Stomatal development in time: the past and the future

Short title: Stomatal Development in Time

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Abstract

Stomata have significantly diversified in nature since their first appearance around 400 million years ago. The diversification suggests the active reprogramming of molecular machineries of stomatal development during evolution. This review focuses on recent progress that sheds light on how this rewiring occurred in different organisms. Three specific aspects are discussed in this review: (i) the evolution of the transcriptional complex that governs stomatal state transitions; (ii) the evolution of receptor-ligand pairs that mediate extrinsic signaling; and (iii) the loss of stomatal development genes in an astomatous angiosperm.

Keywords: stomata; moss; grass; *Arabidopsis*; evolution of development; transcription factors; peptide signaling

Introduction

Stomata, the epidermal valves, have central roles in gas exchange and water loss in plants. It is estimated that stomata evolved only once approximately 400 million years ago to facilitate the colonization of land by plants. As a consequence of their evolutionary trajectory, the morphology, distribution, orientation, and development of stomata are highly diversified in different species [1,2] (Figure 1). For example, *Arabidopsis* stomata consist of two kidney-shaped guard cells (GCs) surrounding a pore. In grass, guard cells are generally dumbbell-shaped and bracketed by subsidiary cells (SCs) (Figure 1g). In contrast to species with paired stomata, *Physcomitrella patens* (*P. patens*) stomata, exhibit incomplete cytokinesis [1-2, 3●●]. The distributions and orientations of stomata vary from species to species as well: *Arabidopsis* presents a scattered distribution of stomata with non-isotropic orientations while grass stomata are arranged strictly in cell files with the identical orientation (Figure 1g and 1h). In *P. patens*, individual stomata spiral at the basal region of the sporophyte, forming a ring-like pattern [1]. Evolution additionally reprogrammed the stomata formation process. One prominent example is the degree of amplifying division, a process in which the stomatal stem cells (meristemoids) divide asymmetrically for self-regeneration as well as increasing the number of surrounding pavement cells (crenulated epidermal cells that protect internal tissues). In the simplest case, *P. patens* stomata develop entirely without amplifying divisions. In *Arabidopsis*, however, meristemoids can divide up to three times before guard mother cell formation (Figure 1f). The

spiral surrounding stomata in *Houttuynia* further suggests that more than three amplifying divisions can occur during stomata formation [1] (Figure 1e). Hence, stomatal patterning is highly diversified in nature.

In the past, our understanding regarding stomatal development have been derived primarily from the model system *Arabidopsis*, where stomata are formed via a series of stereotypical cell divisions and cell-state transitions [4]. At the very beginning, a subset of protodermal cells stochastically acquires the stomatal progenitor cell fate, becoming meristemoid mother cells (MMC). The MMC divides asymmetrically, giving rise to a meristemoid cell and a stomatal-lineage ground cell (SLGC). Both the meristemoid and the SLGC have highly dynamic and stochastic behaviors: the meristemoid either differentiates directly into a guard mother cell (GMC) or undergoes several rounds of amplifying divisions; meanwhile, the SLGC might re-acquire the MMC fate and undergo spacing divisions, or differentiate into a pavement cell. Eventually, the GMC divides symmetrically into paired guard cells (GCs) (Figure 1f). Both extrinsic and intrinsic signals control the stomatal patterning in *Arabidopsis*. The stomatal precursor cell states are specified by heterodimers of basic Helix-Loop-Helix (bHLH) transcription factors. The bHLH subgroup IIIb members, SCREAM (SCRM/ICE1) and SCREAM2 (SCRM2), act as universal components that bind with different subgroup Ia members to execute transitions in a stage-specific manner (Figure 2). In MMC, SCRM/SCRM2 interact with SPEECHLESS (SPCH) to initiate asymmetric cell divisions. Later, MUTE and FAMA act sequentially as SCRM/SCRM2's partners to promote formations of GMC and mature guard cells respectively [5-8] (Figure 1f).

The extrinsic signals are transduced by leucine-rich repeat receptor (LRR) kinase complexes that include members of the ERECTA family (ER), TOO MANY MOUTHS receptor-like protein (TMM) and SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) receptors [9-11]. The receptor complex perceives the secreted cysteine-rich protein ligands of the EPIDERMAL PATTERNING FACTOR (EPF)/EPF-LIKE (EPFL) family and interpret the developmental signal via a mitogen-activated protein kinase (MAPK) cascade to regulate the activity of intrinsic bHLH transcription factors [12-21] (Figure 1f).

The phenotypic diversification of stomata in different species suggests that the genetic network regulating stomatal development is constantly being rewired during evolution (Figure 1). Elucidating a broad spectrum of stomatal development pathways is not only theoretically appealing, but is also beneficial for translational agricultural research, as it provides a rich repertoire of toolkits for creating novel synthetic circuits that can potentially improve photosynthetic efficiency and crop yield. This review focuses on recent progress that promotes our understanding of stomatal development from the evolutionary aspects.

Evolution of the transcriptional complex

The bHLH family genes are widely utilized by animals and plants to regulate developmental processes. In parallel with *Arabidopsis* stomatal development, both tissue-specific bHLH regulators (MyoD, myogenin, Myf5, and MRF4) and non-tissue-restricted bHLH factors (E-like proteins) act at multiple points of cell-state transitions to specify skeletal muscle development in vertebrates [22].

When did plants start to use bHLH modules to regulate stomata formation? Early bioinformatics analysis with the moss *P. patens* genome identified only two genes resembling to SPCH, MUTE, and FAMA, therefore named as SMFs (named after “SPCH, MUTE, and FAMA domain”)(Figure 2)[23]. These two genes were speculated to play roles in stomatal development [1]. A later study partially corroborated this hypothesis by showing that *SMF1* was able to partially rescue *Arabidopsis mute* and *fama* mutants’ defects [23]. The direct *in planta* evidence of *SMF1*’s function in stomatal development was only reported recently. Charter et al. showed that *SMF1* knock-out *P. patens* mutants possess a “loss of stomata” phenotype [3●●]. These results suggest that *SMF* genes emerged early during the evolutionary history of land plants to control stomatal formation, and that ancestral *SMF* genes have a dual function to promote both GMC and GC formation (Figure 1c). Later on, as group Ia bHLH genes expanded, each paralog acquired specialized functions to regulate more sophisticated patterning processes, such as amplifying divisions. For instance, unlike *P. patens*, in which only the small sporophytes possess stomata, the leaf blades of eudicots are large, flat, and efficiently organized for photosynthesis. The evolutionary invention of amplifying divisions, which give rise both additional stomata and pavement cells, enables multiplication of epidermal cells and adjustment of stomatal density as a

broad leaf expands.

Is the partnership of group Ia genes with group IIIb genes evolutionarily conserved? Charter *et al.* confirmed that PpSCRM1 interacts with PpSMF1 in both yeast two hybrid and bimolecular fluorescence complementation assays. Genetic evidence is also consistent with SMF1-SCRM1 heterodimerization as no stomata were found on *ppscrm1* mutant capsules [3●●]. This indicates that subgroup Ia and IIIb bHLHs heterodimers function as an ancient module in stomata development (Figure 1c).

In grass, *SPCH*, *MUTE*, *FAMA* and *SCRM* orthologues were identified in genomes of rice, maize and *Brachypodium distachyon* (*B. distachyon*) [24, 25●●, 26]. While the copy numbers of *MUTE*, *FAMA* and *SCRM* are constant between *Arabidopsis* and grasses, there has been a duplication of *SPCH* in grasses. Current evidence suggests that the two copies of *SPCH* have unequal yet overlapping contribution in grass stomata development. In *B. distachyon*, *spch2* loss-of-function mutants show a more severe reduction in stomatal number compared with *spch1* mutants. Loss of both copies of *BdSPCH1/2* in *B. distachyon* results in a stomataless phenotype, mimicking *spch* knock-out mutant phenotype in *Arabidopsis* [25●●]. Rice *ossppch2-1* mutant has decreased stomata density together with patterning defects. While *ossppch1* mutant phenotype is still unclear, overexpression of only *OsSPCH2*, but not *OsSPCH1*, promotes division in *Arabidopsis* pavement cells, indicating functional divergence of the two *OsSPCH* orthologues [24].

While *SPCH(s)* in general function to initiate the stomatal lineage, they are highly varied in different species. In *Arabidopsis*, *SPCH* is transiently expressed in MMC and early meristemoids to initiate and reiterate asymmetric cell divisions, respectively [6]. The expression of *BdSPCH1* and *BdSPCH2* are prolonged until GMC stage, suggesting that *BdSPCH* genes might have additional roles [25●●]. Rice orthologs *OsSPCH1/2* are expressed before the formation of stomatal files, and *OsSPCH2* fails to rescue *Arabidopsis spch* mutant [24]. Therefore, *SPCH* genes have diverged rapidly to regulate downstream transcription networks in a taxon-specific manner (Figure 1f and 1h).

Compared with *SPCH*, both *MUTE* and *FAMA* appear to be more functionally conserved across different species. Overexpression of *Zm/OsMUTE* and *OsFAMA* in *Arabidopsis* produced excessive stomata and mature single stomata respectively, recapitulating the overexpression phenotype of *Arabidopsis* genes [7,8,24]. *OsMUTE* and *OsFAMA* are additionally shown to be able to partially complement *Arabidopsis mute-1* and *fama-1* mutants [24]. Despite the fact that both *MUTE* and *FAMA* retain conserved roles to promote GMC and GC identities, they have different cell-cycle regulation mechanisms between *Arabidopsis* and the grasses. *AtMUTE* is expressed exclusively in the meristemoid to halt asymmetric amplifying divisions whereas *OsMUTE* is inferred to promote a single asymmetric cell division in stomatal file cells [8,24]. Furthermore, rice loss-of-function mutant *osfama-1* does not show caterpillar-like callus as observed in *Arabidopsis fama-1* mutant [7,24,27]. This discrepancy might be explained by the fact that grasses do not possess meristemoids, where termination of asymmetric cell division is needed for GMC differentiation. Since this transition is governed by *MUTE* and *FAMA*, the interplay between these two genes as well as their downstream cell cycle loops might have been significantly altered during grass speciation.

Do grasses also rely on the SCRM-SMF module for stomatal development? *Arabidopsis SCRM/SCRM2* are expressed throughout the stomatal lineage and form heterodimers with the three bHLHs *SPCH*, *MUTE*, and *FAMA*. The strong dosage effect of *AtSCRM/SCRM2* indicates that they act redundantly to drive the three steps of stomatal differentiation [5]. Despite the dramatic change of leaf pattern between *Arabidopsis* and grasses, the expression pattern of *BdSCRM/SCRM2* remains unchanged in *B. distachyon* [5,25●●]. However, each homolog is functionally distinct. An early nonsense mutation in *BdSCRM/ICE1* is sufficient to produce a stomataless phenotype that can only be observed in *scrm scrm2* double homozygous mutant in *Arabidopsis*. The *bdscrm2* loss-of-function mutant produced stomata complexes with immature GCs, resembling the phenotype of the rice *osfama-1* mutant [24,25●●]. Thus, *BdSCRM1* is specialized to promote GMC formation in stomata files and *BdSCRM2* primarily participates in the GC differentiation (Figure 1h). Although no protein-protein interaction data is currently available, it can be conjectured that the SCRM-SMF module is conserved in grasses based on these genetic data.

In summary, we can conclude that group Ia (*SMF*) and IIb (*SCRM*) bHLH genes are the core intrinsic signaling module throughout land plants and are constantly being rewired to accommodate diverse stomatal development programs throughout evolutionary history (Figure 2).

Evolution of receptor-ligand signaling

Receptor-kinase-mediated information perception and transduction is one of the most fundamental mechanisms across all multicellular organisms. Extensive studies in *Arabidopsis* have revealed that the EPF/TMM/ERECTA module is important for stomatal patterning. However, it had been unknown to what extent this ligand-receptor pair was conserved during the evolutionary history of stomatal development. Recently, Caine et al. showed that the EPF/TMM/ER module functions to regulate stomatal patterning in the model moss *P. patens*, but in a less sophisticated manner [28●●]. For example, the *P. patens* genome harbors a single *EPF1* gene whereas *Arabidopsis* possesses *EPF1*, *EPF2* and *EPFL9*, which encode peptides with unique activities to manipulate the receptor complex. It is important to note that EPF1's function is conserved between these two species, because *Arabidopsis epf1* mutant defects were partially rescued by a *PpEPF1* transgene [28●●]. All this evidence suggests that *EPF1* most closely resembles the ancestral ligand for TMM/ER receptor pairs, and that a later proliferation of ligands might have occurred to fine-tune more complicated stomatal formation processes (Figure 1c).

Unlike the case in *P. patens*, so far it is unclear whether EPF/TMM/ER module is employed to pattern stomata in grass. Instead, two other LRR receptors, PANGLOSS1 (PAN1) and PANGLOSS 2 (PAN2) were shown to be crucial patterning factors of subsidiary mother cells (SMCs) in maize. Strikingly, while *Arabidopsis* TMM and ER proteins do not exhibit polar localization, maize PAN1 and PAN2 are strongly polarized in SMCs [29,30]. A series of studies suggest that the SCAR/WAVE complex polarizes PAN receptors which interact with the small GTPase RHO GTPASE OF PLANTS (ROP) to guide the polarity of SMCs and subsequent division asymmetry [31●,32]. This mechanism is vastly different from the polarity control mechanism in *Arabidopsis*, where the polarity protein BREAKING OF ASYMMETRY IN THE

STOMATAL LINEAGE (BASL) functions as a scaffold for the MAPK kinase cascade [33,34]. It suggests that monocots' subsidiary cells have employed an entirely different machinery for extrinsic signal perception and polarity control so that regular stomatal patterning can be achieved.

It is worth noting, however, that potential orthologs of *EPF/TMM/ER* exist in grass genomes [1,35-37]. In *Arabidopsis*, both EPF-family peptides and ER-family receptor kinases play roles outside of stomatal patterning. For instance, EPF-LIKE4 (EPFL4) and EPFL6 are perceived by ER to promote inflorescence growth [38]. EPFL2 and ER-family constitute a ligand-receptor pair to promote leaf serration depth [39]. Very recently, a mutation in the rice *EPFL1* ortholog, *REGULATOR OF AWN ELONGATION2* (RAE2) was identified as a gene responsible for a key domestication phenotype in Asian rice [40]. Further functional studies of grass *EPF/TMM/ER* orthologs might clarify their roles in stomatal patterning or other developmental processes. Such studies will ultimately unravel the evolutionary co-option and exaptation of these cell-cell signaling modules.

Loss of stomatal development genes in astomatous angiosperms: land plants returning to the sea

Since their appearance around 400 million years ago, stomata have been a key feature of plant adaptation for life on land, but they are not always retained in every plant species. Some marine angiosperms, for instance, have lost stomata after colonizing the sea. The biological development process is ultimately instructed by its genomic information, a principle known as “the hardware decided by the software” [41]. So what has happened to stomatal development genes over evolutionary time when stomata have disappeared? Genomics studies so far suggest that large-scale gene deletion accompanies the loss of stomata. Ran et al. showed that group Ia and IIIb bHLHs are widespread among stomata-producing plants but have not been identified in the genomes of algae, or liverwort and fern species that do not have stomata [26]. The recently published genome of the seagrass *Zostera marina* shows a similar result. In addition to the deletion of transcription factors, a few receptor-module genes are also omitted from the *Z. marina* genome, including the LRR co-receptor *TMM*, the ligands *EPF1*, *EPF2*, and *EPFL9*, and

the polarity controller *BASL*. The loss of genes seems to be highly specific to the stomatal development pathway, because the *ERECTA*-family genes, which play diverse roles in other plant developmental processes as well as stomatal development, are retained [42●●,43]. This phenomenon suggests the interesting hypothesis that the “stomata developing software” is developed by neofunctionalization of both existing and new genes.

Origin and original function of stomata: unsolved questions

The structure and function of stomata are deeply conserved, given the essential role of stomata for plant growth and survival. A similar conservation of the molecular players that regulate stomata development has therefore been anticipated [1,35]. Thus far, as we have shown, this hypothesis has essentially been corroborated by recent evo-devo studies. The biggest unsolved question regarding stomata, however, still remains: what were the origin and original function of stomata? Due to lack of conservation of some of the key genes in astomatous plants, it is currently difficult to trace back exactly how ancestral gene networks neofunctionalize to give rise to stomata. Elucidating the functions of ancestral genes in green algae, if possible, might give some hints. Alternatively, comparing the genetic networks of stomatal development and the development of the air chambers found in many liverworts may provide some insight into convergent evolution.

Did stomata originally evolve to regulate gas exchange and transpiration? Current observations in moss say not. In *P. patens*, stomata are found in sporophytes in which spore capsules are supported by setae whose length are much less than 1mm [44,45]. Unlike many vascular plants, especially trees, mosses do not rely on stomata to pump water for photosynthesis per se. However, the conservation of the ABA signaling network and the presence of thick cuticles in mosses strongly imply that primitive stomata participate in water regulation [45,46]. Interestingly, Chater et al. (2016) reported that moss *ppsmf1* and *ppscrm1* mutants have sporophytes that are deficient in capsule dehiscence, suggesting that the earliest stomata could have evolved to promote drying and lysis of spore capsules thereby aiding spore dispersal [3●●]. It also implies that the “modern” stomatal function as a valve optimizing gas exchange while minimizing water loss might have been an evolutionary co-option of an existing pathway in

stomata. Solving these mysteries requires a deep understanding of functional genomics of stomatous and astomatous species in the future.

Conclusions and Perspective

In the past decade, the model plant *Arabidopsis* has been very successful in elucidating the basic molecular framework that controls stomatal development and patterning. It provides a solid foundation for understanding the evolutionary trajectories of the stomatal patterning pathway as shown by recent studies. The evo-devo studies illustrate a framework that shows the core stomatal development genes are retained in stomatous species, and subfunctionalization and rewiring have occurred during evolution to create different developmental programs. We can optimistically predict that the core stomatal development pathway in more species will be understood fairly soon with the help of the increasing availability of genomic sequences and genetic editing tools, such as the CRISPR-Cas9 system. Meanwhile, we cannot ignore the simple fact that the puzzle of stomata is still not complete in *Arabidopsis*. It is far from fully understood that how the three cell-transition steps are orchestrated at the genomic level, and how each environmental, hormonal, and physiological stimulus impinge on them to dynamically adjust stomatal development in nature. Although a cell-type-specific transcriptome atlas of *Arabidopsis* stomatal lineages has been attempted [47,48], we still do not understand how epigenomes are regulated at distinct stages during stomatal development. Recent studies employing cell-type specific isolation, immunoprecipitation, and high-throughput sequencing have been very fruitful for examining cell-type-specific genome-wide epigenetic modifications in the *Arabidopsis* root, and the same approach is applicable to elucidate the unsolved questions in stomatal development [49]. While a reverse-genetic approach is highly effective in understanding individual genes, a genomes-wide perspective on the evolution of development will be needed to uncover insights into genomics and epigenomics. Our review concludes here, looking forward to the new genomic era of evo-devo research that is just beginning.

Phylogram Construction

Amino acid sequences were found using public databases (<http://www.arabidopsis.org/>; <http://plantfdb.cbi.pku.edu.cn/>; <http://brassicadb.org/>; <http://cisbp.ccbr.utoronto.ca/>;

<http://www.uniprot.org/>; <https://www.ncbi.nlm.nih.gov/>; <http://www.cosmoss.org/>; <http://dendrome.ucdavis.edu/resources/blast/>) and journal articles [50,51]. Reverse blastp or tblastn searches were subsequently performed to additionally support membership in bHLH Ia or IIIb subfamilies. Human MyoD, a distantly related bHLH, was used as the outgroup. MUSCLE alignments [52] were performed using Geneious 9 (Biomatters Ltd., Auckland, New Zealand; <http://www.geneious.com>) [53] with default parameters and visually checked for accuracy. Trees were constructed using RAxML [54] on CIPRES [55] with automatically determined protein evolution model (PROTGAMMAAUTO), 100 rapid bootstraps, and best ML tree inference.

Glossary

MMC (mother meristemoid cell): the precursor protodermal cell that generates the stomatal lineage by producing the meristemoid cell via asymmetric entry division. meristemoid: stomatal stem cell produced by asymmetric entry division of the meristemoid mother cell (MMC); undergoes a number of amplifying divisions (usually two to three in *Arabidopsis*) that reproduce a meristemoid and a larger daughter cell (SLGC); differentiates into a guard mother cell (GMC) when amplifying divisions are completed.

SLGC (stomatal lineage ground cell): the larger daughter cell generated by asymmetric division of MMC or meristemoid; differentiates into a pavement cell or generates a new meristemoid by asymmetric spacing division. GMC (guard mother cell): the final stomatal precursor differentiated from the meristemoid; divides symmetrically to produce a pair of guard cells (GCs). GC (guard cell): terminally differentiated stomatal cell usually produced by symmetric division of the GMC. SMC (subsidiary mother cell): precursor flanking cells that bracket guard cells in the grass stomatal complex; divides asymmetrically to produce one small subsidiary cell (SC) and one large daughter cell. SC (subsidiary cell): mature small flanking cells of the stomata pair. Entry division: asymmetric cell division of MMCs that generates meristemoids. Amplifying division: asymmetric cell division of meristemoids that regenerates a meristemoid and a larger daughter cell (SLGC).

Spacing division: asymmetric cell division of SLGCs that creates new meristemoids that are not adjacent to existing meristemoids (known as the “one-cell spacing rule”).

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Figure Legend

Figure 1. Stomata phenotypes and developmental frameworks in diverse organisms.

(a-b) Examples of primitive stomata in the lycophyte *Selaginella* (a) and the fern *Azolla* (b). (c) Developmental framework of primitive stomata based on recent works in the moss *Physcomitrella patens*. A subset of protodermal cells (white) acquire the MMC identity (purple) and divides asymmetrically to create a meristemoid (blue) and SLGC (white). The meristemoid differentiates into GMC (light green) which divides symmetrically to form the paired guard cells (green). It is currently unknown what transcription factor(s) govern(s) the first transition and the later two transitions are regulated by the SMF1/SCRM1 heterodimer complex. The EPF1/ER/TMM module acts as an extrinsic signal to regulate the stomata patterning, but it is still unknown if the signal is transmitted via a MAPK cascade and relayed on the transcription complex. (d-e) Examples of angiosperms' stomata in *Nymphaea* (d) and *Houttuynia* (e) where the amplifying division exists. (f) Developmental framework of angiosperm stomata based on *Arabidopsis*. A subset of protodermal cells (white) acquire the MMC identity (purple) and divides asymmetrically to create a meristemoid (blue) and SLGC (white). The meristemoid either differentiates directly into a GMC (light green) or undergoes up to three rounds of amplifying divisions. The GMC divides symmetrically into paired guard cells (green). The state transitions are regulated by bHLH heterodimers between SPCH, MUTE, FAMA and SCRM/2 at MMC, meristemoid and GMC stages. The transcription factors are regulated by EPF(L)/ER/TMM receptor ligand module via a MAPK cascade. (g) Example of monocots' stomata in *Oplismenus* where stomata are arranged in specific files. (h) Developmental framework of monocot stomata based on rice and *Brachypodium distachyon*. Stripes of protodermal cells gain the stomata precursor fate (purple) and divides asymmetrically to form GMCs (light green). The GMC divides symmetrically to form immature GC pairs (light green) that differentiates into mature GCs (dark green). The stomata file identity is promoted by both SCRM and SPCH/2. MUTE controls the asymmetric division of stomata precursor cells. The maturation of GCs are regulated by FAMA and SCRM2. During GC formation, subsidiary cells (red) are generated that embrace the GCs.

Figure 2. Phylogenetic analysis of plant bHLH protein subfamilies Ia and IIIb.

(a) Phylogram of plant basic helix-loop-helix transcription factor (bHLH) subfamily IIIb. The ICE1/SCRM and SCRM2 clade is highlighted with a light blue box. Note the highly divergent sequences found in *Marchantia*, a land plant lacking stomata. The scale bar and each * represent 2.0 amino acid substitutions.

(b) Phylogram of plant bHLH subfamily Ia. The angiosperm FAMA, SPCH, and MUTE clades are highlighted with light green, pink, and orange boxes respectively. The moss SMF clade, also demonstrated to have stomatal function, is highlighted with a gradient box. The scale bar represents 2.0 amino acid substitutions. Stars represent bootstrap support values of 90-100; dots represent bootstrap support values of 60-89. At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon*; Br, *Brassica rapa*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Pta, *Pinus taeda*; Ptr, *Populus trichocarpa*; Sl, *Solanum lycopersicon*; Sm, *Selaginella moellendorffii*; Vv, *Vitis vinifera*.