

ABA as a Universal Plant Hormone

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Abstract Absciscic acid (ABA) is a sesquiterpene known to regulate environmental stress responses in angiosperms, such as water-loss-induced stomatal closure, development of seed desiccation tolerance during maturation, and salt-, desiccation-, and freezing-stress tolerance of vegetative tissues. An ABA-induced increase in stress tolerance is also reported in other land plant lineages, including nonvascular bryophytes that diverged from vascular plants more than 420 million years ago. Thus, it is hypothesized that acquisition of sensing and response mechanisms for ABA by land plant ancestors was critical for invasion of and adaptation to land. Because bryophytes are key organisms in plant evolution, clarification of their ABA-dependent processes is important for understanding land plant evolutionary adaptation. Based on past and current studies on ABA in non-seed plants and phylogenetic analysis of genome information from various plant species, we discuss the evolution of ABA function and biosynthesis, transport, and signaling network pathways as well as calcium signaling because of its importance in ABA signaling in angiosperms. Future directions of ABA research in the evo-devo field are also discussed.

1 Introduction

The plant hormone absciscic acid (ABA) can be found in various species across kingdoms, including bacteria, fungi, and animals, as well as plants. The roles or functions of ABA in these non-plant species are largely unknown; however, recent reports suggest a ubiquitous role of the sesquiterpene in the regulation of physiological events in non-plant species (Takezawa et al. 2011). Even in the plant kingdom, our knowledge about ABA function largely depends on studies in angiosperms, and a fundamental understanding of ABA activity has just begun to develop for non-angiosperm species. In vascular plants, ABA is produced under water-stress conditions in vegetative tissues and controls stomatal closure and gene expression related to dehydration tolerance (Finkelstein and Rock 2002; Rock et al. 2010). It also regulates maturation, acquisition of desiccation tolerance, and germination of seeds (Finkelstein et al. 2002). In addition, ABA has diverse functions involved in negative control of growth and development such as inhibition of lateral root growth and inflorescence formation (Milborrow 1974). Recent progress in molecular studies of ABA function in basal land plants has revealed its ancient origin in water-stress responses in land plants (Fig. 1) (Takezawa et al. 2011). We can readily note that ABA functions evolved in land plants as a precursor to the acquisition of stomata, vascular system, and seeds that are its target tissues in angiosperms. Thus, understanding the physiological roles as well as the biosynthesis and metabolic and signaling pathways of ABA in non-seed plants will yield insights into how the ubiquitous compound became a plant hormone that is essential for the water-stress response in land plants.

Bryophytes such as mosses and liverworts are nonvascular plants that first emerged 480 million years ago and are widespread across the world from tropical rain forests to regions with harsh environmental conditions such as the desert or Antarctic.

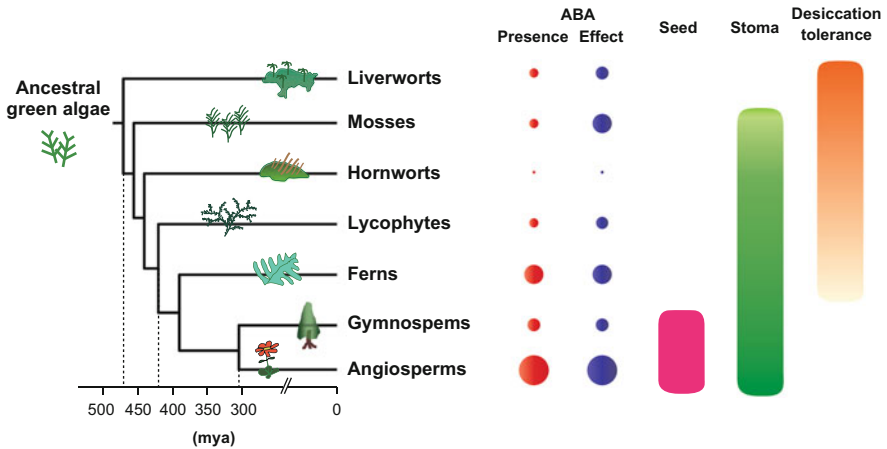


Fig. 1 ABA and land plant evolution. The cladogram shows the evolutionary relationship of land plant groups. Relative effect (blue) and endogenous level (red) of ABA in each plant groups are shown by dot size. ABA effectiveness in seed development, stomatal regulation, and desiccation tolerance is indicated by the bar chart. Color strength shows effectiveness

Because they lack water transport and retention tissues, bryophytes engage in a continual equilibrium with the water potentials of the atmosphere (–100 MPa at 28 °C and 50 % relative humidity), a level most modern-day flowering plants could not tolerate. But when watered, they can equilibrate rapidly with surrounding water potential and are fully hydrated without significant damage. This type of desiccation tolerance is likely an ancestral trait of land plants that has been lost from the vegetative tissues of vascular plants during evolution but “re-evolved” in the *Selaginellas* and the *Leptosporangiate* ferns (Oliver et al. 2000, 2005). The mechanisms underlying desiccation tolerance include (1) limiting damage of cellular components caused by severe dehydration stress; (2) maintaining physiological integrity during desiccation; and (3) provoking the cellular repair mechanism upon rehydration (Bewley 1978).

It is critical to find non-angiosperm plants for which tools are available for gene discovery and functional analysis. The moss *Physcomitrella patens* is one of the few such plants (Quatrano et al. 2007). After findings indicating a high frequency of homologous recombination that enables gene targeting (Schaefer and Zryd 1997), this plant has emerged as a model system for analysis of many aspects of plant biology. In 2008, an assembled genome of *P. patens* (487 Mbp) was released from the Joint Genome Institute in the USA (JGI) (Rensing et al. 2008). The predicted gene number is 32,272 which is comparable to findings in angiosperms, and generation of a linkage map with molecular markers is in progress (Kamisugi et al. 2008), which will be a resource for forward genetics. RNA interference (RNAi) (Bezanilla et al. 2003) and artificial microRNAs (Khraiweh et al. 2008) can be used to downregulate gene expression, an alternative to targeted gene disruption for analysis of gene families in this organism. Taking advantage of this wealth of tools for comparative and functional analysis, molecular dissection of

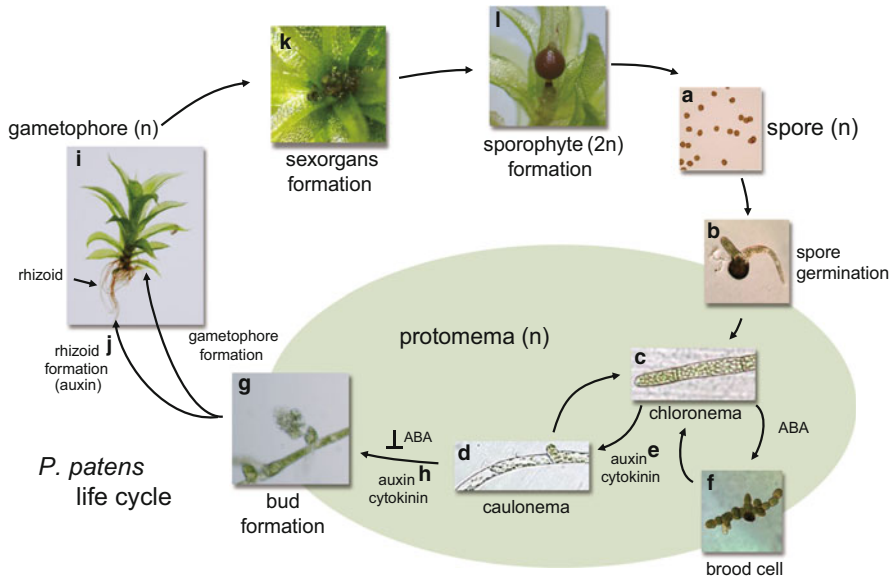


Fig. 2 The life cycle of the moss *Physcomitrella patens*. (a) Spores, (b) spore germination and generating primary chloronemata, (c) chloronema cells, (d) caulonema cells, (e) caulonema differentiation is regulated by auxin and cytokinin, (f) brood cell formation is regulated by ABA, (g) young bud, (h) auxin and cytokinin induce bud formation, and ABA represses the formation, (i) gametophore (leafy shoot) and rhizoid (rootlike tissue), (j) auxin induces rhizoid formation, (k) a short day and a cold treatment (15 °C) induce sex organ formation, (l) after fertilization, the egg cell develops into a sporophyte (diploid) and within its meiosis occurs leading to spore formation. The cycle can be achieved under laboratory conditions in less than 3 months

ABA signaling pathways as well as the biosynthesis pathway in basal land plants is ongoing. For example, recent studies on *P. patens* have revealed developmental and physiological aspects of ABA response in bryophytes (Fig. 2). Gene knockout experiments indicated that desiccation tolerance in the moss involves ABA and the plant-specific transcription factor ABA INSENSITIVE3 (ABI3), which are essential for seed desiccation tolerance of angiosperms (Marella et al. 2006; Khandelwal et al. 2010), suggesting an evolutionarily conserved mechanism for cellular protection from desiccation. Comparative analyses of the common toolkit for ABA functions in land plants will further extend our understanding of the evolution of the signaling machinery that enabled plants to conquer the land.

In this review, we focus on the role of ABA in land plants outside of seed plants including bryophytes, lycophytes, and ferns, and recent progress in the elucidation of an ABA-core toolkit in the model moss *P. patens* and an emerging new model the liverwort *Marchantia polymorpha*. We further discuss the evolution of ABA function, ABA metabolism, and its signaling pathway in relation to calcium signaling that must be related to the innovation of water use efficiency during the evolution of land plants.

2 ABA in Non-seed Plant Lineages

2.1 ABA in Bryophytes

Detection of endogenous ABA in various species of bryophytes by enzyme-linked immunosorbent assays (ELISA) using monoclonal antibodies has been reported. Analysis of endogenous ABA in the desiccation-tolerant liverwort *Exormotheca holstii* has revealed that desiccation of its thalli resulted in a drastic increase in ABA content in comparison with the hydrated thalli (Hellwege et al. 1992). Further analysis indicated that various liverwort species belonging to Marchantiales accumulate endogenous ABA with a large variability in the content ranging from 2 pmol g⁻¹FW to 30 nmol g⁻¹FW (Hartung and Gimmler 1994). In hornworts (*Anthoceros* species), ABA was detected not only in the gametophyte but also in the sporophyte, and stress-induced ABA synthesis was observed (Hartung et al. 1987). In the moss *Funaria hygrometrica*, ABA accumulated during the slow drying process associated with development of desiccation tolerance (Werner et al. 1991). However, these ABA studies require cautious interpretation. Minami et al. (2005) described that the amount of ABA could not be determined precisely by ELISA at least in *P. patens*, probably because of cross-reactivity of the anti-ABA antibody used in the assay with other compounds in crude extracts. Direct measurement of endogenous ABA has been conducted in only limited species of bryophytes. Li et al. (1994) estimated that cultured thalli of *M. polymorpha* contain 4–16 ng of ABA g⁻¹FW by direct measurement by GC-EIMS and GC-NCIMS. In mosses, endogenous ABA has been detected by GC-MS in protonema cells of *P. patens*, and it was found that the cells accumulated more ABA in response to hyperosmotic stress (Minami et al. 2005).

Exogenous application of ABA affects growth and development as well as stress tolerance in bryophytes. In mosses, exogenous ABA inhibits growth of protonema and gametophore (Menon and Lal 1974; Lehnert and Bopp 1983; Chopra and Kapur 1989). ABA also promotes formation of gemma-like structures or spherical “brood cells,” which are forms of vegetative reproduction (Chopra and Kapur 1989; Goode et al. 1993; Schnepf and Reinhard 1997). Another well-documented effect of ABA on protonemata is inhibition of gametophore formation. ABA inhibits caulonema’s differentiation to gametophore by counteracting with cytokinin that promotes bud formation (Valadon and Mummery 1971; Chopra and Kapur 1989; Christianson 2000). There are also reports indicating that ABA inhibits formation of gametangia for sexual reproduction (Bhatla and Chopra 1981; Chopra and Mehta 1987). In comparison to the studies in mosses, there are a relatively limited number of reports describing the effects of ABA on growth and development of liverworts. ABA inhibits growth of gemmae and thalli (Schwabe and Valio 1970) and formation of gametangia in Marchantiales liverworts (Kumra and Chopra 1986). ABA might play a role in heterophyllous switch of liverworts. Hellwege et al. (1992) reported that exogenous ABA converted thalli of aquatic liverworts *Riccia fluitans* and *Ricciocarpus natans* into the land forms.

In addition to above studies, effects of exogenous ABA on tolerance to desiccation and freezing stress in bryophytes are well described. Application of ABA to cultured protonemata of *F. hygrometrica* induces tolerance to rapid drying (Werner et al. 1991). Pretreatment with ABA of gametophores of *Atrichum androgynum* reduces membrane ion leakage caused by desiccation stress (Beckett 1999). ABA appears to prevent reductions in photosynthesis and non-photochemical quenching caused by desiccation in *A. androgynum* (Mayaba et al. 2001). Effects of ABA on freezing tolerance have been also described. ABA pretreatment of protonemata with cryoprotectants such as proline and sucrose effectively enhanced survival after cryopreservation of moss species (Christianson 1998; Burch and Wilkinson 2002). In *P. patens* protonemata, ABA treatment for one day increases freezing tolerance from -2°C to -10°C (Minami et al. 2003). The protonemata accumulate soluble sugars as compatible solutes as well as a number of LEA-like proteins and stress-associated transcripts by ABA treatment (Nagao et al. 2005, 2006).

Exogenous ABA also induces freezing and desiccation tolerance of some liverworts. Pence (1998) showed that ABA pretreatment enhanced survivals after cryopreservation of liverwort species, although the magnitude of the effects of ABA varied among species. ABA effectively increased desiccation tolerance of *Riccia fluitans* while the effect was little on *Plagiochila* sp. and *M. polymorpha* (Pence et al. 2005). The ABA-induced desiccation tolerance appeared to be associated with increased accumulation of total soluble carbohydrates (Pence et al. 2005), though profiles of proteins and gene expression have not been described. These results suggested that effects of ABA on liverworts may vary substantially among different species, but it certainly alters development and desiccation tolerance of some species such as *R. fluitans*. Most of the studies on ABA effects are those on Marchantiales liverworts and very little is known about its effects on other liverwort groups including Jungermanniales.

2.2 ABA in Ferns and Lycophytes

Radioimmunoassays for detection of ABA indicated its distribution in pteridophytes (Weiler 1979). Endogenous ABA has been chemically identified in different species of pteridophytes: protonema of *Anemia phyllitidis* (Cheng and Schraudolf 1974), spores of *Lygodium japonicum* (Yamane et al. 1980), sporophytes of the tree ferns *Cibotium glaucum* and *Dicksonia antarctica* (Yamane et al. 1988), and sporophytes of an aquatic fern *Marsilea drummondii* (Pilate et al. 1989). Recently, detection by more accurate physicochemical methods (GC-MS/MS, GC-SIM, and UPLC-MS/MS) of water-stress-induced foliar ABA levels in ferns (*Pteridium esculentum* and *D. antarctica*) and a lycophyte (*Selaginella kraussiana*) has been reported (Brodrribb and McAdam 2011; McAdam and Brodrribb 2012). These analyses revealed that foliar ABA levels of ferns and lycophytes are quite low (about $10\text{ ng g}^{-1}\text{ FW}$ or below) in unstressed control conditions but are markedly increased (about $200\text{--}600\text{ ng g}^{-1}\text{ FW}$) when exposed to water stress, as observed in seed plants.

Different physiological roles of ABA in growth and environmental responses have been reported.

ABA has a negative effect on growth of gametophyte tissues in ferns. General retardation of growth by ABA of the gametophyte of *L. japonicum* that shows characteristic growth behavior under red and blue light has been reported by Swami and Raghavan (1980). ABA inhibits protonemal elongation, but not spore germination, in *Mohria caffrorum* (Chia and Raghavan 1982). Hickok (1983) showed that ABA stimulates rhizoid production of the *Ceratopteris* gametophyte at concentrations of 10^{-6} – 10^{-5} M but is inhibitory at higher concentrations. ABA has been shown to regulate sex determination in *Ceratopteris*. The fern *Ceratopteris* produces either hermaphroditic or male gametophytes, and this developmental fate is determined by the antheridiogen (possibly a gibberellin-like substance) produced by the hermaphroditic gametophytes. Hickok (1983) showed that ABA inhibits antheridiogen-induced formation of male gametophytes. Analysis of ABA-insensitive mutants indicated that these plants develop as males in the presence of antheridiogen and ABA (Warne and Hickok 1991).

ABA also has been shown to control heterophyllous switch in aquatic fern plants. The sporophyte of *Marsilea quadrifolia* produces different types of leaves above and below the water level. It has been shown that its submerged type shoots are modified by ABA, and the development of aerial-type leaves is induced (Liu 1984). Here ABA promoted growth of both leaves and roots but inhibited growth in the internodes. ABA-induced developmental changes returned to the default mode when ABA was removed. A number of ABA-regulated genes, designated *ABRH* for ABA-responsive heterophyly, have been identified from the shoot apex tissues of *M. quadrifolia* (Hsu et al. 2001). Of interest, unnatural R(–)-ABA has a greater effect on induction of heterophyly and the *ABRH* gene expression than natural S(+)-ABA, which is possibly due to slow 7'-hydroxylation of R(–)-ABA for its metabolism (Lin et al. 2005). The heterophyllous switch between aquatic and land forms by ABA has been observed in a wide range of plant species from angiosperms to liverworts (Anderson 1978; Hellwege et al. 1992). Whether or not the mechanisms for these morphogenetic responses by ABA are conserved among different classes of land plants of distantly related taxa is yet to be determined.

The role of ABA in desiccation tolerance of both ferns and lycophytes has been reported. Detached fronds of the desiccation-tolerant fern *Polypodium virginianum* survive slow drying but are severely damaged by drying with silica gel due to rapid water loss. When the fronds are treated with ABA, the amount of water lost is reduced, resulting in better survival after silica gel drying (Reynolds and Bewley 1993a). This process accompanied de novo synthesis of specific proteins (Reynolds and Bewley 1993b). The effect of exogenous ABA on survival after open drying followed by liquid nitrogen storage for cryopreservation of gametophyte tissues of six fern species was examined, and preculture with 10 μ M ABA for one week increased survival of the tissues of all species (Pence 2000). Liu et al. reported that ABA functions in desiccation tolerance in the lycophyte. Dehydration treatment of the sporophyte of *Selaginella tamariscina* causes a threefold increase in endogenous

ABA content, associated with upregulation of expression of genes involved in ABA signaling and cellular protection (Liu et al. 2008).

3 Evolution of ABA-Related Genes

With the advance of sequencing technology of DNA, genomic information of various plant species is now available (Phytozome; <http://www.phytozome.net>). Here we summarize the comparison of numbers of ABA-related genes that are involved in ABA biosynthesis and catabolism and ABA transport and the signaling among plant lineages from green algae to angiosperms (Table 1) and describe insights into the evolution of ABA-related genes in plants. We note that the sequenced plant species are still biased and genomic information from representatives of gymnosperms, ferns, hornworts, and liverworts will be required to complete an evolutionary comparison of ABA-related genes.

3.1 ABA Biosynthesis and Metabolism

The rates of ABA biosynthesis and catabolism are critical to determining the accumulation of ABA as well as the strength of the response. Although a variety of organisms synthesize ABA, the carotenoid pathway in angiosperms is the only defined pathway for ABA biosynthesis (Nambara and Marion-Poll 2005). The Arabidopsis *ABA1* encodes zeaxanthin epoxidase (ZEP), which catalyzes the initiation step of the carotenoid pathway. The cyanobacterium *Synechocystis* genome lacks genes for ABA1 (Yoshida 2005). Our phylogenetic analysis also showed no *ZEP* in the primitive red alga *Cyanidioschyzon merolae* (Takezawa et al. 2011) but identified one *ZEP* ortholog in *C. reinhardtii* (Table 1). The genome of the basal land plant *P. patens* contains a gene set for the carotenoid pathway except for *ABA2*, suggesting that the carotenoid pathway was completed in ancestral land plants. *ABA2* appeared only in angiosperms, suggesting the presence of alternative enzymatic pathway to convert xanthoxin to ABA aldehyde. Land plants from bryophytes to angiosperms increase ABA accumulation upon water stresses (Takezawa et al. 2011; McAdam and Brodribb 2012). *NCED* (9-cis-epoxy-carotenoid dioxygenase) catalyzes the rate-limiting step of water-stress-induced ABA biosynthesis in angiosperms (Nambara and Marion-Poll 2005), and *NCED* expression is upregulated by water stress in angiosperms (Xiong and Zhu 2003). The moss *P. patens* is currently the most basal fully sequenced land plant and accumulates ABA upon osmostress (Minami et al. 2005). The *P. patens* genome encodes two *NCED* genes (Table 1) with expression upregulated by dehydration, salinity, and exogenous ABA (Richardt et al. 2010), as observed in vascular plants. These data suggest that water-stress-controlled biosynthesis of ABA was established in the last common ancestor of land plants. Disruption of these genes for the carotenoid pathway of ABA biosynthesis in the basal land plant *P. patens*

Table 1 Comparison of number of abscisic acid (ABA)-related genes among plants

Function	Gene	Green algae		Bryophytes		Lycophytes		Angiosperms	
		<i>C. reinhardtii</i>		<i>P. patens</i>		<i>S. moellendorffii</i>		<i>O. sativa</i>	<i>A. thaliana</i>
ABA metabolism	ABA1/ZEP	1		1		1		1	1
	ABA4	0		1		1		1	1
	NCEDs	0		2		1		3	5
	ABA2	0		0		0		1	1
	AAO3	0		0		0		0	1
	ABA3	1		1		1		0	1
	BG1	0		0		0		0	1
ABA transport	CYP707As	0		0		2		3	4
	ABCG25	0		0		0		1	1
	ABCG40	0		0		0		10	1
	AIT	0		0		0		1	4
	PYR/PYL/RCARs	0		4		5		11	14
	Group A PP2C	0		2		3		10	9
ABA signaling	Subclass III SnRK2	0		4		2		3	3
	ABI3	0		3		3		1	1
	ABI4	0		0		0		1	1
	ABI5	0		2		4		5	7
	SLAC1	0		4		3		9	5
	CIPK/SnRK3	0		7		4		29	25
	CDPK	20		30		10		31	34
Ca ²⁺ -dependent factor	CBL	2		4		3		10	10
	CaM/CML	9		29		26		37	57

The Arabidopsis ABA1, ABA4, NCED3, ABA2, AAO3, ABA3, BG1, CYP707A3, ABCG25, ABCG40, AIT1, PYR, ABI1, SRK2E, ABI3, ABI4, ABI5, SLAC1, CIPKs, CDPKs, CBLs, and CMLs amino acid sequences were used for a BLASTP search to determine the putative orthologs in five species, and the neighbor-joining (NJ) trees were drawn using MEGA5.0. The number of potential orthologs was counted with the NJ-trees. The database used was Phytozome v8.0 (<http://www.phytozome.net>)

via gene targeting will uncover the significance of the carotenoid pathway for ABA biosynthesis as well as the physiological roles in nonvascular plants.

Several genes involved in ABA catabolism have been identified in Arabidopsis. ABA hydroxylation at the 8' position to give phaseic acid is the main irreversible catabolic pathway in angiosperms (Nambara and Marion-Poll 2005). The metabolites 7'- and 9'-hydroxy ABA are also reported in some angiosperm species (Zhou et al. 2004). To date, only CYP707A genes for 8'-hydroxylation of ABA have been reported in Arabidopsis (Kushiro et al. 2004), and these genes are involved in drought tolerance (Umezawa et al. 2006) and seed dormancy as well as germination (Okamoto et al. 2006). Another catabolic pathway is conjugation of ABA to glucose-ester (GE), which is subsequently sequestered into vacuoles (Boyer and Zeevaert 1982; Bray and Zeevaert 1985). ABA-GE is reversibly converted to ABA by BETA GLUCOSIDASE (BG) in Arabidopsis. To date, two Arabidopsis BG genes, endoplasmic reticulum-localized AtBG1 and vacuole-localized AtBG2, have been reported to catalyze ABA-GE hydrolysis (Lee et al. 2006; Xu et al. 2012). *AtBG1* disruption results in reduced ABA accumulation in seeds (about 40 % of WT) and in water-stressed leaves (about 80 % of WT). The *atbg1* plants show not only ABA-deficient phenotypes but also a dwarf phenotype with yellow leaves that can be rescued by exogenous ABA (Lee et al. 2006). These data suggest roles for released ABA in the stress response as well as in normal development of Arabidopsis. The authors suggested that this reversible pathway is involved in water-stress-induced rapid ABA accumulation that does not require gene activation. However, a relatively small impact of loss of AtBG1 on ABA accumulation in water-stressed leaves compared to the other phenotypes may suggest roles for AtBG1 beyond ABA release. We also note that no direct evidence of ABA production is provided for the *atbg2* plants that exhibit phenotypes similar to those of the *atbg1* plants (Xu et al. 2012).

An unexpected result was that *P. patens* does not possess genes for any of these enzymes for ABA catabolism. CYP707A genes are present only in vascular plants, and BG1 is present only in angiosperms (Table 1 and Fig. 3). Our preliminary experiment to detect ABA catabolites showed that protonemata of *P. patens* do not accumulate either ABA-GE or 8'-hydroxylation catabolites. Instead, we detected only 9'-hydroxylation catabolites (neoPA) (Sakata et al. unpublished results). *P. patens* actively exports synthesized ABA to the extracellular environment (Minami et al. 2005). The extracellular export system, rather than inactivation and sequestration, is likely to function as the major system to reduce intercellular ABA level in bryophytes. Recently, Okamoto et al. (2011) reported that ABA 9'-hydroxylation is catalyzed by CYP707A as a side reaction in Arabidopsis. Bryophytes may possess other CYPs that catalyze 9'-hydroxylation of ABA as the main reaction.

3.2 ABA Transporters

ABA of angiosperms has been considered as a root-derived signaling molecule that induces physiological changes in shoots in response to dry soil conditions. In fact, expression of Arabidopsis AAO3, AtABA2, and AtANCED3 genes is observed in

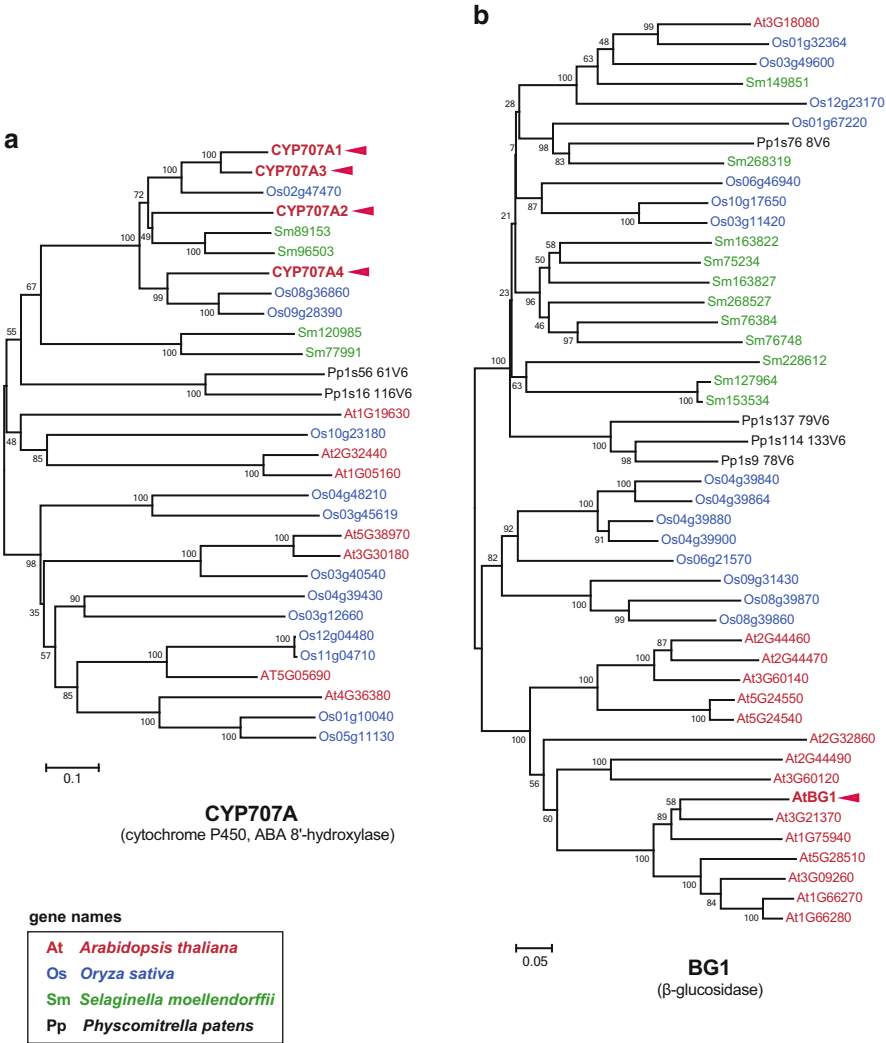


Fig. 3 Phylogenetic relationship among orthologs of the CYP707A family and BG1 glucosidase. The phylogenetic tree of CYP707A family (a) and BG1 glucosidases (b) was generated by MEGA5.0 by using the neighbor-joining method. Genes of *A. thaliana*, *O. sativa*, *S. moellendorffii*, and *P. patens* are indicated by red, blue, green, and black, respectively. Previously reported Arabidopsis ABA 8'-hydroxylase CYP707A family and ABA glucosidase BG1 are indicated by arrowheads

vascular tissues (parenchyma cells) in response to water stress (Endo et al. 2008; Koiwai et al. 2004; Seo and Koshiba 2011), indicating that ABA produced in vascular tissues must be delivered to the cells such as guard cells that require water-stress responses. The molecular basis of ABA transportation was only recently clarified. Two independent research groups simultaneously made the breakthrough for ABA transporters using Arabidopsis. Kang et al. (2010) reported that a

pleiotropic drug resistance transporter (PDR)-type ABC transporter PDR12 (AtPDR12/AtABCG40) mediates uptake of ABA into plant cells, and expression of AtPDR12/AtABCG40 in yeast cells and BY2 cells increases ABA uptake. Furthermore, protoplasts derived from *atabcg40* knockout mutant plants showed reduced uptake of ABA. AtPDR12/AtABCG40 is membrane localized and expressed ubiquitously with higher expression in guard cells. The *atabcg40* plants show a delay in gene expression in response to ABA application and are impaired in stress tolerance. Kuromori et al. (2010) isolated an Arabidopsis mutant line with an ABA-sensitive phenotype in the germination and seedling stages from *activator (Ac)/dissociation (Ds)* transposon-tagged mutant collection. The *Ds* element was inserted into the *At1g71960 (AtABCG25/AtWBC26)* gene. Expression of *AtABCG25* was also ubiquitous with the most obvious expression in the hypocotyls, roots, and vascular veins of leaves and enhanced by ABA treatment. Subcellular localization of AtABCG25 was observed at the plasma membrane. ATP-dependent ABA transport activity was observed in membrane vesicles derived from AtABCG25-expressing insect cells, suggesting that AtABCG25 functions as an ABA exporter. Kuromori et al. (2011) also suggested the involvement of AtABCG22—which is closely related to AtABCG25 in the phylogenetic tree—in ABA function in guard cells, although direct evidence for ABA transport activity is lacking. More recently, Kanno et al. (2012) identified a novel ABA transporter using an elegant experimental design with Arabidopsis cDNAs capable of inducing interactions between PYR/PYL/RCAR and PP2C in yeasts under low ABA concentrations. The isolated gene, designated ABA-IMPORTING TRANSPORTER (AIT), encoded the low-affinity nitrate transporter NRT1.2, belonging to the NRT1/PTR transporter family, which consists of 53 related sequences (Tsay et al. 2007). AIT1 shows a higher affinity for the natural (+)-ABA than for the synthetic (–)-ABA as its substrate, and is insensitive to an ABA agonist, pyrabactin, indicating specific recognition of ABA structure. We note that AtABCG40 and AtABCG25 had no effect in this assay. AIT1 was localized at the plasma membrane of Arabidopsis cells, and the promoter activity was observed in imbibed seeds and in vascular tissues in cotyledons, true leaves, hypocotyls, roots, and inflorescence stems after germination. The *ait1/nrt1.2* plants showed a lower surface temperature in the inflorescence stems than those of the WT due to the excess water loss from open stomata. The finding of an ABA transporter that also transports nitrate partly explains the cross talk between ABA signals and nitrate (Matakiadis et al. 2009).

Of interest, none of these ABA transporters can be found in either the genome of the moss *P. patens* or that of the basal vascular plant *S. moellendorffii* (Table 1 and Fig. 4). We know that *P. patens* exports intercellular cytosolic ABA outside of cells (Minami et al. 2005) and that membrane proteins likely drive this action. Arabidopsis ABA transporters described here might evolve after emergence of seed plants, and there must be other ABA transporters that are yet to be identified.

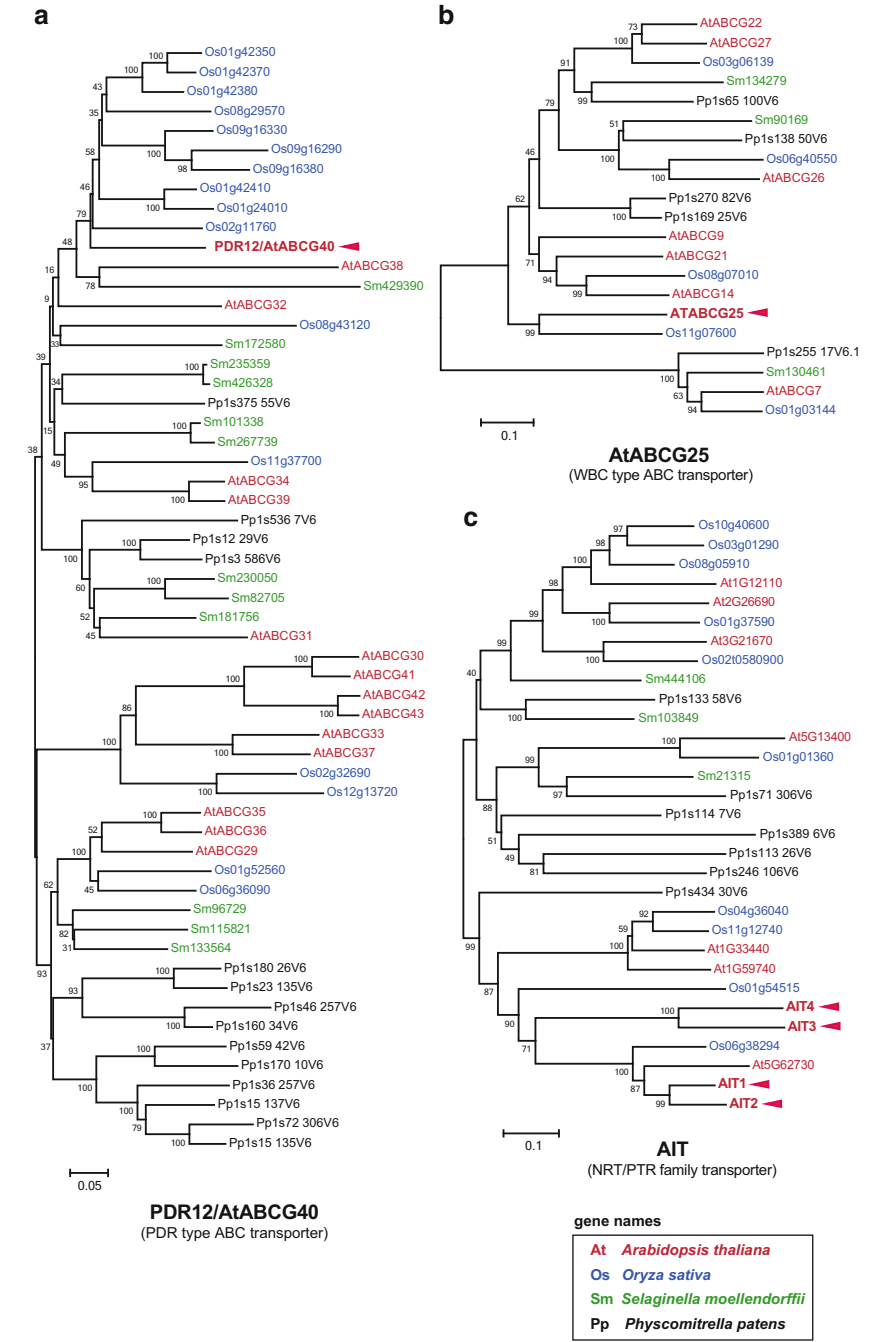


Fig. 4 Phylogenetic relationship among orthologs of ABA transporters. The phylogenetic tree of PDR12/AtABCG40 (a), AtABCG25 (b), and AITs (c) was generated by MEGA5.0 by using the neighbor-joining method. Genes of *A. thaliana*, *O. sativa*, *S. moellendorffii*, and *P. patens* are indicated by red, blue, green, and black, respectively. Previously reported ABA transporters, PDR12/AtABCG40, AtABCG25, and AITs, are indicated by arrowheads

3.3 ABA Signaling Core Components

Compared to enzymes for ABA metabolism and transporters, ABA signaling molecules are well conserved among land plants. Studies in *Arabidopsis* identified several candidates of ABA receptors including membrane-integrated G-protein-coupled receptors (GPCRs) (Liu et al. 2007), GPCR-type G proteins (Pandey et al. 2009), and the chloroplast-localized Mg chelatase H subunit (Shen et al. 2006); however, their functions as ABA receptors have been questioned or disproven (Risk et al. 2009; Tsuzuki et al. 2011). Only the cytosolic pyrabactin resistance 1/pyrabactin resistance 1-like/regulatory component of ABA receptor (PYR/PYL/RCAR) (Ma et al. 2009; Park et al. 2009) establishes a robust link to central components of the ABA signaling pathway: plant-specific SNF-related protein kinases (SnRK2s) as positive regulators and ABI1-related type-2C protein phosphatases (PP2Cs) as negative regulators (Takezawa et al. 2011; Umezawa et al. 2010). The three ABA-core components are considered to form an ABA receptor complex to trigger the ABA signaling cascade. ABA-triggered phosphorylation of the transcription factor (Fujii and Zhu 2009) and S-type anion channel activation (Brandt et al. 2012) by the ABA-core components have been reconstructed in vitro. Moreover, an *Arabidopsis* sextuple mutant of PYR/PYL/RCAR genes exhibits extreme ABA insensitivity in almost all known ABA-related responses, as is expected from their roles as ABA receptors (Gonzalez-Guzman et al. 2012). These results indicate that the cytosolic ABA receptors play a major role in sensing the hormone in *Arabidopsis*. The ABA-core components described here are well conserved only in land plants, although their functions outside angiosperms are yet to be elucidated.

Recent studies on model bryophytes indicate that group A PP2C, a member of the ABA receptor core components, plays a role in ABA responses in basal land plants. Komatsu et al. (2009) reported a disruption of one of two *PpABII* genes (*PpABIIA* and *PpABIIIB*) encoding Group A PP2Cs in *P. patens*. Single disruption of *PpABIIA* by gene targeting causes an ABA-hypersensitive phenotype such as increased stress tolerance and enhanced expression of ABA-inducible genes, suggesting that *PpABII* also functions as a negative regulator of ABA signaling in *P. patens* (Komatsu et al. 2009). More recently, we carried out homology search for signaling molecules for ABA responses in *M. polymorpha* and isolated the *MpABII* gene that encodes an ABI1-like PP2C (Tougane et al. 2010). To analyze functions of *MpABII*, a transient assay system by particle bombardment of cultured cells of *M. polymorpha* was established and used for analysis of ABA-induced gene expression. In this assay, the cells bombarded with the *Em-GUS* construct showed ABA-induced GUS expression, and co-expression of the *MpABII* with *Em-GUS* resulted in suppression of the ABA-induced GUS expression. Furthermore, transgenic *P. patens* plants stably expressing *MpABII* showed a reduced sensitivity to osmotic and freezing stress and did not undergo typical morphological changes by ABA treatment (Tougane et al. 2010). These results indicated that *MpABII* functions as a negative regulator of ABA signaling.

3.4 Transcription Factors

To date, many transcription factors have been described that are involved in ABA responses of angiosperms. Among them, ABI3 (Giraudat et al. 1992) and ABI5 (Group A bZIP) (Finkelstein and Lynch 2000) isolated from the analysis of Arabidopsis *ABA-insensitive* (*abi*) mutants (Koornneef et al. 1984) are considered to be central to conducting ABA signaling during seed maturation and germination. Of interest, the two transcription factors arose co-instantaneously with other ABA-related genes (Table 1). The shared feature of the transcription factors is that they target ABA-responsive elements (ABREs), with ACGT-core sequences present in the promoters of many ABA-responsive genes of angiosperms (Marcotte et al. 1989; Carles et al. 2002; Hobo et al. 1999; Vasil et al. 1995; Casaretto and Ho 2003). Knight et al. (1995) found that a wheat ABA-inducible *Em* gene promoter is activated by exogenous ABA in the moss *P. patens*, demonstrating evolutionarily conserved machinery from ABA perception to gene activation between *P. patens* and angiosperms. ABI3, also known as VP1 in maize, is an indispensable transcription factor in concert with ABA that regulates maturation and desiccation tolerance of seeds (Giraudat et al. 1992; McCarty et al. 1989). It was unexpected that ABI3/VP1, known as a seed-specific transcription factor, originated in the ancestral land plants. Marella et al. (2006) first reported the function of ABI3 from the moss *P. patens* (PpABI3). PpABI3 can activate the *Em* promoter in both *P. patens* and barley alleurone cells, and partially complements the phenotypes of Arabidopsis *abi3-6* mutant plants. These findings indicate an evolutionarily conserved function of ABI3 in land plants. Sakata et al. (2010) performed comparative analysis of Arabidopsis ABI3 and PpABI3 regarding the activation of the *Em* promoter in *P. patens*. PpABI3 showed more dependency on the other *cis*-element RY sequence, to which the conserved B3 domain of ABI3 binds (Suzuki et al. 1997), while Arabidopsis ABI3 activated the *Em* promoter either in an ABRE- or RY sequence-dependent manner. The difference might be attributed to less-conserved N-terminal regions (B1 and B2) of PpABI3, which are considered to be involved in interaction with other transcription factors (Nakamura et al. 2001; Zhang et al. 2005). These findings suggested that B3 domain-dependent transcriptional regulation of ABI3 is evolutionarily conserved between the moss and angiosperms, whereas angiosperm ABI3 has evolved to activate ABA-inducible promoters independently from the B3 DNA binding domain. Khandelwal et al. (2010) showed that expression of most of the ABA-inducible genes examined was not affected in *abi3* null *P. patens* plants, suggesting that ABI3 is not an essential component for ABRE-mediated gene expression in *P. patens*, as observed in the ABA response of vegetative tissues of angiosperms.

ABI5 belongs to Group A bZIP, which has 13 genes in Arabidopsis (Jakoby et al. 2002). These bZIPs can be further divided into two subgroups based on the conserved N-terminal domains. Nine bZIPs, including ABI5 and AREB/ABFs (ABA-RESPONSIVE ELEMENT BINDING)/(ABA-RESPONSIVE ELEMENT BINDING FACTOR), contain three N-terminal conserved regions termed C1, C2, and C3, and one C-terminal conserved region designated as C4, whereas the

other four bZIPs lack the C1 domain (Bensmihen et al. 2002). The former group (ABI5/AREB/ABF family) is characterized by being involved in ABA signaling in seed development and germination (ABI5, EEL, DPBF2/AtbZIP67, DPBF4, and AREB3) or vegetative tissues (AREB1/ABF2, AREB2/ABF4, ABF1, and ABF3) (Bensmihen et al. 2005; Finkelstein et al. 2005; Fujita et al. 2005; Yoshida et al. 2010) through binding to the ABRE of the ABA-regulated genes via the bZIP DNA binding domain (Choi et al. 2000; Carles et al. 2002; Uno et al. 2000). In contrast to ABI3, Group A bZip has been reported to be absent in the *P. patens* genome and suggested to have first appeared in the most recent common ancestor of spermatophytes; thus it may be related to seed formation (Correa et al. 2008). However, our phylogenetic analysis identified two genes that encode putative Group A bZIPs (Table 1). This fact suggests that the origin of the Group A bZIP is older than proposed. Functional analysis of these genes in *P. patens* will shed light on elucidation of the core component of the toolkit for ABA response in land plants.

It is also known that different types of bZIP transcription factors recognize the ACGT-containing sequences found in seed-specific gene promoters. Opaque2 (O2), a Group C bZIP in maize, binds to TCCACGTAGA in the 22-kDa α -zein promoter (Schmidt et al. 1992). Orthologous genes from wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) are also reported to play the same roles as O2 in their corresponding species (Albani et al. 1997; Oñate et al. 1999). The Group C bZIP is evolutionarily conserved among land plants (Correa et al. 2008), the Arabidopsis genome encodes four Group C bZIP genes, and bZIP10 and bZIP25 have been characterized in association with ABI3. In contrast to maize O2, bZIP10 and bZIP25 are ubiquitously expressed in Arabidopsis tissues (Lara et al. 2003), suggesting an acquired broader range of function outside seeds. These two bZIP proteins physically interact with ABI3 in yeast cells, and co-expression of AtbZIP10/25 with ABI3 results in a remarkable increase in transactivation of the *At2S1* promoter (Lara et al. 2003). A recent study has indicated that heterodimerization of AtbZIP10/25 with another class of bZIP protein (Group S) is also involved in the ABI3-mediated regulation of seed maturation-specific genes in Arabidopsis (Alonso et al. 2009). Richardt et al. (2010) reported in their microarray analysis that two bZIP genes belonging to Group E and G are ABA inducible in *P. patens*. These observations suggest the possibility for the involvement of other classes of bZIPs in the regulation of ABA responses in basal land plants.

4 Evolutionarily Conserved Function of ABA

4.1 Desiccation Tolerance

The definition of desiccation tolerance (DT) is referred as the ability to survive drying to the absolute water contents less than about $0.1 \text{ g H}_2\text{O g}^{-1}$ dry matter or to a water potential of less than about -100 MPa (Farrant 2010). Several moss species

such as *Tortula caninervis* are known to survive more than -540 MPa (Oliver et al. 2005). Phylogenetic analyses have suggested that DT in vegetative tissue, as expressed in bryophytes, was lost in the vegetative body of vascular plants during the evolution; instead, reproductive organs such as seeds have acquired the trait (Rensing et al. 2008). To survive severe water deficits, plant cells must maintain physiological integrity during dehydration and repair mechanisms must exist that function upon rehydration (Bewley 1979). Some moss species such as *T. ruralis* show constitutive DT without any preconditioning. *T. ruralis* constitutively accumulates soluble sugars and late embryogenesis abundant proteins (LEAs), both of which are suggested to play a major role in protecting cellular integrity upon desiccation (Charron and Quatrano 2009). The desiccated *T. ruralis* produces a set of mRNAs as well as polypeptides whose synthesis is unique to the rehydrated state (Oliver 1991; Scott and Oliver 1994), suggesting that changes in gene expression are also important for cellular repair mechanisms upon the rehydration phase.

Desiccation tolerance of seeds in angiosperms strongly associates with ABA (Finkelstein et al. 2002). ABA is also implicated in regulation of DT in bryophytes. In contrast to the desiccation-tolerant mosses, several moss species such as *P. patens* and *Funaria hygrometrica* are drought tolerant but not tolerant to rapid drying. Slow desiccation treatment of *F. hygrometrica* leads to loss of almost all of their water after 24 h with an induced level of endogenous ABA of about sixfold and recovery after rehydration. Application of exogenous ABA at greater than $10\text{ }\mu\text{M}$ confers tolerance to rapid drying, and exogenous ABA has no effect on the rate of water loss (Werner et al. 1991). These data suggest that the DT of *F. hygrometrica* is not constitutive but inductive and that ABA plays a key role in the induction. This ABA-induced desiccation tolerance has been further analyzed at the molecular level using the model moss *P. patens*. Without preconditioning *P. patens* does not survive water potentials lower than about -13 MPa; however, $50\text{ }\mu\text{M}$ ABA treatment prior to desiccation increases survival against at least -273 MPa (Koster et al. 2010). Application of several micromolar ABA also establishes tolerance to rapid desiccation by exposing to an atmosphere of about -100 MPa (about 50 % relative humidity) in a laminar flow cabinet for 24 h (Khandelwal et al. 2010). Osmostress also induces accumulation of endogenous ABA in *P. patens* (Minami et al. 2005). These facts also indicate a role for ABA in induction of DT in *P. patens*. Furthermore, exogenous ABA induces accumulation of boiling-soluble proteins in *P. patens*, a characteristic feature of hydrophilic LEA proteins which specifically accumulate in developing seeds of angiosperms (Knight et al. 1995). ABA treatment of *P. patens* also induces accumulation of soluble sugars such as sucrose (Oldenhof et al. 2006; Nagao et al. 2006) and a trisaccharide theandrose (Nagao et al. 2006). Oldenhof et al. (2006) reported that exogenous ABA preserves membrane phase behavior in the dried state and that the membranes do not undergo a gel to liquid membrane phase transition during dry–rehydration process. It is likely that ABA functions to elicit cellular protection mechanisms upon the rehydration phase to regain integrity of membranes.

The *Arabidopsis abi3* mutant lacks expression of a set of seed-specific genes including *LEA* and desiccation tolerance of seeds (Nambara et al. 1995; Marella et al.

2006), indicating that ABA- and ABI3-mediated gene expression is key for desiccation tolerance of seeds. The ABA- and ABI3-mediated regulation of LEA gene expression is evolutionarily conserved between *P. patens* and *Arabidopsis* (Marella et al. 2006). Moreover, disruption of ABI3 results in loss of ABA-induced DT of *P. patens* (Khandelwal et al. 2010). These data collectively suggest that the mechanism that confers desiccation tolerance of plant cells was established in the last common ancestor of bryophytes and angiosperms. During evolution, land plants acquired a way to execute the induction of desiccation tolerance in an ABA-dependent manner, and the system independently has been used to provide desiccation tolerance of vegetative tissues of bryophytes and angiosperms seeds.

4.2 Low Temperature Responses

Overwintering plants in the temperate and sub-frigid zones of earth in general can acclimate to low temperatures, and these plants develop freezing tolerance in response to pre-exposure to nonfreezing low temperature. It has been suggested that ABA plays a role in this cold acclimation process. Endogenous ABA levels increase during cold acclimation (Daie and Campbell 1981; Lalk and Dorffling 1985; Smoleńska-Sym et al. 1995; Jouve et al. 2000), and exogenous ABA treatment at ambient temperatures increases freezing tolerance (Chen and Gusta 1983). Analysis of transcripts has indicated that ABA treatment increases expression of a number of stress-responsive genes that are also induced by cold (Zeevaart and Creelman 1988). These results suggest that the endogenous ABA accumulated in response to low temperature exposure plays a role in enhanced freezing tolerance. Accordingly, analysis of cold-induced genes of *Arabidopsis* has revealed the frequent occurrence of ABRE in the promoters (Yamaguchi-Shinozaki and Shinozaki 1994; Seki et al. 2002).

Despite these facts, the role of ABA in cold acclimation has yet to be conclusively established (Gusta et al. 2005). Studies on ABA-deficient *aba1* and ABA-insensitive *abil-1* of *Arabidopsis* have indicated that the contribution of ABA to cold acclimation might be limited. Both ABA-deficient *aba1* and ABA-insensitive *abil-1* mutants showed accumulation of cold responsive (COR) transcripts by low temperature treatment (Gilmour and Thomashow 1991). In addition, cold-induced gene expression is mediated by CBF/DREB transcription factors that bind to the distinct *cis*-element C-repeat, also known as the dehydration-responsive element (DRE), independent of ABA (Baker et al. 1994). These results suggest that endogenous ABA has little or no role in the induction of cold tolerance. However, the issue is not simple because the C-repeat/DRE can serve as a coupling element (CE) with ABRE; thus, the C-repeat/DRE contributes to the ABA-induced gene expression when present with ABRE (Narusaka et al. 2003). In addition, expression of genes for CBFs/DREBs is induced by ABA (Knight et al. 2004). In contrast to molecular studies of cold responses in *Arabidopsis*, such studies on other plant species with various cold tolerances are limited. Recent global promoter analyses indicate that both ABRE and C-repeat/DRE are conserved in cold-induced promoters of *Arabidopsis* and soybean. ABRE

is also conserved in the cold-induced promoter of rice, but the C-repeat/DRE is not (Maruyama et al. 2012). These results indicate that the mechanism for cold-induced gene expression might vary even among angiosperm species, though implicating the presence of evolutionarily conserved *cis*-promoter elements regulated by ABA.

Even nonvascular plants can undergo cold acclimation. Moss species growing in their natural habitat have a higher freezing tolerance in winter than in summer (Rütten and Santarius 1992). Laboratory experiments using *P. patens* have indicated that its protonemata and gametophore develop freezing tolerance in response to exposure to nonfreezing cold temperatures for several days (Minami et al. 2005; Sun et al. 2007). The increase in freezing tolerance accompanies an increase in various LEA-like transcripts, which are also inducible by ABA treatment. *P. patens* genes, which are remarkably induced by cold acclimation, contain putative ABREs but not C-repeats/DRE in the promoter, indicating a possible role of evolutionarily conserved *cis*-elements in cold-induced gene expression (Cuming et al. 2007; Bhyan et al. 2012). In contrast to these facts, analysis of endogenous ABA levels has suggested that obvious increases in ABA levels are not observed during up to one week of cold acclimation (Minami et al. 2005). Thus, the contribution of endogenous ABA in cold acclimation of moss is not yet conclusive.

Our recent results using ABA-insensitive AR7 lines of *P. patens* indicate that the ABA signaling process is necessary for cold responses in mosses. The AR7 mutant that has been isolated by ultraviolet mutagenesis and the D2-1 line that overexpresses the catalytic domain of Group A PP2C show an ABA-insensitive growth and cannot develop freezing and desiccation tolerance. Of interest, these lines did not acclimate to low temperature to enhance freezing tolerance, with reduced accumulation of stress-associated transcripts (Bhyan et al. 2012). These results indicate that cold and ABA signal transduction share a common mechanism regulated by reversible phosphorylation. Identification of the molecule under such regulation as well as transcription factors responsible for the cold-induced gene expression would clarify how ABA signaling molecules modify the cold-response pathway.

4.3 Stomatal Closure

Stomata are widely distributed among land plants except liverworts, indicating that the epidermal apparatus evolved after the separation of liverworts and mosses from a common ancestor more than 400 million years ago (Fig. 1). The role of ABA in seed plant stomatal closure is well documented (Schroeder et al. 2001; Rock et al. 2010; Mori and Murata 2011). Water-stressed seed plants increase accumulation of ABA, which effectively closes the stomatal aperture by reducing the turgor pressure of guard cells. This physiological event is considered as one of the most important functions of ABA to protect plants from water deficiency. However, ABA signaling can be found in liverworts (Tougane et al. 2010), suggesting that the role of ABA in stomatal closure is an evolutionarily acquired feature. It is still a matter of debate as to when land plants acquired the ABA-dependent closure system of the stomatal aperture in their evolutionary history; one group has reported that ABA

involvement in regulating plant water loss and hydration through stomata is found only in seed plants, but another group has suggested that ABA influences the guard cell aperture of the basal vascular plants. Brodribb and McAdam (2011) examined the ABA response of the stomata of lycophytes and ferns by adding a high concentration of ABA to the transpiration stream. Even with the high concentration of foliar ABA (7,000 ng g⁻¹ FW), stomata of fern (*Pteridium esculentum*) and lycophyte (*Lycopodium deuterodensum*) species lacked the closure response, although angiosperm (*Helianthus annuus*) and conifer (*Callitris rhomboidea*) species showed rapid stomatal closure at leaf ABA levels of 1,500–2,000 ng g⁻¹ FW. Moreover, they showed a strong correlation between leaf water content and stomatal aperture in lycophytes and ferns. These authors suggested that stomatal closure of lycophytes and ferns is passively controlled and insensitive to ABA and that active metabolic control of stomatal closure evolved after the divergence of ferns about 360 million years ago. However, this hypothesis has been challenged (Ruszala et al. 2011). They examined stomatal responses of the lycophyte *Sellaginella uncinata* to ABA as well as CO₂ in comparison with Arabidopsis. Exogenous ABA from 0 to 25 μM inhibited the light-induced opening of pre-closed stomata and promoted stomatal closure of pre-opened stomata dose dependently, although McAdam and Brodribb (2012) specifically examined this finding and could not repeat it (see below). This ABA effect was blocked in the presence of EGTA and verapamil, which inhibit ABA-induced calcium transient in angiosperm guard cells. ABA also induced an increase of reactive oxygen species (ROS) in *S. uncinata*, the intracellular second messenger of the Ca²⁺ transient in angiosperms guard cells. These authors further demonstrated that the *S. uncinata* gene encoding an SnRK2 similar to Arabidopsis OST1 could complement the Arabidopsis *ost1* mutant phenotype. The stomata of *S. uncinata* also exhibited CO₂-induced stomatal closure as observed in seed plants. These data suggested that evolutionarily conserved mechanisms operate stomatal closure between *S. uncinata* and angiosperms. According to this report, active stomatal control emerged in ancestral vascular plants about 420 million years ago. However, an evolutionarily conserved function of OST1-like SnRK2 may not necessarily be related to the conserved mechanism of stomatal closure because this type of kinase can be found in the liverwort *M. polymorpha*, which lacks stomata (data not shown).

The conflict between the reports may be attributed to the method or plant materials they employed; however, guard cells of ferns and lycophytes might possess a signaling pathway that can be activated by ABA. In fact, a set of genes that encode signaling components and biosynthesis enzymes for ABA in angiosperms is evolutionarily conserved among land plants including the basal land plant bryophytes that diverged from vascular plants 420 million years ago. It is intriguing that the known ABA transporter genes can be found only in seed plants (Table 1). Acquisition of these genes in seed plants might contribute to the rapid reaction of the stomatal aperture in response to environmental water conditions. As mentioned first in this section, liverworts, which represent the oldest extant lineage of land plants, lack stomata but possess the ABA signaling pathway, indicating that ABA evolved as a phytohormone to regulate physiological aspects other than

stomatal control. A key question is when endogenous ABA acquired a role in controlling the turgor pressure of guard cells. McAdam and Brodribb (2012) further investigated the function of water-stress-induced endogenous ABA in stomatal closure of the lycophyte (*S. kraussiana*) and ferns (*P. esculentum* and *Dicksonia antarctica*) in comparison with seed plants (*Pisum sativum*, *P. radiata*, and *Callitris rhomboidea*). They exposed plants to water stress that induced stomatal closure and increase of endogenous ABA levels in all plant species examined. Rapid rehydration of excised, drought-treated leaves quickly restored their water potential while maintaining high concentration of ABA. This manipulation clearly discriminated the stomatal response of seed plants from that of lycophyte and ferns. Stomata of the lycophyte and ferns rapidly opened their stomata upon rehydration while stomata of seed plants failed to reopen upon rehydration probably because of a high level of foliar ABA. These authors suggested that endogenous ABA is not involved in the regulation of stomatal closure of lycophytes and ferns. Under these circumstances, we need more studies to draw any conclusions about the role of endogenous ABA in stomatal closure in the basal vascular plants. Ultimately, elucidation of the role of ABA in basal vascular plants will require genetic modification of ABA signaling or biosynthetic pathways, which is still difficult to perform in these species at this stage.

The basal land plants bryophytes occupy an important place in elucidating the evolution of stomatal control in land plants. However, the physiology of stomata in basal land plants is poorly understood. Among bryophytes, stomata are found in mosses and hornworts but not in liverworts. The current understanding is that stomata arose only once during land plant evolution after the divergence of liverworts from other bryophytes (Vatén and Bergmann 2012). Stomata occur in the sporophytes of mosses and hornworts, and those of *Sphagnum*, sister to other mosses, are considered to function in facilitating sporophyte desiccation (Duckett et al. 2009). Stomata from the hornwort *Anthoceros* species have been reported to close in response to exogenous ABA (Hartung et al. 1987). Lucas and Renzaglia (2002), however, examined the behavior of stomata from several hornworts including *A. caucasicus* and observed no responses to light/dark conditions and ABA (Lucas and Renzaglia 2002). These authors suggested that the stomata of hornworts function in providing a passageway for gas exchange. Further analyses will be required to evaluate these reports. Stomata of mosses of the Funariaceae including *P. patens* are located only in the ring around the base of the diploid sporophyte structure and have been confirmed to close in response to a light-to-dark shift, atmospheric CO₂ levels, and exogenous ABA (Chater et al. 2011). The fungal toxin fusicoccin, which activates the H⁺-ATPase pump essential for turgor-driven stomatal opening in angiosperms, also induced stomatal opening of *P. patens* and *F. hygrometrica*. Responsiveness of the ABA-inducible *PpLEA1* promoter was observed at the base of the sporophyte around the stomatal ring, suggesting activation of ABA signaling in this region. They also demonstrated that a gene encoding *Physcomitrella* SnRK2 (PpOST1) functionally complements the phenotype of the Arabidopsis *ost1* mutant and disruption of *PpOST1* led to attenuation of ABA-induced stomatal closure of *P. patens*. Molecular genetic analysis suggests that ABA can change the stomatal aperture in *P. patens* and *F. hygrometrica* through a

functionally conserved OST1-like SnRK2; however, this ABA response may be nonadaptive because the stomata of the bryophyte sporophyte are considered to act to divert water and solute from the parental gametophytes or in the desiccation of sporophytes (Ligrone et al. 2012a, b).

5 Role of Cytosolic Calcium and Calcium-Binding Proteins in ABA Signaling

It has been discussed that calcium, a ubiquitous second messenger, plays a role in ABA-induced signaling processes in plants. Calcium concentrations in cytosol ($[Ca^{2+}]_{cyt}$) maintained at sub-micromolar levels can be increased in response to extracellular signals through opening of plasma membrane and endomembrane-located calcium channels. The generated calcium signals are decoded by various types of calcium-binding proteins (CaBPs) that undergo drastic conformational changes upon calcium binding to a calcium-binding loop called the EF hand. Many biotic and abiotic stress responses are thought to be mediated by $[Ca^{2+}]_{cyt}$ and CaBPs by regulation of functions of intracellular signaling molecules as well as gene expression driven by transcription factors (Trewavas and Malhó 1998; Kudla et al. 2010; Reddy et al. 2011).

More than 200 EF-hand CaBPs are encoded in the Arabidopsis genome (Day et al. 2002). A major group of these are low molecular weight CaBPs which do not have domains with catalytic functions but interact with other enzymes to modulate their functions; the other groups have a distinct catalytic domain with activity regulated by the calcium-binding domain within the same polypeptide. The former group includes calmodulin (CaM), CaM-like proteins (CMLs), and calcineurin B-like proteins (CBLs), and the representative of the latter is calcium-dependent protein kinases (CDPKs).

5.1 CDPKs

The role of calcium in ABA responses has been demonstrated in guard cells of stomata for their stress-induced closure process (Schroeder and Hagiwara 1990; Leckie et al. 1998). During stomatal closure, ABA inhibits plasma membrane hyperpolarization and influx of potassium ion to cytosol, while facilitating the release of potassium by triggering membrane depolarization. The influx of calcium to cytosol has been characterized as one of the initial events occurring during the stomatal ABA response. The initial $[Ca^{2+}]_{cyt}$ elevations serially induce further Ca^{2+} release from the vacuoles (McAinsh et al. 1995), thus generating specific oscillation patterns of cytosolic calcium distribution (McAinsh et al. 1990, 1995). The calcium oscillation induced by ABA causes depolarization of plasma membrane, facilitated by S-type anion currents in guard cells leading to opening of the outward rectifier potassium channel (Pei et al. 1997; Siegel et al. 2009).

In Arabidopsis, a central guard cell S-type anion channel (SLAC1) has been identified as a key regulator facilitating calcium-induced membrane depolarization during stomatal closure (Vahisalu et al. 2008). Recent reports suggest the involvement of CDPKs in the regulation of SLAC1 activity. CDPKs, belonging to the SNF1 protein kinase family, have a serine–threonine protein kinase domain fused to a CaM-like domain with EF hands. Binding of calcium to the EF-hand motifs results in release of autoinhibition of the catalytic domain and makes the enzyme active (Harmon et al. 2000). An Arabidopsis *atcpk3/atcpk6* double mutant that shows impaired ABA and Ca^{2+} -induced stomatal closure has reduced S-type anion channel activity (Mori et al. 2006). ABA-induced activation of SLAC1 channels has been reconstituted in *Xenopus* oocytes using the ABA receptor PYR1 and Group A PP2Cs with either CPK6 or OST1 (Brandt et al. 2012). These studies indicate that for stomatal regulation, CDPK can activate the SLAC1 channel in parallel with activation by the subclass III SnRK.

CDPKs appear to play a role in ABA-induced gene expression. Mutations of two Arabidopsis CDPKs, *AtCPK4* and *AtCPK11*, result in ABA-insensitive phenotypes in seed germination and seedling growth, and the double mutants have stronger ABA insensitivity than the single mutants (Zhu et al. 2007), indicating that the two CDPKs function as positive regulators of ABA signaling. Group A bZIPs (ABF1, ABF2, ABF3, and ABF4), responsible for ABA-induced gene expression in the vegetative tissues of Arabidopsis, are activated by ABA-induced phosphorylation. In vitro, AtCPK32 interacts with and phosphorylates the ABA-induced transcription factor ABF4, which directly binds ABA-inducible promoters. The CPK4 and CPK11 kinases both phosphorylate two ABA-responsive transcription factors, ABF1 and ABF4 (Zhu et al. 2007). Lynch et al. (2012) reported that CPK11 very efficiently phosphorylates the N-terminal halves of both ABF1 and ABF3 but not of ABF2 and ABF4. ABFs are also direct targets of phosphorylation by the subclass III SnRK, and the ABA-activated phosphorylation of the ABF2 fragment is completely impaired in the *srk2d/e/i* triple mutant in vitro. The ABA insensitivity of the *cpk4 cpk11* double mutant is also limited compared to that of the *srk2d/e/i* triple mutant; thus, CDPKs might be partially involved in ABA signaling through the phosphorylation of specific ABFs in parallel with or downstream of SnRK2s, although how ABA activates the kinases is yet to be elucidated.

The Arabidopsis genome encodes 34 CDPKs, and these kinases are also conserved in non-seed plants as well as in green algae, the latter of which lack ABA-related genes (Table 1 and Fig. 5a). The number of CDPK genes appears to be unrelated to the evolution of green plants, suggesting their fundamental roles in plant life, although their roles in the non-seed plants are largely unknown.

5.2 Other CaBPs

CBLs with sequence similarity to mammalian calcineurin B have targeted a family of protein kinases referred to as CIPKs (CBL-interacting protein kinases)

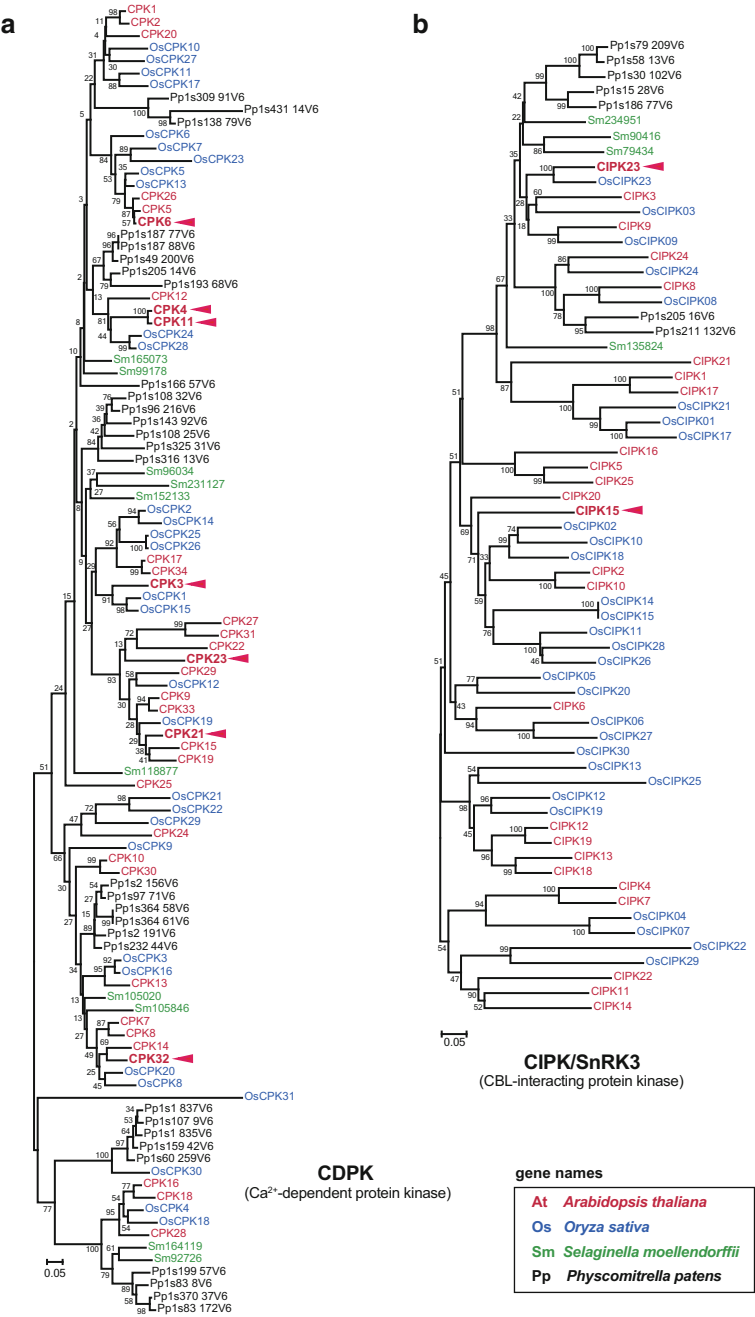


Fig. 5 (continued)

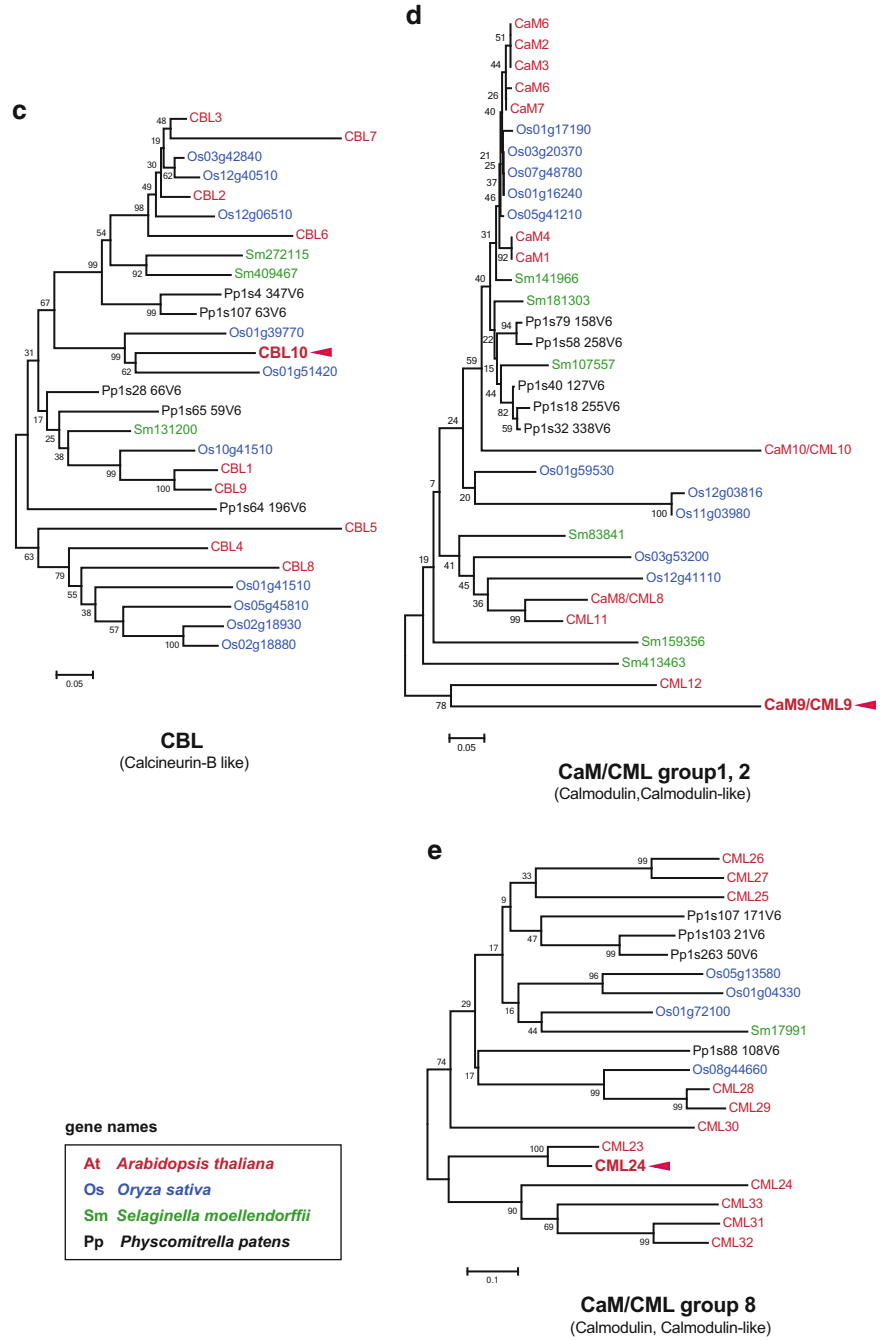


Fig. 5 Phylogenetic relationship among orthologs of Ca^{2+} -dependent components. The phylogenetic tree of CDPK (a), CIPK (b), CBL (c), and CML (d, e) was generated by MEGA5.0 by using the neighbor-joining method. Genes of *A. thaliana*, *O. sativa*, *S. moellendorffii*, and *P. patens* are indicated

(Luan 2009; Kudla et al. 2010). By genetic analysis of salt-overly-sensitive mutants of Arabidopsis, *SOS3* (*AtCBL4*) and *SOS2* (*AtCIPK24*) have been identified as loci for salt stress sensing (Halfter et al. 2000). Sequencing of the Arabidopsis genome has revealed 25 CIPKs targeted by 10 CBLs (Table 1 and Fig. 5b, c). These proteins have diverse functions in plant responses to environmental stresses as well as to light, hormone, and sugars (Batistic et al. 2010). Expression of Arabidopsis *AtCIPK3* is responsive to exogenous ABA and cold, salinity, and wounding. Disruption of *AtCIPK3* and its interacting *AtCBL9* results in an ABA-hypersensitive phenotype in seed germination, indicating that the *AtCIPK3/AtCBL9* is a negative regulator of ABA signaling (Kim et al. 2003; Pandey et al. 2004, 2008). In contrast, transgenic plants lacking *AtCBL1* are sensitive to drought, cold, and salt stress independent of ABA, indicating its role in positive regulation of stress responses (Albrecht et al. 2003; Cheong et al. 2003). Both *AtCBL1* and *AtCBL9* can target to *AtCIPK1*, and ABA-dependent and -independent signaling processes are regulated (D'Angelo et al. 2006). In rice, Xiang et al. (2007) reported that among 20 CIPK genes (*OsCIPKs*) tested, the expression of the majority of CIPKs was found to be induced by ABA (16 genes), drought (15 genes), salt (12 genes), and cold (3 genes). Transgenic plants overexpressing the transgenes *OsCIPK03*, *OsCIPK12*, and *OsCIPK15* showed improved stress tolerance with increased proline contents compared to wild type (Xiang et al. 2007). These results suggested that CBL/CIPKs have functions in ABA signaling and stress tolerance in angiosperm species. CBLs are found not only in angiosperms but also in *P. patens* and green algae (Table 1 and Fig. 5b, c). On the other hand, CIPK appears to be unique in land plants, although angiosperms have a greater number of CBL/CIPKs compared to non-seed plants. This pattern indicates that the numbers increased with increasing morphological and physiological complexity in land plant evolution.

CaM targets multiple proteins through calcium-dependent binding to basic amphipathic amino acid stretches in the regulatory domain. Protein kinases are among important targets of CaM, based on information obtained in animal studies of calcium/CaM-dependent protein kinases (Hanson and Schulman 1992). A screen using ³⁵S-labeled CaM identified a receptor-like kinase (RLK) CRCK1 from Arabidopsis. The expression of *CRCK1* was increased in response to cold and salt stress, H₂O₂, and exogenous ABA (Yang et al. 2004). Although targets of CaM-binding RLKs have not been identified, these RLKs might regulate the stress-signaling pathway by affecting the MAP kinase cascade (Yang et al. 2010a, b). In addition, transcriptional activators can be targets of CaM for the control of stress-induced gene expression mediated by calcium. Overexpression of GmCaM4, a divergent CaM isoform that specifically binds to the MYB2 transcription factor, resulted in

Fig. 5 (continued) by red, blue, green, and black, respectively. Previously reported ABA signaling-related components, CPK3, CPK4, CPK6, CPK11, CPK21, CPK23, CPK32, SLAC1, CIPK15, CIPK23, CBL10, CML9, and CML24, are indicated by arrowheads. CML groups according to the phylogenetic analysis of Arabidopsis CaM and CML (McCormack et al. 2005)

salt stress tolerance and increased expression of *P5CS1*, *ADH1*, and *RD22* (Yoo et al. 2005). MYB2 is known as a transcription factor induced by dehydration, salt, and ABA, and its overexpression resulted in enhanced ABA sensitivity (Urao et al. 1993); thus, CaM interaction adds another regulatory mechanism of calcium. CaM also interacts with other transcription factors such as TGA3, GT-2-like1 (GTL1), WRKY, and NAC domain transcription factors as well as ABF2/AREB1, potentially being a target of CaM regulation (Popescu et al. 2007a, b) (for review, see Kim et al. 2009; Galon et al. 2010). These transcription factors might also play a role in calcium-dependent modification of the ABA signaling pathway.

The Arabidopsis genome encodes 50 CMLs according to classification by (Day et al. 2002). Physiological functions of most CMLs have not been identified, but some CMLs are likely to be regulating ABA signaling. In Arabidopsis, *AtCML24* transcripts were induced by multiple environmental signals. *AtCML24*-underexpressing transgenics are less sensitive to ABA in germinating seeds and seedlings (Delk et al. 2005). Another CML, *AtCML9*, is rapidly induced by abiotic stress and ABA in young seedlings, and in the *cml9* knockout mutants showed a hypersensitive response to ABA with enhanced tolerance to salt and drought stress (Magnan et al. 2008). Targets of most CMLs, including *AtCML9* and *AtCML24*, are yet to be identified.

5.3 The Role of Calcium in Basal Land Plants

Circumstantial evidence indicates that calcium plays a role in bryophytes' responses to ABA and environmental stresses. Cold-, touch-, and blue/UV-A light-induced increase in cytosolic calcium has been demonstrated in *P. patens*, suggesting that calcium is a ubiquitous intracellular messenger in land plants (Russell et al. 1996, 1998; Tucker et al. 2005). The role of cytosolic calcium in stress responses in mosses has been suggested by the study of the *P. patens PCA1* gene encoding a PIIB-type Ca^{2+} -ATPase whose transcript accumulation is induced by dehydration, NaCl, and ABA. The study indicated that disruption of *PCA1* gene results in elevated cytosolic calcium levels upon salt stress. Furthermore, *PCA1* knockout plants showed reduced transcript levels of *PpCOR47* in response to ABA and salt stress and were more susceptible to salt stress than the wild type (Qudeimat et al. 2008). These results indicate that homeostasis of cytosolic calcium levels is important for ABA and stress sensing and signaling processes. Calcium-binding proteins that sense changes in calcium concentrations in *P. patens* remain to be elucidated. The *P. patens* genome encode 29 CaMs, four CBLs, seven CIPKs, 30 CDPKs, and probably more than two dozen CMLs, which might play roles in ABA and stress responses in mosses (Table 1 and Fig. 5).

Regulation of ABA signaling by $[\text{Ca}^{2+}]_{\text{cyt}}$, a universal second messenger, might have provided land plants with novel mechanisms to flexibly alter their stress responses, which change moment by moment in the terrestrial environment. Identification of target molecules for regulation by $[\text{Ca}^{2+}]_{\text{cyt}}$ is key to understanding acquisition of such

mechanisms. Genes of CBLs/CIPKs, CDPKs, and CaM-binding transcription factors are likely to have appeared long before the emergence of ABA-related genes, suggesting that the integration of calcium signaling into ABA signaling might occur during evolution of land plants. Clarification of the role of $[Ca^{2+}]_{cyt}$ in ABA signaling of bryophytes will further extend our knowledge about stress adaptation in land plants.

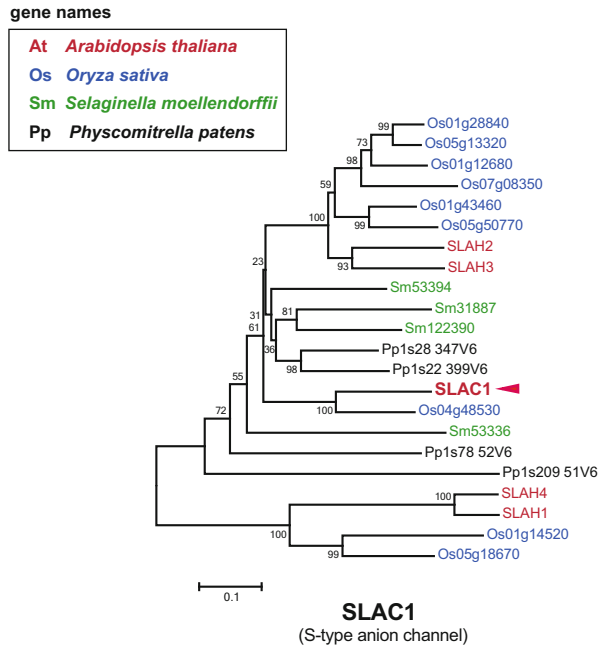
6 Conclusions and Perspectives

Since pioneering studies in the 1960s, ABA had been held as a plant hormone that regulates water-stress responses mainly in vegetative stomata and reproductive seeds of angiosperms. Recent progress in sequencing technology as well as physiological and molecular analyses of basal land plants has demonstrated the origin of ABA in the last common ancestor of extant land plants. Key issues for future evo-devo studies of ABA include pinpointing the origin of ABA signaling and how this signaling has been modified to establish seed development and stomatal regulation.

To address how ABA signaling evolved during plant evolution, gibberellin (GA) would be a good model. GA signaling observed in angiosperms is evolutionarily conserved in the lycophyte *S. moellendorffii* but not in the bryophyte *P. patens* (Hirano et al. 2007). A recent study suggested that the GA perception system evolved to regulate a preexisting GAMYB-based system for spore and sexual organ development during land plant evolution (Aya et al. 2011). The GA studies indicate that the ABA perception system might have merged during evolution of algae to land plants to regulate an as-yet unknown preexisting system in a water-stress-dependent manner. Charophyceae species are the closest relatives of the green land plants. Although the genome sequences of the Charophyceae species are not yet released, mRNA sequences are available in public databases. We search the Charales sequences for ABA-related genes and identified some that encode SnRK2-like kinase and Group A-like bZIP transcription factors. This preliminary result suggests that several key genes for ABA signaling already emerged before the evolution of land plants. A complete genome sequence as well as functional analysis of the conserved molecules of the Charophyceae species would deepen our understanding of how ABA signaling evolved in land plants.

Recent progress in ABA studies has highlighted a toolkit for ABA function that is absent in algae, suggesting that emergence of the toolkit in the ancestral land plants was key to using the ubiquitous molecule as a stress hormone, enabling adaptation to the terrestrial environment. Thus, the current picture of ABA function in angiosperms must have resulted from the modification and incorporation of new molecules into the toolkit established in the ancestral land plants. For example, SLAC1 is crucial for the control of stomatal apertures by ABA in seed plants in concert with calcium signaling; however, genes encoding SLAC1-like anion channels can be found in basal land plants (Table 1 and Fig. 6). Because of the lack of genomic information from *M. polymorpha*, we cannot currently draw conclusions about whether SLAC1 coevolved with stomata; however, integration

Fig. 6 Phylogenetic relationship among orthologs of SLAC1. The phylogenetic tree of SLAC1 was generated by MEGA5.0 by using the neighbor-joining method. Genes of *A. thaliana*, *O. sativa*, *S. moellendorffii*, and *P. patens* are indicated by red, blue, green, and black, respectively. The *Arabidopsis* SLAC1 is indicated by an arrowhead



of the anion channel and calcium signaling under the control of ABA-core components must be considered as a key event allowing land plants to achieve permanent hydration (homoiohydry).

The evolution of ABA signaling also enabled acquisition of seed desiccation tolerance. It appears that a common system operates vegetative (gametophyte) desiccation tolerance of bryophytes and seed (sporophyte) desiccation tolerance; however, ABA signaling in seeds has developed a novel function to operate seed dormancy and germination, which is known to involve not only ABA signaling but also GA signaling as well as light signaling. Because GA signaling evolved after ABA signaling, evolution of a cross talk between the two pathways in the reproductive organ would be an issue to address in future studies. The same is true for ABA signaling and pathogenesis. Salicylic acid (SA) plays a central role in pathogen response in angiosperms. Interaction between SA signaling and ABA signaling for pathogen responses has been suggested (Rock et al. 2010). Involvement of ABA in pathogenesis is largely unknown, although ABA and SA increase in *P. patens* upon infection by *Botrytis cinerea* (Ponce de León et al. 2012). The role of ABA in pathogen responses of basal land plants is an open question.

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