate the *MML7/MYB25* promoter, but only when associated with GhHD-1. Such results suggest the presence of a transcriptional complexes, involving MML(s) and GhHD-1, necessary for fiber development that are similar to the sorts of complexes seen in Arabidopsis trichome development (Ishida et al., 2008), although clearly involving different factors. Here, we identified some potential new players in fiber development that belong to the same phylogenetic group of MYBs and suggest a very subtle networking involving both protein-protein and protein-DNA interactions. The next steps involve the functional characterization of these novel cotton *MML* genes by silencing and over-expression in transgenic cotton and the study of their interactions in both cotton protoplasts and yeast to confirm their roles in generating the fibers of commercially important cotton species.

AUTHOR CONTRIBUTIONS

Phylogeny analysis (**Figure 1A**): Frank Bedon. Genomic organization of *MMLs* (**Figure 1B**): Danny J. Llewellyn. Gene expression studies (**Figures 1D–F**): Frank Bedon and Lisa Ziolkowski, (**Figure 1C**): Sally A. Walford. Transient assays in protoplast (**Figure 1G**): Frank Bedon and Lisa Ziolkowski. Wrote the paper: Frank Bedon, Sally A. Walford, Elizabeth S. Dennis and Danny J. Llewellyn.

ACKNOWLEDGMENTS

This work was supported in part by funding from the Monsanto Company (Frank Bedon, Lisa Ziolkowski), as well as Cotton Breeding Australia, a joint venture between CSIRO and Cotton Seed Distributors (Sally A. Walford, Danny J. Llewellyn).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00179/full

Supplementary data 1 | Sequence information.

Supplementary data 2 | Gene expression data.

Supplementary data 3 | Transient assays.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 February 2014; accepted: 14 April 2014; published online: 01 May 2014.
*Citation: Bedon F, Ziolkowski L, Walford SA, Dennis ES and Llewellyn DJ (2014) Members of the MYBMIXTA-like transcription factors may orchestrate the initiation of fiber development in cotton seeds. Front. Plant Sci. **5**:179. doi: 10.3389/fpls.2014.00179*
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 18 February 2014
doi: 10.3389/fpls.2014.00050

The role of lipid post-translational modification in plant developmental processes

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Most eukaryotic proteins are post-translationally modified, and modification has profound effects on protein function. One key modification is the attachment of a lipid group to certain amino acids; this typically facilitates subcellular targeting (association with a membrane) and protein–protein interactions (by virtue of the large hydrophobic moiety). Most widely recognized are lipid modifications of proteins involved in developmental signaling, but proteins with structural roles are also lipid-modified. The three known types of intracellular protein lipid modifications are S-acylation, N-myristoylation, and prenylation. In plants, genetic analysis of the enzymes involved, along with molecular analysis of select target proteins, has recently shed light on the roles of lipid modification in key developmental processes, such as meristem function, flower development, polar cell elongation, cell differentiation, and hormone responses. In addition, while lipid post-translational mechanisms are generally conserved among eukaryotes, plants differ in the nature and function of target proteins, the effects of lipid modification on target proteins, and the roles of lipid modification in developmental processes.

Keywords: prenylation, farnesylation, geranylgeranylation, myristoylation, acylation, palmitoylation

INTRODUCTION

With the rapid advancement of genome sequencing technologies, it is now possible to know virtually all the genes, and thus the full complement of proteins, in an increasing number of organisms. The next frontier involves learning more about these proteins: their activation/deactivation, their localization, and their function. Most proteins are post-translationally modified, and these modifications have profound implications on protein activity. This review focuses on lipid modifications of proteins. Intracellular protein lipid modifications include prenylation, N-myristoylation, and S-acylation and serve a primary role of facilitating targeting and association of the lipidated protein to various cellular membranes and subdomains of membranes, along with a secondary role in promoting protein–protein interactions between the hydrophobic chain and a hydrophobic region of an accessory protein (Thompson and Okuyama, 2000). Hundreds of proteins are known or thought to be lipid modified in plants; this review focuses on the role of protein lipid modifications in developmental signaling and plant growth processes.

PROTEIN PRENYLATION

Of the three types of known intracellular lipid modifications of proteins in plants, protein prenylation is the most well-studied. Prenylation involves the addition of a single 15-carbon farnesyl or single or dual 20-carbon geranylgeranyl moieties to one or two cysteines near the C-terminus of target proteins (Zhang and Casey, 1996). Prenylation is conserved among Eukaryotes (Yalovsky et al., 1999; Galichet and Gruissem, 2003; Maurer-Stroh et al., 2003). Three separate heterodimeric enzymes perform prenylation: protein farnesyltransferase (PFT), protein geranylgeranyltransferase-I (PGGT), and Rab geranylgeranyltransferase (Rab-GGT, also called geranylgeranyltransferase-II; Maurer-Stroh et al., 2003; McTaggart, 2006).

Protein farnesyltransferase and protein geranylgeranyltransferase-I share a common α subunit but have distinct β subunits that are distantly related in sequence (usually 25–35% similarity) and that determine substrate specificity. PFT recognizes a C-terminal CaaX box, where C is the prenylated cysteine, a is usually aliphatic, and X is usually alanine, cysteine, glutamine, methionine, or serine. PGGT recognizes a similar sequence, except the X is almost always leucine. A wide range of critical signaling proteins is prenylated by PFT and PGGT, including members of the Ras superfamily of small GTPases, heterotrimeric G protein γ subunits, certain classes of protein kinases, and many others (McTaggart, 2006; Maurer-Stroh et al., 2007; Nguyen et al., 2010). Mutations in the Ras family are implicated in at least 15% of human cancers, and farnesyltransferase inhibitors have undergone Phase III trials as chemotherapy agents (Nguyen et al., 2010).

Rab geranylgeranyltransferase also consists of α and β subunits, which are distantly related (typically 20–30% similarity) to the PFT/PGGT α and β subunits, respectively, and also requires an additional protein, Rab escort protein (REP), for activity (Leung et al., 2006). Mammalian and yeast Rab-GGT is thought to exclusively prenylate Rab GTPases, a family of Ras-related small GTPases involved in organelle biogenesis and vesicle transport (Leung et al., 2006). Rab-GGT contain a more diverse array of C-terminal target sequences, which includes dual-modified CC, CXC, CCX, CCXX, CCXXX, and the singly modified CXXX, with C as the modified cysteine (Maurer-Stroh and Eisenhaber, 2005). Defects in Rab-GGT prenylation are also implicated in a number of human diseases (McTaggart, 2006).

Many studies over the years confirm that prenylation mechanisms are broadly conserved in plants (Galichet and Gruissem, 2003; Crowell and Huizinga, 2009; Sorek et al., 2009; **Table 1**). The β subunits and shared α subunit of PFT and PGGT are present in single copy in *Arabidopsis* (Cutler et al., 1996; Ziegelhoffer et al., 2000; Caldelari et al., 2001; Maurer-Stroh et al., 2003; Running et al., 2004; Johnson et al., 2005). Rab-GGT is less well characterized in plants, but Rab-GGT activity has been shown to be present in extracts of tomato, tobacco, and *Arabidopsis* (Yalovsky et al., 1996; Hala et al., 2005). Interestingly, the *Arabidopsis* genome contains two putative Rab-GGT α and β subunits, along with a REP homolog (Lange and Ghassemian, 2003; Maurer-Stroh et al., 2003; Hala et al., 2005, 2010; Crowell and Huizinga, 2009; **Table 1**); single copies of the α and β subunits are present in yeast and animals examined to date (Leung et al., 2006).

PFT AND PGGT FUNCTION IN PLANTS

Genetic analysis in *Arabidopsis* has revealed important roles of PFT and PGGT in plant growth, development, and environmental responses. Mutations in the *Arabidopsis* PFT β subunit, called *enhanced response* to *abscisic acid1* (*era1*, also called *wiggum*), show increased sensitivity to abscisic acid (ABA) in the maintenance of seed dormancy and guard cell function (Cutler et al., 1996; Pei et al., 1998; Allen et al., 2002; Brady et al., 2003). Modulation of *era1* levels has been shown to preserve yield of *Brassica napus* (canola) under drought conditions in field trials (Wang et al., 2005). *era1* mutants also have several developmental defects (Running et al., 1998; Bonetta et al., 2000; Yalovsky et al., 2000a). Shoot meristems are increased in size, resulting in altered phyllotaxy, and floral meristems are wider, resulting in extra organs, particularly extra sepals and petals. In addition, *era1* mutants are slightly shorter in stature and slightly slower growing. Still, the mild phenotype of *era1* is in contrast to homologous mutants in animals, where PFT mutants are lethal (Therrien et al., 1995; Mijimolle et al., 2005), and in yeast, where PFT is required for growth (Schafer et al., 1990).

Surprisingly, mutations in the *Arabidopsis* PGGT β subunit (termed *GGB*) do not cause developmental defects and in fact are completely indistinguishable from wild type under normal growth conditions. Under exogenous ABA, *ggb* mutants show slightly enhanced responses in stomata closure, and under exogenous auxin, there is a slight enhancement of lateral root formation, but no other phenotypes are apparent (Johnson et al., 2005). This is in stark contrast to animal and yeast PGGT β subunit knockouts, which are invariably lethal (Ohya et al., 1991; Diaz et al., 1993; Trueblood et al., 1993; Therrien et al., 1995).

Mutations in the *Arabidopsis* α subunit shared between PFT and PGGT, termed *PLURIPETALA* (PLP), cause severe developmental phenotypes, including much larger shoot meristems, stem fasciation, and extra floral organs (Running et al., 2004). Flowers of *plp* mutants show about one extra organ per whorl, with the exception of petals, of which there can be up to 12 instead of the normal four found in wild type. The large meristems seen in *plp* appear to result from a defect in differentiation: primordia that are initiated from the shoot meristem occasionally fail to differentiate into a primordia fate and retain a partial or full meristem fate, initiating their own primordia and eventually fusing to the primary shoot meristem, resulting in compound shoot meristems harboring multiple central zones (Running et al., 2004). *plp* mutants also flower a month late under long days. *plp* mutants are sensitive to ABA in inhibition of seed germination, though they are not as sensitive as *era1* mutants, and also show enhanced drought tolerance (Running et al., 2004; Wang et al., 2009). Adaxial leaf pavement cells have fewer lobes in *plp* (Sorek et al., 2011). Remarkably, *plp* mutants remain viable and fertile, though with reduced seed set, while PFT/PGGT α subunit mutants are lethal in yeast (He et al., 1991) and have not been reported in non-plant eukaryotes.

It is likely that *ERA1* and *GGB* represent the only PFT/PGGT β subunits in plants, as the *era1 ggb* double mutant phenotypically resembles *plp* (Johnson et al., 2005). The mild phenotypes of *era1* and especially *ggb* compared to *plp* suggest that there is a great deal of functional overlap in *Arabidopsis* prenylation enzymes compared to those of other eukaryotes. Cross-specificity of PFT and PGGT have been reported for a small number of target proteins in mammals and yeast, but in most cases proteins that are substrates for PFT are poor substrates for PGGT and vice versa (Zhang and Casey, 1996; Maurer-Stroh and Eisenhaber, 2005; McTaggart, 2006). Additional genetic, cytological, and biochemical evidence supports considerable cross-specificity of PFT and PGGT in plants. Overexpression of *GGB* in *era1* mutants can significantly rescue the *era1* phenotype (Johnson et al., 2005). Recombinant *Arabidopsis* PFT can prenylate several PGGT targets, including the heterotrimeric G protein γ subunits AGG1 and AGG2, several Rop-GTPases, and selected membrane-anchored ubiquitin-fold (MUB) proteins, and farnesylation is sufficient for extensive membrane localization of most of these proteins *in vivo* (Downes et al., 2006; Zeng et al., 2007; Sorek et al., 2011). *In vitro* studies show that *Arabidopsis* PFT has nearly as high affinity for leucine as it does for the standard alanine, cysteine, glutamine, methionine, and serine in the X position of the CaaX target sequence, while PGGT shows weak but measurable affinity for non-leucine terminal amino acids (Andrews et al., 2010), which helps explain both the weak *era1* and very weak *ggb* mutant phenotypes compared to *plp*. Plant prenylation enzymes appear to be less

Table 1 | Putative protein prenylation components in *Arabidopsis*.

Enzyme	α subunit	α subunitgene name(s)	β subunit	β subunitgene name(s)
Farnesyltransferase	PFT/PGGTα	*PLP* (At3g59380)	PFTβ	*ERA1* (At5g40280)
Geranylgeranyltransferase	PFT/PGGTα	*PLP* (At3g59380)	PGGTβ	*GGB* (At2g39550)
Rab geranylgeranyltransferase	Rab-GGTα	*RGTA1, RGTA2* (At4g24490, At5g41820)	Rab-GGTβ	*RGTB1, RGTB2* (At5g12210, At3g12070)

stringent in protein target sequence in general: an aromatic amino acid in the a_2 position of the CaaX box does not hinder prenylation of the Rho-related GTPases Rac13 from cotton and ROP2 from *Arabidopsis* (Trainin et al., 1996; Johnson et al., 2005; Lane and Beese, 2006), while similar sequences act as potent inhibitors of mammalian PFT (Lane and Beese, 2006).

There are several possible explanations for the viability of *plp* mutants. One is the presence of non-prenylated proteins that can compensate for the loss of function of prenylated proteins. Another is that other lipid modifications, such as acylation (Hemsley and Grierson, 2008), may be enough to confer proper localization to a subset of prenylated proteins. Both of these may be true for ROP proteins, the Rho-related GTPases in plants (Vernoud et al., 2003). *Arabidopsis* contains 11 ROPs, 10 of which contain a CaaX motif; of these, three are either not prenylated or show only weak prenylation *in vitro* (Lavy et al., 2002; Zheng et al., 2002). Instead, these three (termed type II ROPs) are palmitoylated, and palmitoylation is necessary for their membrane localization and function (Lavy et al., 2002; Sorek et al., 2011).

PFT AND PGGT TARGET PROTEINS

Perhaps the most surprising aspect of the viability and fertility of *plp* is the sheer number of putative PFT and PGGT protein targets that would be affected in the mutant plants. Our analysis and those of others show that over 250 *Arabidopsis* proteins fit ideal criteria for prenylation, while as many as 700 fit minimal criteria (the presense of the cysteine four residues from the C terminus; Galichet and Gruissem, 2003). Some of the functional classes of proteins found in these database searches include transcription regulation, signaling, polar cell growth, cell cycle regulation, cell wall modification, hormone synthesis and response, metal ion homeostasis, pathogen defense response, protein folding, and many of unknown function (Galichet and Gruissem, 2003; Zeng and Running, 2008; Crowell and Huizinga, 2009; Sorek et al., 2009). Some functional classes, such as prenylated transcription factors, are not found in other eukaryotes, while some proteins that are prenylated in other systems lack prenylation motifs in plants, like nuclear lamins and inositol triphosphate 5′-phosphatases (Crowell and Huizinga, 2009; Sorek et al., 2009).

An increasing number of plant proteins have been shown to be prenylated *in vitro*, and there is direct or indirect evidence for the prenylation of several proteins *in vivo* as well. Of the aforementioned ROP proteins, Class I (ROP1–ROP8) contain a classical CaaL motif and are likely prenylated *in vivo*; indeed, ROP6 has been shown to be geranylgeranylated *in vitro* and *in vivo* (Sorek et al., 2011). *Arabidopsis* ROP1, ROP2, ROP3, and ROP7 were also shown to be geranylgeranylated (Sorek et al., 2009). In the absence of PGGT, ROP6 can also be farnesylated *in vitro* and *in vivo*. ROP proteins from other species have also been shown to be prenylated, including ROP1Ps from pea and Rac13 from cotton (Trainin et al., 1996).

The *Arabidopsis* heterotrimeric G protein γ subunits AGG1 and AGG2 have been shown to be prenylated (Adjobo-Hermans et al., 2006; Zeng et al., 2007). Like ROP6, both AGG1 and AGG2 contain a CaaL motif for geranylgeranylation, but they can also be farnesylated *in vitro*. Their membrane localization is only slightly affected in a *ggb* mutant, indicating again a compensation for the loss of PGGT by PFT, but is drastically altered in a *plp* mutant (Zeng et al., 2007). Interestingly, a third, atypical heterotrimeric G protein γ subunit, AGG3, was recently identified, but, despite its plasma membrane localization, it lacks a prenylation motif and is likely not prenylated (Chakravorty et al., 2011).

There are six MUB protein family members in *Arabidopsis*, five of which have a CaaX or CaaL box and can be prenylated *in vitro* (Downes et al., 2006). All six *Arabidopsis* MUBs are associated with the plasma membrane *in vivo*. One difference observed with MUB6, which has a CaaL box, is that its plasma membrane localization is lost in a *ggb* mutant, suggesting that PFT cannot compensate for the loss of PGGT for all target proteins.

The *Arabidopsis* nucleosome assembly protein AtNAP1;1 has been shown to be farnesylated *in vivo* (Galichet and Gruissem, 2006). AtNAP1;1 also provides the only known example for which farnesylation is developmentally regulated in plants, and non-farnesylated AtNAP1;1 promotes cell expansion versus cell division in later stages of leaf development (Galichet and Gruissem, 2006).

Plants also harbor the only known prenylated transcription factors, including APETALA1 protein family members (Yalovsky et al., 2000b). *Arabidopsis apetala1* mutant flowers show a lack of petals and a conversion of sepals to leaf-like bracts, with production of additional flowers within the flower (Mandel et al., 1992). This is different than the *plp* floral mutant phenotype, making it unclear what the role of prenylation is for APETALA1 function. However, prenylation of APETALA1 is required for the overexpression phenotype of a conversion of apical meristem to floral meristem fate (Yalovsky et al., 2000b).

A variety of other plant proteins have been shown to be prenylated *in vitro*, including the *Arabidopsis* metal binding domain proteins ATFP3 and HIPP26, the tobacco disease resistance-related protein NTGP4, tobacco and *Arabidopsis* homologs of the yeast v-SNARE protein Ykt6p, petunia calmodulin protein CaM53, the *Arabidopsis* AUX/IAA family member IAA4, and *Arabidopsis* AtIPT3 (Barth et al., 2009; Crowell and Huizinga, 2009). Disappointingly, however, there is no indication that any of the currently known prenylated proteins play any role in the striking developmental phenotype of *plp* mutants, suggesting that as-yet-unidentified target proteins play specific roles in meristem function and flower development.

Rab-GGT FUNCTION IN PLANTS

While PFT and PGGT mutants have been available for many years, only recently have mutants in one of the Rab-GGT subunits been characterized. Mutations in the Rab-GGT beta subunit RGTB1 gene result in a striking series of phenotypes, including smaller, epinastic leaves, loss of apical dominance (extreme branching), greatly delayed senescence, and infertility, though both male and female gametes can be effectively used in crosses (Hala et al., 2010). The mutant results in the accumulation of the unprenylated form of at least one Rab protein, Rab A2a. The mutants also show shoot gravitropic defects and a constitutive photomorphogenic

phenotype in the dark, but surprisingly, little or no effects on root growth (Hala et al., 2010).

The effects of mutations in the other beta subunit, RGTB2, and in either of the alpha subunits RGTA1 and RGTA2, have not been reported. It would be interesting to see, for instance, if the *rgtb1, rgtb2* double mutant is lethal. RGTB2 mRNA is present but is one-tenth as abundant as RGTB1 mRNA (Hala et al., 2010), suggesting that residual Rab-GGT activity may be responsible for the survivability of *rgtb1* mutants. Another intriguing possibility is if some Rab proteins with a CaaX motif can be efficiently prenylated by PFT or PGGT, compensating for the loss of Rab-GGT function. There is some precedence for this: mammalian Rab8, with a CVLL motif can be efficiently prenylated by PGGT *in vitro* (Wilson et al., 1998), and Rab11 (CQNI) can be prenylated by PFT (Joberty et al., 1993). Indeed, 32 of the 56 *Arabidopsis* Rab proteins contain a CXXX motif that can in theory act as a PFT target. Interestingly, at least one Rab protein, ARA6, is not prenylated in *Arabidopsis*; instead, it is myristoylated and palmitoylated (Ueda et al., 2001) and is likely to function in the absence of Rab-GGT activity. While RGTA1 is expressed everywhere, RGTA2 is expressed only in the pollen (Hala et al., 2010), suggesting that, if indeed Rab-GGT is required for survival, viable *rgta1* complete loss of function mutants may be more difficult to isolate.

N-MYRISTOYLATION

N-myristoylation involves the addition of a 14 carbon saturated myristate group to the N-terminal glycine of target proteins (Gordon et al., 1991; Johnson et al., 1994). It is carried out by a monomeric enzyme, *N*-myristoyltransferase (NMT; Duronio et al., 1989). The absolute requirement for enzymatic activity is an N-terminal glycine on the target protein, while amino acid residues two through six are used in substrate recognition, residues 7 through 10 are less restricted, and residues 11 through 17 are hydrophilic (Maurer-Stroh et al., 2002; Utsumi et al., 2004). Interestingly, experimental evidence as well as homology remodeling of NMT protein structures suggest that there are substrate specificity differences among different species (Maurer-Stroh et al., 2002).

Arabidopsis contains two homologs of NMT, termed *AtNMT1* (At5g57020) and *AtNMT2* At2g44170 (Qi et al., 2000), and AtNMT1 protein has been shown to have myristoylation activity on N-terminal glycine-containing peptides (Qi et al., 2000; Boisson et al., 2003; Podell and Gribskov, 2004). In fact such studies have allowed a refinement of the predicted *Arabidopsis* myristoylation target sequence, which is somewhat distinct from that of animals and fungi (Boisson et al., 2003; Podell and Gribskov, 2004).

Several lines of evidence indicate that AtNMT1 is the primary N-myristoylation enzyme in *Arabidopsis*. Its mRNA is expressed at a higher level than that of *AtNMT2* (Pierre et al., 2007), and indeed it remains unclear if AtNMT2 has any myristoylation activity, despite showing 80% identity to AtNMT1 (Boisson et al., 2003). *AtNMT1* knockouts are lethal at an early stage (Pierre et al., 2007), suggesting that AtNMT2 cannot compensate for loss of AtNMT1 activity in *Arabidopsis*. In addition, constitutive expression of AtNMT2 in an *AtNMT1* mutant also fails to rescue (Pierre et al., 2007), indicating that *AtNMT2* truly has a different function than *AtNMT1*. Unlike *AtNMT1*, *AtNMT2* cannot complement a yeast *nmt1* mutant, and AtNMT2 cannot myristoylate at least one AtNMT1 target, SALT OVERLY SENSITIVE3 (Boisson et al., 2003).

AtNMT1 AND AtNMT2 FUNCTION IN PLANTS

A knockout of the *AtNMT1* gene results in a seedling lethal phenotype: seedling arrest occurred at day 4 of germination (Pierre et al., 2007). In addition, seedlings were hypersensitive to high glucose concentration. Interestingly, the roots and root meristem were normal in this mutant, but the rib zone of the shoot meristem was completely disorganized, suggesting a role in shoot meristem establishment for *AtNMT1* (Pierre et al., 2007). Lack of AtNMT1 expression at later time points shows that AtNMT1 is required for most aspects of post-embryonic development as well, including flower differentiation, fruit maturation, and fertility (Pierre et al., 2007). A weak allele of *AtNMT1* shows defects in Golgi trafficking and integrity (Renna et al., 2013). The loss of *AtNMT2* was much less severe than the loss of *AtNMT1* and resulted strictly in a delay of flowering (Pierre et al., 2007). However, expression of *AtNMT2* under the *AtNMT1* promoter resulted in shoot meristem defects.

MYRISTOYLATION TARGETS IN PLANTS

As is the case for prenylation, proteins shown or predicted to be myristoylated by NMT1 *Arabidopsis* include several protein classes that are also myristoylated in other eukaryotes, as well as novel classes (Boisson et al., 2003; Podell and Gribskov, 2004; Martinez et al., 2008). Of the 319 Arabidopsis proteins predicted to be myristoylated by Podell and Gribskov (2004), 77 are kinases (including most of the known calcium-dependent protein kinases), 13 are phosphatases, 15 are GTP-binding proteins, 10 are transcription factors, and 132 are unknown proteins.

In recent years the number of plant proteins shown experimentally to be N-myristoylated, either directly (by *in vitro* myristoylation assays) or indirectly (by mislocalization of the protein when the N-terminal glycine is mutated) has increased significantly (**Table 2**). The developmental arrest of *Atnmt1* knockouts appears to be due primarily to the loss of myristoylation on SnRK1 kinase (Pierre et al., 2007), but in other cases it is unclear how these myristoylated proteins contribute to the *Atnmt1* phenotype.

PROTEIN S-ACYLATION

Protein S-acylation is the covalent attachment of a fatty acid to a cysteine residue. Commonly the acyl group involved is palmitate, a 16-carbon saturated fatty acid, but other groups, including shorter or longer or unsaturated groups, can be added (Hallak et al., 1994). Unlike prenylation and N-myristoylation, S-acylation is reversible (Resh, 2006). Also unlike prenylation or myristoylation, which have specific target sequences at the C- and N-terminus of the protein, the prenylated cysteine can be localized anywhere in the protein, though it is common to find acylated cysteines in close proximity to myristoylated glycines on the N-terminus or prenylated cysteins on the C-terminus (Resh, 2006).

Table 2 | Myristoylated proteins.

Protein	Species	Function	Reference
SnRK1	*Arabidopsis*	Protein kinase	Pierre et al. (2007)
ARA6	*Arabidopsis*	RabGTPase	Ueda et al. (2001), Boisson et al. (2003)
SOS3	*Arabidopsis*	Calcinurin B-like protein	Ishitani et al. (2000)
PTO	Tomato	Protein kinase	De Vries et al. (2006)
CBL1	*Arabidopsis*	Calcinurin B-like protein	Batistic et al. (2008)
AtPCaP1	*Arabidopsis*	Cation-binding protein	Nagasaki et al. (2008)
NtCDPK2	Tobacco	Calcium-dependent protein kinase	Witte et al. (2010)
CPK3	*Arabidopsis*	Calcium-dependent protein kinase	Mehlmer et al. (2010)
AtTrx*h*9	*Arabidopsis*	h-type thioredoxin	Meng et al. (2010), Traverso et al. (2013)
BON1	*Arabidopsis*	Copine protein	Li et al. (2010)
CBL4	*Arabidopsis*	Calcium sensor	Held et al. (2011)
CAST AWAY	*Arabidopsis*	Cytoplasmic receptor kinase	Burr et al. (2011)
RPP1-WsB	*Arabidopsis*	Leucine-rich repeat	Takemoto et al. (2012)
RPS5	*Arabidopsis*	Nucleotide-binding	Takemoto et al. (2012)
PBS1	*Arabidopsis*	Protein kinase	Takemoto et al. (2012)
POLTERGEIST	*Arabidopsis*	2C-type protein phosphatase	Gagne and Clark (2010)
POLTERGEIST-LIKE	*Arabidopsis*	2C-type protein phosphatase	Gagne and Clark (2010)
PP2C74	*Arabidopsis*	2C-type protein phosphatase	Tsugama et al. (2012b)
PP2C52	*Arabidopsis*	2C-type protein phosphatase	Tsugama et al. (2012a)
PCaP1	*Arabidopsis*	Cation binding protein	Vijayapalani et al. (2012)
CAN1	*Arabidopsis*	Nuclease	Lesniewicz et al. (2012)
CAN2	*Arabidopsis*	Nuclease	Lesniewicz et al. (2012)
GPA1	*Arabidopsis*	Heterotrimeric G protein alpha subunit	Boisson et al. (2003), Feng et al. (2013)

In contrast to prenylation or N-myristoylation, a large protein family is responsible for S-acylation. *Arabidopsis* harbors 24 putative protein S-acyltransferases (PATs), which are characterized by having four to six transmembrane domains and a DHHC amino acid motif within a cysteine rich domain (Hemsley and Grierson, 2008; Hemsley, 2009; Batistic, 2012). Several of these 24 have been confirmed to have acyltransferase activity (Batistic, 2012). Interestingly, the 24 PATs are localized to a variety of different intracellular membranes, although the majority is localized at the plasma membrane (Batistic, 2012).

PAT FUNCTION IN PLANTS

Despite the high number of PATs in *Arabidopsis*, they do not act completely redundantly; two of the PATs have been reported so far to have visible phenotypes when mutated. The first mutation in a PAT, called *tip1*, was originally identified in a screen for altered root hair phenotypes (Schiefelbein et al., 1993; Ryan et al., 1998). The root hairs of *tip1* mutants are wider, shorter and often branched at their base. Pollen, another cell type that undergoes polar elongation, is affected in both germination and growth, resulting in reduced male fertility (Schiefelbein et al., 1993; Ryan et al., 1998). *tip1* mutants also show general growth defects, including shorter stature and smaller rosettes (Ryan et al., 1998). Interestingly, *TIP1* (At5g20350) encodes the only PAT in *Arabidopsis* that also contains ankryin repeats, a total of six at the N-terminus; ankryin repeats play a role in protein–protein interactions (Hemsley et al., 2005).

Mutations in a second PAT, *PAT10* (At3g51390), has recently been described. The PAT10 protein is localized to the tonoplast and possibly the Golgi and trans-Golgi network, and mutations in *PAT10* result in pleiotropic effects on growth and environmental response (Qi et al., 2013; Zhou et al., 2013). *pat10* mutants are smaller and grow much more slowly than wild type, with much smaller rosette leaves and inflorescence stems, a consequence of both reduced cell division and cell expansion (Qi et al., 2013; Zhou et al., 2013). Vascular development is also affected (Qi et al., 2013). Fertility is much reduced, a consequence of short stamens, pollen coat defects, pollen tube elongation and targeting defects, and disorganized embryo sacs (Zhou et al., 2013). *pat10* mutants are also salt sensitive (Zhou et al., 2013).

S-ACYLATION TARGETS IN PLANTS

Identification of S-acylated proteins in plants using bioinformatics is not feasible due to the lack of a consensus sequence for acylation. However, a recent study using a combined proteomics/bioinformatics approach identified about 600 proteins putatively palmitoylated, much more than previously thought

(Hemsley et al., 2013). These include a proteins with a diverse array of biochemical functions, including MAP kinases, leucine-rich repeat receptor kinases, integral membrane transporters, ATPases, and SNAREs, many of which were hitherto not suspected of being acylated. This study also confirmed that S-acylation is important for the function of FLS2, a leucine-rich repeat receptor like kinase involved in pathogen perception (Hemsley et al., 2013).

Previous studies have focused on the role of S-acylation of particular target proteins. In some cases S-acylation compensates for the lack of prenylation in targeting proteins to the membranes. The three type II ROPs of *Arabidopsis*, AtROP9, AtROP10, and AtROP11 are S-acylated in lieu of prenylation, and S-acylation is required for their correct membrane localization (Lavy et al., 2002). AtMUB2 also is likely S-acylated via its cysteine-rich C-terminus and is targeted to the membrane (Downes et al., 2006).

More commonly, though, S-acylation occurs in conjunction with either prenylation or myristoylation. In some examples, either prenylation or myristoylation is sufficient to target proteins to an internal membrane, while further S-acylation is required to confer plasma membrane localization. For instance, in the case of AGG2, disruption of a putative S-acylated cysteine site two amino acids upstream of the prenylated cysteine causes localization to the trans-Golgi, while full plasma membrane localization is restored when both cysteines are intact (Zeng et al., 2007). Interestingly, disruption of the putatively acylated cysteine in the same position in AGG1 does not cause a change in membrane localization (Zeng et al., 2007). Similarly, in the case of h-type thioredoxin, myristoylation alone confers localization to the ER/Golgi, and S-acylation is required for localization to the plasma membrane (Traverso et al., 2013).

Several other proteins previously mentioned are dual modified, including ARA6 (Ueda et al., 2001), type I ROPs (Sorek et al., 2007, 2011), CBL1 (Batistic et al., 2008), NtCDPK2 (Witte et al., 2010), POLTERGEIST and POLTERGEIST-LIKE (Gagne and Clark, 2010), CBL4 (Held et al., 2011), RPS5 (Qi et al., 2012), and CAN1 and CAN2 (Lesniewicz et al., 2012).

Since PAT10 and TIP1 act non-redundantly, and the other PATs cannot completely compensate for their function, it follows that at least some of their respective target proteins are unique. In the case of PAT10, it was noted that certain *CBL1*-related genes also have putative S-acylation sites, are also localized to the tonoplast and, like *PAT10*, are involved in abiotic stress responses (Zhou et al., 2013). Indeed, CBL2, CBL3, and CBL6 are all cytoplasmically localized instead of tonoplast-localized both in *pat10* mutants and when wild type plants are treated with the palmitoylation inhibitor 2-bromopalmitate, making these excellent candidates for PAT10 targets (Zhou et al., 2013). In the case of TIP1, it is possible to compare the relative S-acylation levels of a protein between wild type and *tip1* mutant samples. One hundred three proteins showed a greater than or equal to 1.5 fold decrease in S-acylation in the mutant, indicating that they are either targets of TIP1 or they may show reduced acylation indirectly as a consequence of the developmental effects of *tip1* mutants (Hemsley et al., 2013).

CONCLUSIONS AND FUTURE PROSPECTS

Much progress has been made in understanding the processes of protein lipidation in *Arabidopsis* in the past decade. In the case of prenylation and N-myristoylation, *Arabidopsis* target sequences have been refined, which is important as they vary somewhat from non-plant eukaryotes, and a large scale proteomics effort have uncovered a wealth of S-acylation targets, though target sequences for S-acylation remains elusive. We now have in hand *Arabidopsis* mutations for many of the key lipidation enzymes, though it would be a welcome advance to differentiate a role for putative Rab-GGTase subunits RGTB2, RGTBA1, and RGTBA2, and it will be interesting to see if other PATs have phenotypes other than the two reported. It is likely that further information will come from double mutant studies; for instance, whether *rgtb1 rgtb2* double mutants are lethal, and to uncover putative redundancies within the PAT family. Now that information for expression and subcellular localization of PATs are at hand (Batistic, 2012), it may be easier to identify possible redundant factors.

A wealth of information is now available for lipidated proteins; however, those that have been confirmed via *in vitro* and *in vivo* assays and subcellular localization studies still represent only a small percentage of proteins that are likely to be lipidated. More high-throughput studies on identification of prenylated or N-myristoylated proteins would be helpful. Further characterization of additional target proteins will help clarify the multiple roles of lipidation in subcellular localization and protein function.

One gaping hole in our knowledge on plant protein lipidation is that almost all the studies of knockout and knockdown mutants in lipidation enzymes in plants has been in *Arabidopsis*. It is not known, for instance, if the lack of both farnesylation and geranylgeranylation (represented by *plp* mutants) is more generally lethal in plants (as it is in other eukaryotes), and *Arabidopsis* represents an outlier in its function. Similarly with the other prenylation, N-myristoylation, or S-acylation enzymes, it is not known whether their biological function is conserved among plants. Knockouts or knockdowns of lipidation enzymes in species other than *Arabidopsis* will help answer these questions.

ACKNOWLEDGMENT

Work in the Running laboratory is currently funded by KSEF 2841-RDE-016 to Mark P. Running.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 December 2013; paper pending published: 20 January 2014; accepted: 01 February 2014; published online: 18 February 2014.

*Citation: Running MP (2014). The role of lipid post-translational modification in plant developmental processes. Front. Plant Sci. **5**:50. doi: 10.3389/fpls.2014.00050*

This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science.