the plant journal

SEB Society for Experimental Biology

The Plant Journal (2012) **72**, 947–959 doi: 10.1111/tpj.12005

Studies of *Physcomitrella patens* reveal that ethylene-mediated submergence responses arose relatively early in land-plant evolution

Yuki Yasumura[1], Ronald Pierik[2], Mark D. Fricker[1], Laurentius A. C. J. Voesenek[2] and Nicholas P. Harberd[1,*]

[1]*Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK, and*

[2]*Plant Ecophysiology, Institute of Environmental Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*

Received 18 April 2012; revised 20 August 2012; accepted 22 August 2012; published online 18 October 2012.

*For correspondence (e-mail nicholas.harberd@plants.ox.ac.uk).

SUMMARY

Colonization of the land by multicellular green plants was a fundamental step in the evolution of life on earth. Land plants evolved from fresh-water aquatic algae, and the transition to a terrestrial environment required the acquisition of developmental plasticity appropriate to the conditions of water availability, ranging from drought to flood. Here we show that extant bryophytes exhibit submergence-induced developmental plasticity, suggesting that submergence responses evolved relatively early in the evolution of land plants. We also show that a major component of the bryophyte submergence response is controlled by the phytohormone ethylene, using a perception mechanism that has subsequently been conserved throughout the evolution of land plants. Thus a plant environmental response mechanism with major ecological and agricultural importance probably had its origins in the very earliest stages of the colonization of the land.

Keywords: evolution, *Physcomitrella patens*, ethylene, submergence, water relations, phytohormones.

INTRODUCTION

The land plants evolved from charophytic algae 430–470 million years ago (Kenrick and Crane, 1997; Turmel *et al.*, 2006; Finet *et al.*, 2010). The transition from an aquatic to a terrestrial environment made it essential to develop adaptive mechanisms to survive fluctuating water availability, mechanisms which were subsequently diversified. For example, flood-resistant rice cultivars exhibit increased tolerance of submergence. Whilst deepwater rice varieties undergo rapid stem elongation as a means to escape complete submergence (Hattori *et al.*, 2009), the Sub1 variety employs a quiescence strategy to conserve carbohydrate reserves until floodwaters recede (Fukao *et al.*, 2006; Xu *et al.*, 2006; Bailey-Serres and Voesenek, 2008). In both cases, the phytohormone ethylene plays a key role. Ethylene diffuses more slowly in water than in air, resulting in the entrapment of ethylene in submerged plant tissues (Jackson, 1985). The resultant accumulation of ethylene triggers submergence responses in a range of angiosperms (flowering plants) (English *et al.*, 1995; Van der Straeten *et al.*, 2001; Voesenek *et al.*, 2003, 2004; Hattori *et al.*, 2009).

Ethylene and submergence escape responses were first linked in studies of coleoptile growth in rice (Ku *et al.*, 1970) and *Callitriche platycarpa* (Musgrave *et al.*, 1972), and this linkage has subsequently been observed in many angiosperms (Jackson, 2008). Submergence escape also occurs in non-angiosperms, suggesting that this is an evolutionarily conserved mechanism. For example, in the fern *Regnillidium diphyllum* and in the aquatic liverwort *Riella helicophylla* escape is induced by treatment with ethylene (*Regnillidium diphyllum*) or by a combination of ethylene and auxin (*Riella helicophylla*) (Musgrave and Walters, 1974; Stange and Osborne, 1988).

The angiosperm ethylene signalling pathway is well understood. Ethylene is perceived by the ETR1 receptor (and homologues). In the absence of ethylene, ETR1 maintains activation of CTR1, which in turn represses ethylene signalling. Binding of ethylene to ETR1 inactivates CTR1, thus de-repressing the activity of EIN3 and EIN3-like transcription factors, which regulate the expression of ethylene-inducible genes such as *ERF1* and *ERF2* of the AP2/ERF gene family (Stepanova and Alonso, 2009; Yoo *et al.*, 2009). Many ethylene-responsive genes are regulated by AP2/ERF transcription factors. For example, two deepwater rice ERFs, SNORKEL1 and SNORKEL2, trigger rapid stem elongation in response to submergence or ethylene, via increased accumulation of the growth-promoting phytohormone gibberellin (GA) (Hattori

et al., 2009). In contrast, submergence-induced expression of the tolerance-specific SUB1A ERF confers adaptive responses by regulating metabolic activities and limiting the responsiveness of the plant to GA (Voesenek *et al.*, 2004; Fukao *et al.*, 2006; Xu *et al.*, 2006; Fukao and Bailey-Serres, 2008). Thus, despite employing distinct submergence tolerance strategies, these two different rice strains both use ethylene to regulate submergence response.

Here we investigate the evolutionary conservation of ethylene-mediated submergence responses in land plants, by focusing on the submergence responses of bryophytes and using the moss *Physcomitrella patens* as a genetic model (Schaefer and Zryd, 1997; Rensing *et al.*, 2008). Because the bryophytes diverged relatively early from the main land-plant lineage (Kenrick and Crane, 1997) they are ideally positioned to assess the conservation of molecular mechanisms. The bryophytes produce ethylene, via an uncertain biosynthetic pathway. Whilst 1-aminocyclopropane-1-carboxylic acid (ACC) is the ethylene precursor in angiosperms, the ethylene precursor in bryophytes is unclear (Rohwer and Bopp, 1985; Osborne *et al.*, 1995; Banks *et al.*, 2011). However, in addition to the evidence from the older literature (e.g. Stange and Osborne, 1988), there are several recent indications that ethylene is biologically relevant to bryophytes. For example, the *P. patens* genome encodes proteins resembling angiosperm ethylene signalling components (Rensing *et al.*, 2008; Ishida *et al.*, 2010). In addition, an ethylene-binding activity comparable to that of other land plants is observed in bryophytes and in charophytic (but not chlorophytic) algae (Wang *et al.*, 2006). Sequences potentially encoding ethylene signalling components have also been recognized in charophytic expressed sequence tag (EST) collections (Timme and Delwiche, 2010). This possible development of ethylene function in charophytes may have been crucial for colonization of the land.

Here we show that *P. patens* exhibits a characteristic submergence response that results in plants that are morphologically distinct from non-submerged controls. By transgenically expressing a mutant form of a putative *P. patens* ethylene receptor, we first show that *P. patens* responds to ethylene via an ethylene perception mechanism that has been substantially conserved during the evolution of land plants. Second, we show that the *P. patens* submergence response is dependent on a functional ethylene perception mechanism. Ethylene-mediated submergence responses are therefore likely to have arisen relatively early in the evolution of land plants.

RESULTS

Physcomitrella patens exhibits a characteristic submergence response

To determine how bryophytes respond to submergence, we grew the moss *P. patens* for a prolonged time under water. In air, growth of *P. patens* from small explants of filamentous tissue on an agar-based solid medium results in a radial spread of filaments consisting of a densely packed core region and a peripheral colonizing frontier (Figure 1a). Most of the core comprises photosynthetically active chloronema, whereas the peripheral filaments are faster-growing and less photosynthetically active caulonema. Both types of filament

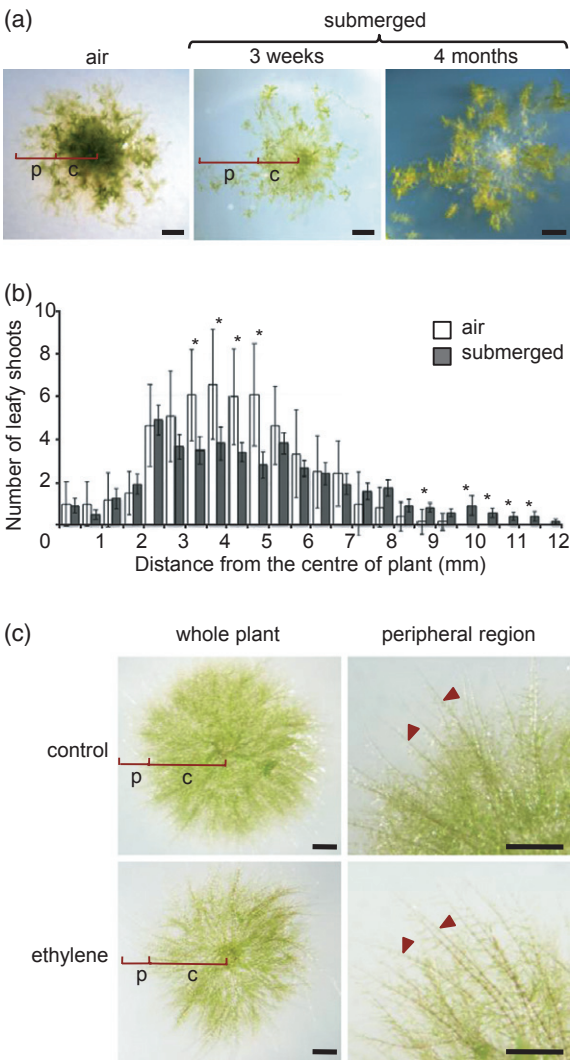

Figure 1. Responses of *Physcomitrella patens* to submergence and ethylene.
(a) *Physcomitrella patens* grown on agar-based media with no added water for 3 weeks (air) and with added water (complete submergence) for 3 weeks and 4 months. Water was added on the fifth day following inoculation. c, core region; p, peripheral zone. Scale bar, 2 mm.
(b) Leafy shoot distribution measured against distance from the centre of the plant. Plants were grown on agar-based media with no added water for 3 weeks (air), and with added water (submerged) for 3 weeks. Water was added on the fifth day following inoculation. Error bars represent standard deviation and *Data points where the differences between air-grown and submerged plants were statistically significant.
(c) Views of 14-day-old *P. patens* (whole plant and close up of peripheral region) transferred on the fifth day following inoculation to a chamber filled with air (control) or with air containing 3 p.p.m. ethylene (ethylene) and incubated for a further 10 days. c, core region; p, peripheral zone. Red arrows identify individual caulonemal filaments. Scale bar, 1 mm.

form branches, which mostly develop into chloronema but sometimes into caulonema. Gametophores (leafy shoots) develop on caulonemal filaments during the later stages of plant development. Here we show that *P. patens* survived months of complete submergence, and growth of *P. patens* under water causes a pronounced change in this filamentous structure (Figure 1a). Following submergence, growth of core filaments was reduced, resulting in a more sparsely packed core, whereas filamental extension in the periphery was enhanced. The distribution of later-stage leafy shoots reflects this change: in non-submerged plants, leafy shoots form preferentially within the core region whereas in submerged plants, leafy shoots form mainly at the periphery (Figure 1a,b). This shift in growth pattern towards colonizing activity may be an 'escape' response to submergence, a response that presumably increases the chances of finding a less flood-prone environment.

Ethylene responses and submergence responses in *P. patens* share common features

To determine whether ethylene is involved in bryophyte submergence responses, we cultured *P. patens* in an atmosphere containing ethylene. In these conditions, the core region of the plant was less densely packed with filaments than the control plants (Figure 1c). At the periphery of ethylene-treated plants, filament density was also reduced (Figure 1c). These modifications of filamentous architecture are features shared between ethylene-treated and submerged plants, suggesting that ethylene may be involved in the *P. patens* submergence response. However, there are subtle differences in appearance between submergence- and ethylene-treated plants. These differences are most likely due to the fact that submerged plants are also exposed to physiological changes such as reduced concentrations of oxygen and carbon dioxide. To investigate the role of ethylene in the submergence response we focused on the common effects of ethylene and submergence.

Bryophyte genes encode proteins homologous to angiosperm ethylene signalling components

To determine how bryophytes perceive and process the ethylene signal, we sought to identify DNA sequences encoding proteins with high amino acid sequence similarity to components of the angiosperm ethylene signalling pathway. We interrogated the fully sequenced genome of *P. patens*, EST libraries of the liverwort *Marchantia polymorpha* (K. Ishizaki and T. Kohchi (University of Kyoto, Japan), personal communications.), and genome sequences of the lycophyte, *Selaginella moellendorffii* (Banks *et al.*, 2011) (Figures 2 and S1–S4 in Supporting Information). Phylogenetic analysis of the identified sequences resolved their relationship to angiosperm homologues (Figures 2 and S1–S4).

BLAST (Altschul *et al.*, 1997) database searches identified sequences encoding seven putative ethylene receptor proteins in *P. patens* (Ishida *et al.*, 2010), two in *M. polymorpha* and four in *S. moellendorffii*. Two partial EST sequences (tentatively called Kf1 and Sp1) from charophytic algae

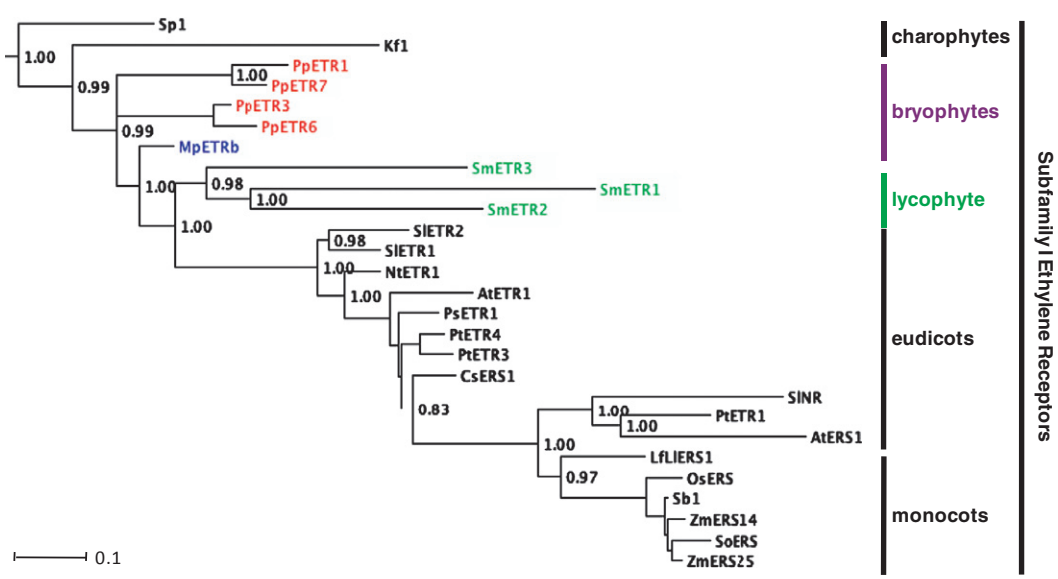

Figure 2. Phylogenetic relationships between subfamily I ethylene receptors.
Extract from a broader phylogenetic tree of ethylene receptors and putative ethylene receptors (Figure S1), showing the relationships between subfamily I ethylene receptors. Four putative ethylene receptor protein amino acid sequences from *Physcomitrella patens* (PpETR1, -3, -6 and -7; red), MpETRb from *Marchantia polymorpha* (blue) and SmETR1-3 of *Selaginella moellendorffii* (green) are included in the subfamily I group, as are two partial amino acid sequences from the charophytic algae *Klebsormidium flaccidum* (Kf1) and *Spirogyra pratensis* (Sp1), along with multiple angiosperm sequences (in eudicots and monocots). The original tree (shown in Figure S1) was constructed using Bayesian methods on aligned amino acid sequences. Numbers indicated are Bayesian posterior probability. Accession numbers and names of the species from which sequences were obtained are listed in Table S6. Scale bar, 0.1 substitutions.

(Timme and Delwiche, 2010; J. Thierer and C.F. Delwiche (University of Maryland, MD, USA), personal communications.) were also found to encode proteins with amino acid sequence similarity to angiosperm ethylene receptors (Figures 2 and S1). These non-seed plant sequences and algal sequences were included in the phylogenetic analysis to resolve their relationship with the two distinct subfamilies (I and II) of angiosperm ethylene receptors (Hua *et al.*, 1998) (Figure S1). The analysis placed four PpETR sequences, MpETRb and three SmETR sequences within subfamily I, whilst PpETR2, -4 and -5, MpETRa and SmETR4 exhibited greater sequence divergence. This diverged group of ETR sequences are more closely related to subfamily I than to subfamily II (Figure S1b).

The sequences were then analysed in greater detail to assess the presence of two differential subfamily characteristics (Hua *et al.*, 1998); namely, five well-conserved motifs within the histidine kinase domain of subfamily I receptors (Figure 3a), and an N-terminal extension of 10–20 hydrophobic amino acids found in subfamily II receptors. All of the non-seed plant ETR sequences had the well-conserved five signature motifs of subfamily I histidine kinases, and all except MpETRa lacked the N-terminal extension characteristic of subfamily II. This suggests that these sequences identify two classes of non-seed plant ethylene receptors:

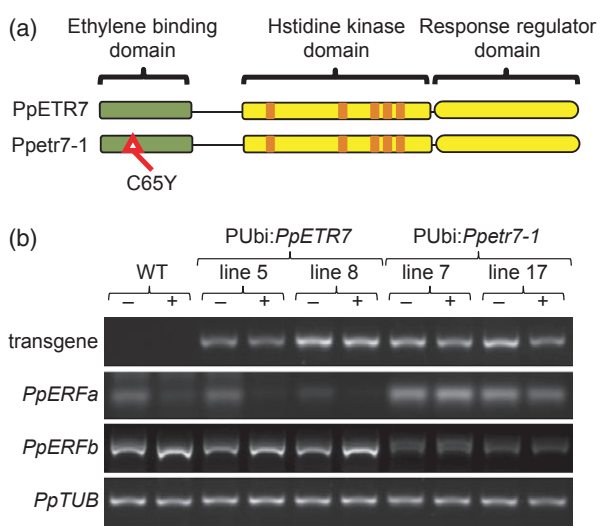

Figure 3. Expression of a mutated candidate ethylene receptor confers ethylene insensitivity on *Physcomitrella patens*.
(a) Schematic diagram of PpETR7 and Ppetr7-1 (mutant) candidate ethylene receptors. DNA sequences encoding these candidate receptors were expressed from a maize ubiquitin promoter in transgenic PUbi:*PpETR7* and PUbi:*Ppetr7-1 P. patens* lines. The triangle shows where the amino acid substitution (C65Y) was introduced into the ethylene-binding domain of the mutant candidate ethylene receptor. Orange bars indicate the location of conserved histidine kinase signature motifs.
(b) Semi-quantitative RT-PCR showing levels of transgene transcripts, of putative *PpERF* transcripts (*PpERFa* and *PpERFb*) in wild type (WT), PUbi:*PpETR7* and PUbi:*Ppetr7-1* lines, using tubulin (*PpTUB*) as a control. The RNA was harvested from 7-day-old protonema treated in an air-flow chamber with air (–) or air containing 3 p.p.m. ethylene (+) for 3 days.

one belonging to subfamily I, the other being detectably diverged from subfamily I but not having the characteristics of subfamily II. The apparent absence of genes encoding subfamily II ethylene receptors in the fully sequenced genomes of *P. patens* and *S. moellendorffii* may suggest that subfamily II was established after the divergence of the lycophytes. The C-terminal receiver domain, found in some (but not all) angiosperm ethylene receptors, was present in all of the non-seed plant sequences analysed here, except for SmETR2.

Figure 2 shows a clade of subfamily I ethylene receptors extracted from the full analysis shown in Figure S1a. The tree topology broadly relates to the overall plant phylogeny, with the algal (charophyte) sequences at a basal position, and bryophyte sequences placed basal to vascular plant sequences. Among the *P. patens* putative subfamily I ethylene receptors, PpETR7 was selected for the functional analysis described in subsequent sections of this paper. Further phylogenetic analysis provided strong evidence for the existence of genes encoding EIN3, EIL and ERF-type components (Figures S3 and S4), and less compelling evidence for the existence of genes encoding the CTR1 component of ethylene signalling in early land plants (see supplementary text in the Supporting Information legends file (Figures S2–S4) online for further explanation, and Figure S2) (Banks *et al.*, 2011). We next determined whether, despite the possible lack of CTR1 function, a candidate *P. patens* ethylene receptor (PpETR7) facilitates the response of *P. patens* to ethylene.

Mutation of the presumed ethylene binding site of PpETR7 inhibits the *P. patens* ethylene response

To determine if *PpETR* gene sequences confer the *P. patens* ethylene responses we took the following approach. The dominant *Atetr1-1* mutation confers ethylene insensitivity to AtETR1 (and hence to *Arabidopsis thaliana* plants) (Bleecker *et al.*, 1988; Schaller and Bleecker, 1995; Hall *et al.*, 1999). The causal amino acid substitution (C65Y) encoded by *Atetr1-1* lies in the ethylene-binding domain of AtETR1 and blocks ethylene binding. Since this ethylene-binding domain is highly conserved in *PpETR* gene sequences, an identical mutation could be introduced at the corresponding position of *PpETR7* to generate a *Ppetr7-1* allele (Figure 3a). *Physcomitrella patens* was subsequently transformed with gene constructs expressing the wild-type *PpETR7* and mutated *Ppetr7-1* sequences under the control of a maize ubiquitin promoter (Harwood *et al.*, 2008), which acts as a strong promoter in *P. patens* (Figure S5a). Multiple independent transgenic lines were obtained, and genomic integration and expression of transgenes was confirmed (Figure S5b,c).

We next showed that *Ppetr7-1* expression altered the accumulation of ethylene-regulated *P. patens* transcripts (Figure 3b). Wild-type (WT), *PpETR7* (PUbi:*PpETR7*) and *Ppetr7-1* (PUbi:*Ppetr7-1*) expressing lines were cultured in

an airflow system with or without ethylene (this treatment having little effect on transgene expression; Figure 3b). Two of the *PpERF* transcripts identified in Figure S4 were ethylene regulated. *PpERFa* transcript levels were repressed by ethylene, and whilst this expression pattern was commonly observed in WT and PUbi:*PpETR7* lines, it was abolished in PUbi:*Ppetr7-1* lines (Figure 3b). *PpERFb* transcripts, on the other hand, were possibly upregulated by ethylene and accumulated to much lower levels in PUbi:*Ppetr7-1* lines (Figure 3b). These effects of ethylene and *Ppetr7-1* expression suggest that ethylene regulates *PpERF* expression via PpETR7 proteins.

We next compared the effect of *PpETR7* and *Ppetr7-1* expression on the morphology of *P. patens* (Figure 4). Whilst ethylene caused PUbi:*PpETR7* plants to develop with reduced filamentous density (as described earlier for WT; Figures 1a and 4a), PUbi:*Ppetr7-1* lines failed to show such morphological changes and thus appeared to have reduced ethylene sensitivity (Figure 4a,b). Given that PUbi:*PpETR7* lines remained ethylene sensitive, it is reasonable to conclude that reduced ethylene sensitivity in PUbi:*Ppetr7-1* lines is conferred by the point mutation they contain. This point mutation alters the likely ethylene-binding domain of PpETR7 and dominantly confers reduced ethylene sensitivity. PpETR7 therefore functions as an ethylene receptor in *P. patens*.

In addition to reduced ethylene sensitivity, the *Ppetr7-1* mutation also conferred a morphological phenotype. PUbi:*Ppetr7-1* plants exhibited a significantly higher proportion of caulonemal filaments than WT or PUbi:*PpETR7* plants (Figure 4c). At the moment we are unable to suggest an explanation for this phenomenon. Furthermore, PUbi:*Ppetr7-1* caulonemal filaments were inhibited in branch development, as their branches were much shorter than those of the WT (Figure 4d–f) while cell size remained unaltered (Figure S6). This property might affect filamentous expansion of the plant as a whole, because PUbi:*Ppetr7-1* plants tended to be more compact than WT

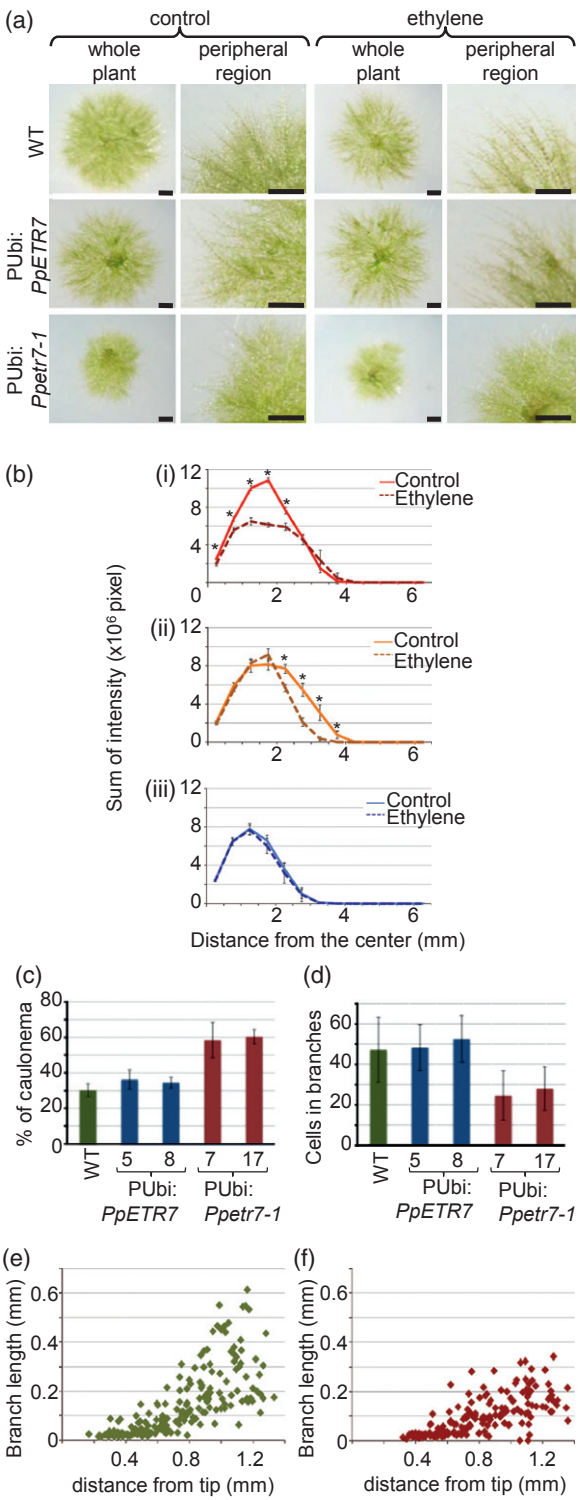

Figure 4. Effect of PUbi:*Ppetr7-1* on the ethylene response and filamentous growth of *Physcomitrella patens*.

(a) Fourteen-day-old wild type (WT), PUbi:*PpETR7* and PUbi:*Ppetr7-1* plants, previously transferred (on the fifth day following inoculation) to a chamber filled with air (control) or with air containing 3 p.p.m. ethylene (ethylene) and incubated for a further 10 days. Scale bar, 1 mm.

(b) Radial intensity was measured and the sum of intensity plotted against distance from the centre of the plant for ethylene-treated (dotted line) and control (bold line) 14 day-old WT (i), PUbi:*PpETR7* (ii) and PUbi:*Ppetr7-1* (iii) plants. $n = 7$ for each graph. The asterisk (*) indicates data points where the differences between control and ethylene-treated plants were statistically significant (bar represents standard deviation).

(c) Proportion of caulonema in the peripheral filaments of *P. patens*. For each of WT, two independent PUbi:*PpETR7* (five and eight) and PUbi:*Ppetr7-1* lines (seven and 17), over 100 peripheral filaments visible in the field of vision of a stereomicroscope were counted and classified into chloronema and caulonema to calculate the proportion of caulonema among total peripheral filaments. Eight samples were taken from three independent plants and the result was shown as the mean (bar represents standard deviation).

(d) Number of cells found in the branches formed on a peripheral caulonemal filament within 15 cells from the tip. Twenty samples were taken for each of WT, PUbi:*PpETR7* (five and eight) and PUbi:*Ppetr7-1* lines (seven and 17), and the result is shown as the mean (bars represent standard deviation).

(e, f) Length of individual chloronemal branches found on peripheral caulonemal filaments was measured for WT (e) and PUbi:*Ppetr7-1* line 7 (f) and plotted against the position of the branch from the tip of the caulonema. Branches formed within 1.3 mm of the tip of the caulonema were considered.

or PUbi:*PpETR7* plants (Figure 4a). These observations suggest that *PpETR* function may regulate the colonization ability of *P. patens*.

Treatment with 1-methylcyclopropene alters the filamentous architecture of WT and PUbi:*PpETR7* but not PUbi:*Ppetr7-1* plants

The effect of reduced ethylene signalling on the filamentous architecture of *P. patens* was confirmed when 1-methylcyclopropene (1-MCP) was used to chemically block the ethylene binding of ethylene receptors (Sisler and Serek, 1997; Figure 5). This experiment was carried out in a sealed container, in which WT plants spread out their filaments to form an elaborate colonizing apparatus at their periphery. In the presence of 1-MCP, peripheral growth of WT plants was reduced (Figure 5). Thus, treatment of WT plants with 1-MCP resulted in a phenotype resembling that resulting from expression of *Ppetr7-1*. A similar effect of 1-MCP was observed in PUbi:*PpETR7* lines (Figure 5). In contrast, PUbi:*Ppetr7-1* lines did not detectably change their filamentous architecture when treated with 1-MCP (Figure 5). Possibly, the expression of *Ppetr7-1* masks the effect of 1-MCP on the endogenous ethylene receptors of *P. patens* by saturating the inhibitory effect. Importantly, blocking the binding of ethylene to the receptor, by molecular or chemical means, results in altered filamentous morphology in *P. patens*, confirming the role of ethylene receptors in regulating the filamentous architecture of *P. patens*.

Impaired ethylene signalling perturbs the bryophyte submergence response

We next determined if the impaired ethylene signalling conferred by *Ppetr7-1* is associated with an alteration in the submergence response of *P. patens*. Wild type and transgenic *P. patens* were grown under water (Figure 6a). As previously described (Figure 1a), WT and PUbi:*PpETR7* plants responded to submergence by focusing their growth at the peripheral part of the plant (Figure 6a). In contrast, the PUbi:*Ppetr7-1* plant continued, following submergence, to form densely packed filaments in the core part of the plant. These differences became particularly clear once leafy shoots had appeared (Figure 6b). Fully expanded leafy shoots were found in the peripheral regions of the submerged WT and PUbi:*PpETR7* plants and not in the centre, whilst in non-submerged controls leafy shoots formed at the centre and smaller leafy shoots were found at the periphery (Figure 6b). In contrast, both the submerged and non-submerged PUbi:*Ppetr7-1* plants formed leafy shoots throughout the plant structure (leafy shoots remained small in the submerged PUbi:*Ppetr7-1*). Quantification of the radial growth of plants also supported the reduced effect of submergence stress on PUbi:*Ppetr7-1* plants (Figure 6c). Thus the normal submergence-induced change in filamentous architecture exhibited by WT *P. patens*, a response which may be considered to be an 'escape' strategy, is blocked by the reduced ethylene sensitivity conferred by *Ppetr7-1*. We conclude that ethylene is involved in the regulation of the submergence response in *P. patens*.

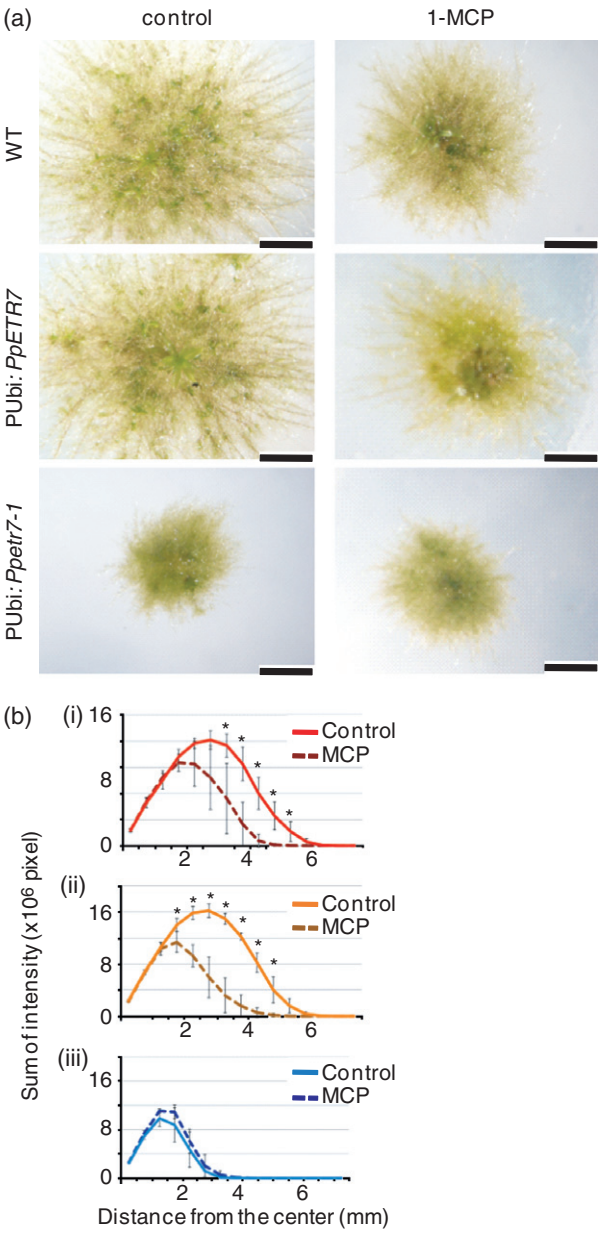

Figure 5. Effect of the ethylene-binding inhibitor 1-methylcyclopropene (1-MCP) on the morphology of wild type (WT), PUbi:*PpETR7* and PUbi:*Ppetr7-1* *Physcomitrella patens*.
(a) Fourteen-day-old WT, PUbi:*PpETR7* and PUbi:*Ppetr7-1* plants, previously transferred (on the fifth day following inoculation) to a sealed desiccator filled with air (control) or with air containing 1-MCP and incubated for a further 10 days. Scale bar, 2 mm.
(b) Radial intensity was measured and the sum of intensity plotted against distance from the centre of the plant for 1-MCP-treated (dotted line) and control (bold line) 14-day-old WT (i), PUbi:*PpETR7* (ii) and PUbi:*Ppetr7-1* (iii) plants. $n = 4$ for each graph. The asterisk (*) indicates data points where the differences between control and 1-MCP-treated plants were statistically significant (bar represents standard deviation).

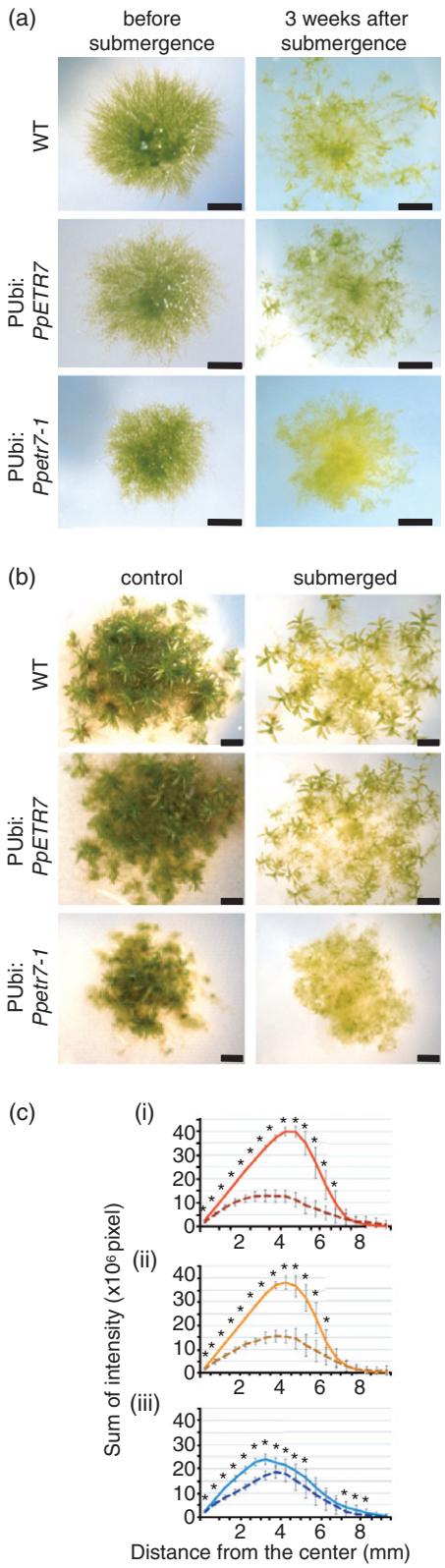

Figure 6. Effect of submergence on growth and transcript abundances in wild type (WT), PUbi:*PpETR7* and PUbi:*Ppetr7-1*.
(a) Seven-day-old *Physcomitrella patens* plants (before submergence) were grown under water for three additional weeks (3 weeks after submergence). Scale bar, 2 mm.
(b) Eight-week-old plants grown on agar medium (control), compared with plants grown on agar, then submerged on the eighth day following inoculation and incubated for a further 7 weeks (submerged). Scale bar, 2 mm.
(c) Radial intensity was measured and the sum of intensity plotted against distance from the centre of the plant for each of submergence-treated (dotted line) and control (bold line) 28-day-old plants. Submergence-treated plants were submerged on the eighth day after inoculation. Graphs for WT plants are shown in (i), PUbi:*PpETR7* line 8 in (ii), PUbi:*Ppetr7-1* line 7 in (iii). $n = 16$ for each graph. The asterisk (*) indicates data points where the differences between air-grown and submerged plants were statistically significant (bars represent standard deviation).

We next investigated the possibility that submergence stimulates ethylene-mediated changes in the regulation of internal water status. Although vascular plants have structural adaptations such as roots and stomata for the regulation of internal water status, bryophytes lack such features in their vegetative gametophytic tissues. Instead, *P. patens* is thought to regulate internal water status mostly at the cellular level. In plants, movement of cellular water across membranes is regulated by proteins known as aquaporins (Hachez *et al.*, 2006; Lienard *et al.*, 2008; Maurel *et al.*, 2008). Lienard *et al.* (2008) characterized three *PIP2* genes in *P. patens*, which encode the plasma membrane intrinsic protein (PIP) group of aquaporins, and reported the role of *PpPIP2;1* and *PpPIP2;2* (but not *PpPIP2;3*) in the regulation of water permeability of the cell membrane through mutant analysis. They also showed that *PpPIP2;1* and *PpPIP2;2* are involved in the desiccation tolerance of leafy shoots. Since it is known that aquaporin activity in vascular plants can be regulated at the level of transcript accumulation (as well as at the protein level) (Hachez *et al.*, 2006), we tested *PpPIP2* transcript levels in WT and transgenic plants with or without submergence stress. We found that *PpPIP2;2* was upregulated in submerged plants of WT and PUbi:*PpETR7* lines, but that *PpPIP2;2* transcripts were not detectable in PUbi:*Ppetr 7-1* lines (Figure 7a). In contrast, *PpPIP2;3* was negatively regulated by submergence in WT and PUbi:*PpETR7* lines, whilst increased levels of *PpPIP2;3* transcript were observed in both submerged and non-submerged PUbi:*Ppetr7-1* lines (Figure 7a). Notably, this effect of submergence on *PpPIP2;2* and *PpPIP2;3* was comparable with that of ethylene (Figure 7b). Expression of *PpPIP2;2* and *PpPIP2;3* is therefore regulated by both ethylene and submergence, and this regulation is perturbed in PUbi:*Ppetr7-1* lines. These observations suggest that, upon submergence, ethylene regulates cellular water content through aquaporins, and that this effect is blocked in *Ppetr7-1* lines with reduced ethylene sensitivity.

On the other hand, the level of some transcripts appeared to be regulated by submergence stress but not by ethylene. For example, whilst transcripts encoding the basic helix–loop–helix transcription factor PpRSL4 (Menand *et al.*, 2007) accumulated to a higher level in submerged than in control

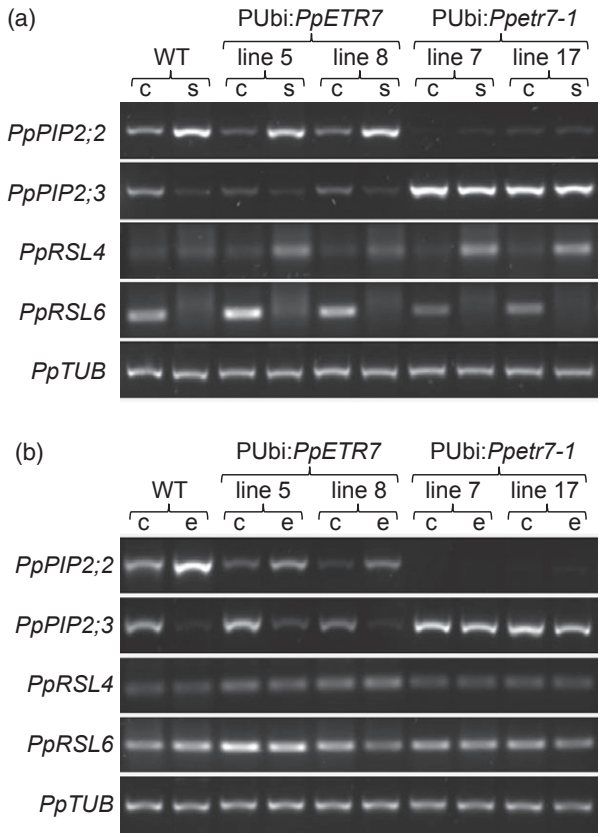

Figure 7. The effects of submergence and ethylene on transcript accumulation.
Semi-quantitative RT-PCR analysis of levels of aquaporin-encoding (*PpPIP2;2* and *PpPIP2;3*) transcripts and transcripts encoding RSL proteins (*PpRSL4* and *PpRSL6*) using tubulin (*PpTUB*) as a control in wild-type (WT), PUbi:*PpETR7* and PUbi:*Ppetr7-1* lines.
(a) The RNA was harvested from 8-day-old protonema, which were grown on agar medium for 5 days and then treated (s) or untreated (c) with submergence stress for 3 days.
(b) The RNA was harvested from 7-day-old protonema treated in an air-flow chamber with air (c) or air containing 3 p.p.m. ethylene (e) for 3 days.

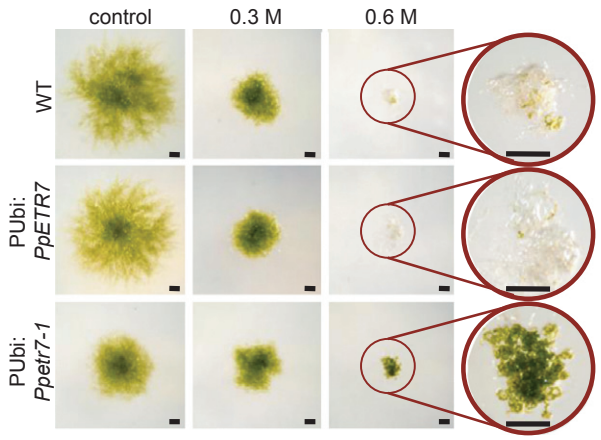

Figure 8. PUbi:*Ppetr7-1* confers increased tolerance of osmotic stress.
Ten-day-old wild-type (WT), PUbi:*PpETR7* and PUbi:*Ppetr7-1* lines grown on media with no added mannitol (control), 0.3 and 0.6 M mannitol. Scale bar, 0.5 mm.

plants, levels of transcripts encoding PpRSL4 remained unaltered by ethylene treatment or by expression of the *Ppetr7-1* transgene (Figure 7). Similarly, the level of transcripts encoding the related PpRSL6 protein (Menand *et al.*, 2007) is reduced by submergence treatment and unaffected by ethylene (Figure 7). These observations suggest that whilst ethylene is involved in the submergence response, some aspects of the submergence response are regulated independently of ethylene.

Bryophyte ethylene signalling mediates diverse water-stress responses

Since aquaporins contribute to the maintenance of water status in water deficit and osmotic stress (Lienard *et al.*, 2008; Maurel *et al.*, 2008), we tested the osmotic stress tolerance of WT, PUbi:*PpETR7* and PUbi:*Ppetr7-1 P. patens*, using various concentrations of mannitol. Mannitol caused a reduction of growth in all lines (Figure 8). However, the PUbi:*Ppetr7-1* lines displayed increased resistance to osmotic stress at the cellular level. PUbi:*Ppetr7-1* cells survived 0.6 M and higher concentrations of mannitol, concentrations at which WT and PUbi:*PpETR7* cells exhibited almost complete senescence (Figures 8 and S7). Thus reduced ethylene signalling alters the response to osmotic stress, suggesting that ethylene is involved in normal regulation of water status.

Abscisic acid is another phytohormone known to increase the tolerance of bryophytes to desiccation and osmotic stress (Mayaba *et al.*, 2001; Cuming *et al.*, 2007; Khandelwal *et al.*, 2010). To test whether ABA sensitivity was altered in the PUbi:*Ppetr7-1* lines, WT and transgenic lines were treated with 0 or 10 μM ABA and RNA was extracted for RT-PCR analysis (Figure 9). These studies revealed that the levels of *PpPYR1* transcripts (encoding the putative *P. patens* ABA receptor; Umezawa *et al.*, 2010; Chater *et al.*, 2011) were indistinguishable in WT and transgenic lines. We found that regulation of *PpABIA* transcripts (which encodes a type 2C protein phosphatase; Komatsu *et al.*, 2009) in response to ABA was unaffected by the expression of *PpETR7* or *Ppetr7-1* (Figure 9). Similarly, ABA-inducible expression of *PpLEA1* transcripts (Kamisugi and Cuming, 2005) was observed in all the lines tested (though the extent of induction is considerably lower in PUbi:*PpETR7* lines, Figure 9). Thus, the effect of reduced ethylene-sensitivity on cellular osmotic tolerance does not seem to be due to effects on ABA signalling or response, suggesting that ethylene and ABA may have independent and distinctive roles in the regulation of the response of *P. patens* to water-deficit stress.

As for the regulation of cellular water content, a tonoplast-localized aquaporin has been shown to be inducible by ABA (Cuming *et al.*, 2007). In our experiments the level of

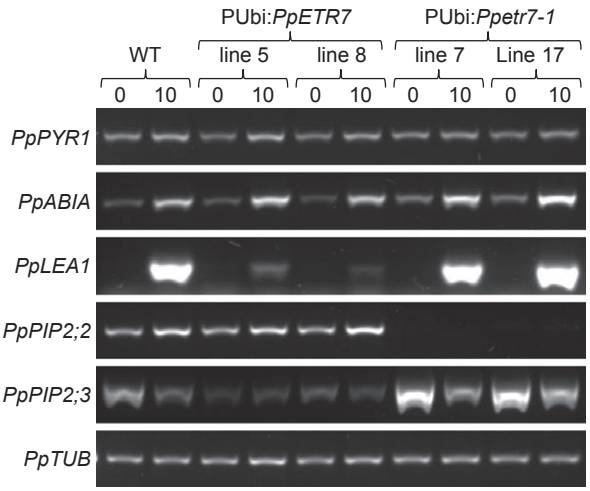

Figure 9. Effect of ABA on ABA-responsive gene expression and aquaporin transcript levels in wild type (WT), PUbi:*PpETR7* and PUbi:*Ppetr7-1*.
Semi-quantitative RT-PCR showing levels of transcripts encoding ABA-signalling components (putative *PpPYR1* and *PpABI1A*), of ABA-inducible *PpLEA1* transcripts and of transcripts encoding aquaporins (*PpPIP2;2* and *PpPIP2;3*), using tubulin as a control. The RNA was harvested from 8-day-old protonema which were transferred on the fifth day to media containing no added ABA (0) or media containing 10 μM ABA (10) and incubated for a further 3 days.

transcripts encoding plasma membrane-localized PpPIP2;3 was down-regulated by ABA in both WT and PUbi:*Ppetr7-1* plants (Figure 9). This observation further supports the conclusion that the reduced ethylene sensitivity conferred by expression of *Ppetr7-1* does not alter the ABA response. Thus, both ABA and ethylene influence the *PpPIP2;3* transcript level, suggesting some overlap in the downstream targets of these two signalling pathways.

DISCUSSION

Although the bryophytes have long been known to produce ethylene (Rohwer and Bopp, 1985; Osborne *et al.*, 1995), the physiological functions of ethylene in bryophyte biology remained poorly understood. Here we show that ethylene regulates protonemal growth in *P. patens*, thus modulating filamentous architecture, and that *P. patens* perceives ethylene using PpETR7, a homologue of angiosperm ethylene receptors. This conclusion is supported by showing that the ethylene-insensitivity mutation *etr7-1* blocks ethylene responses in *P. patens*, and also by a recent report that PpETR1c binds ethylene (Ishida *et al.*, 2010). We also show that expression of the *Ppetr7-1* allele alters the ethylene-regulated expression of *PpERF*s. In Arabidopsis, *AtERF1* and other family members regulate ethylene-responsive genes and are themselves regulated by ethylene through the activity of transcription factors such as EIN3 (Solano *et al.*, 1998; Fujimoto *et al.*, 2000). Database searches (see above) revealed that EIN3 is well conserved in bryophytes. Thus, *P. patens* perceives ethylene via ETR receptors, with resultant regulation of *PpERF* expression. Future studies will determine the extent to which signalling downstream of the *P. patens* ethylene receptor is mediated by proteins related to the angiosperm CTR1, EIN3 and ERF proteins.

We next demonstrate the involvement of ethylene in submergence responses in *P. patens*. We show that ethylene has effects on filamentous architecture and on the expression of aquaporin-encoding *PpPIP2;2* and *PpPIP2;3* genes in common with the effect of submergence. We also show that the impaired ethylene perception conferred by *Ppetr7-1* inhibits submergence responses. However, whilst the PUbi:*Ppetr7-1* lines failed to produce morphological changes associated with the submergence stress, they survived prolonged submergence similarly to the WT and the PUbi:*PpETR7* lines. Additional changes in the WT in response to submergence, such as reduced pigmentation, were also observed in the *PUbi:Ppetr7-1* lines. We conclude that the submergence response in *P. patens* is regulated in part by ethylene. Since the bryophytes are the most distantly related land plants to angiosperms, the link between ethylene and submergence may exist widely among the land plants (see also Musgrave and Walters, 1974; Stange and Osborne, 1988). Thus the regulation of submergence responses by ethylene may be an ancient mechanism that was first established in the land-plant ancestor, and subsequently conserved amongst the land plants.

Physcomitrella patens may also rely on ethylene perception for detecting general water-related stress. Whilst *Ppetr7-1* confers altered submergence responses, it also increases cellular osmotic tolerance. It is possible that external water levels are detected via ethylene signalling, such that increased signalling is required for the submergence response and reduced signalling is associated with water deficit stress. Whilst ABA is known to improve tolerance against drought and osmotic stress in *P. patens* (Cuming *et al.*, 2007; Khandelwal *et al.*, 2010), expression of *Ppetr7-1* did not result in altered ABA responses, as shown with ABA-regulated expression of *PpABIA* and *PpLEA1* genes. In angiosperms, ethylene mediates water deficit responses, often through interaction with ABA. For example, ethylene production is repressed by ABA as part of the drought response (Hussain *et al.*, 2000; Spollen *et al.*, 2000), and ABA and ethylene interact antagonistically in *Leontodon hispidus* to regulate the plant's sensitivity to water deficit stress (Wilkinson and Davies, 2010). Fukao *et al.* (2011) demonstrated in rice that an ethylene-inducible ERF protein SUB1A, which, as previously described, confers submergence tolerance, also enhanced tolerance against drought by increasing ABA sensitivity. Given such variation, the interaction between ethylene and ABA may be adaptable and species-specific. In this study, ethylene-insensitivity improved cellular osmotic tolerance of *P. patens* without altering tested ABA responses, suggesting that ethylene may play a role in osmotic tolerance in *P. patens* independently of ABA.

However, since both ethylene and ABA regulate water relations in *P. patens*, the precise relationship between these two regulators awaits further study.

Survival of water stress, including submergence and water deficit, will have been a fundamental adaptation required upon land colonization. Since ethylene appears to have regulated the water stress response in ancestral land plants, ethylene signalling could have been one of the mechanisms that enabled plants to colonize the land (Timme and Delwiche, 2010). Plant ethylene receptors have their origins in plastids (Mount and Chang, 2002), but ethylene-binding activity was found only in land plants and charophytes, and not in other algae (Wang *et al.*, 2006). Thus, the biological function of ethylene in plants might have originally evolved in the charophytic algal ancestor of the land plants. The presence of ethylene signalling in charophytes is supported by the finding of EST sequences encoding ethylene signalling components and biosynthesis enzymes in two charophycean species (Timme and Delwiche, 2010). Since the land plants are thought to have evolved from a charophytic algal lineage, recruitment of ethylene to the regulation of the water stress response could have been a pre-requisite for colonization of the land. Subsequently, the adaptability of the ethylene signalling pathway enabled the evolution of strategies to cope with fluctuating water availability, ranging from desiccation to submergence, and other abiotic stresses during the evolution of the land plants.

Diverse adaptations and the physiological and developmental innovations that the land plants evolved are often coordinated with plant growth through the activity of phytohormones. Recent studies are beginning to unravel the degree of evolutionary conservation and modification found in the molecular mechanisms by which phytohormones regulate plant growth. Previous work suggested that GA, a key growth regulator of angiosperms, was not involved in growth regulation in early land plants and evolved relatively recently in the vascular plant lineage (Hirano *et al.*, 2007; Yasumura *et al.*, 2007). On the other hand, Prigge *et al.* (2010) showed that auxin regulates developmental processes in *P. patens*, and suggested that the basic auxin perception mechanism was present in the ancestor of land plants. Our present study suggests that the ethylene perception mechanism was also present in that ancestor. These ancient hormonal regulatory systems (auxin and ethylene) have their core functional perception mechanisms well conserved, whilst downstream events are often co-opted to regulate the structures or developmental programs specific to bryophyte or vascular plant lineages.

EXPERIMENTAL PROCEDURES

Plant strains and growth conditions

Physcomitrella patens subsp. *patens* (Gransden2004 strain) was provided by Yasuko Kamisugi (University of Leeds, UK). Cultures were grown at 25°C with continuous light (60 μmol m^{-2} sec^{-1}) on BCD medium (Grimsley *et al.*, 1977; without ammonium tartrate supplement), with or without supplements/submergence treatment as indicated. Plants were grown from small explants of filamentous tissue (1 mm diameter) for morphological observation. Tissues for DNA/RNA extraction were grown from fragmented protonema.

Ethylene and 1-MCP treatment

Ethylene was applied at 3 μl L^{-1} with continuous airflow (1 L min^{-1}) in cuvettes (24 L). Ethylene concentrations in the cuvette were verified by analysing air samples with a gas chromatograph with a photo-ionization detector (Syntech Spectras Analyzer GC955-100; Synspec, http://www.synspec.nl/). To treat plants with 1-MCP, 5 μl L^{-1} 1-MCP (SmartFresh, Rohm and Haas Trading Europe, http://www.rohmhaas.com/) was added in a closed glass container. The growth chamber was kept at 21°C with continuous light of 60 μmol m^{-2} sec^{-1}.

Leafy shoot measurement

The distance of individual leafy shoots from the centre of the plant was measured on plant pictures with the program ImageJ (http://rsbweb.nih.gov/ij/). Student's *t*-test was applied for the statistical test.

Quantification of filamentous growth

The blue channel of the red–green–blue image was used to segment the plant structure as it provides a high-contrast image that is dependent on chlorophyll absorption as a measure of tissue density. A shading correction was used to compensate for uneven illumination across the field and the image inverted to provide a positive estimate of tissue density. Plant structure was accentuated using contrast-limited histogram equalisation (CLAHE; Zuiderveld, 1994) and automatically segmented using an intensity threshold that minimizes interclass variance between the object and background (Otsu, 1979). The intensity-weighted centroid was used to determine the centre of the plant. The integrated intensity and pixel count determined in expanding 500-μm rings following background subtraction of the original intensity image or the segmented image, respectively, provided a profile of the tissue density and surface coverage with radius (Figure S8). Student's *t*-test was applied for the statistical test.

Gene identification and isolation

Physcomitrella patens and *S. moellendorffii ETR1-*, *CTR1-*, *EIN3-* and *ERF*-related sequences were obtained by a BLAST search of the *Physcomitrella* genome website (http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html), NCBI (http://www.ncbi.nlm.nih.gov/) and *Selaginella* Genomics (http://selaginella.genomics.purdue.edu/) databases. *Marchantia polymorpha* sequences were obtained from EST collections held by the Kohchi laboratory (University of Kyoto, Japan). A *MpETRa* EST fragment was extended to obtain the remaining coding sequences by rapid amplification of cDNA ends or by thermal-asymmetric-interlaced PCR (see Table S1 for primer sequences). Sequences for *Spirogyra pratensis* and *Coleochaete orbicularis* were obtained from a database provided by Ruth Timme (Timme and Delwiche, 2010) and those for *Klebsormidium flaccidum* were obtained from EST collections held by the Delwiche laboratory (University of Maryland, College Park, MD, USA).

Phylogenetic analysis

Amino acid sequence alignments were generated using the programs JALVIEW (http://www.jalview.org/) and BIOEDIT (http://

www.mbio.ncsu.edu/bioedit/bioedit.html). Automatically generated alignments using the MAFFT Multiple Sequence Alignment tool (Katoh *et al.*, 2002) were manually adjusted to refine the alignment and remove the regions containing unalignable sequences (see Tables S2–S5 for unedited versions of sequence alignments). The sequence alignments were used to infer Bayesian trees using the program MRBAYES v.3.1.2 (http://mrbayes.csit.fsu.edu/index.php), using the WAG substitution matrix. For more information see Methods S1.

Transformation of *P. patens*

Wild-type *PpETR7* coding sequences were PCR-amplified with *Pfu* turbo polymerase (http://www.genomics.agilent.com/), and cloned into pGEM® T easy (Promega, http://www.promega.com/). This whole plasmid was PCR-amplified using the primers Ppetr7.3F and Ppetr1-1R (Table S1), which resulted in integration of the *Ppetr7-1* point mutation within the *PpETR7* sequence. The PCR product was then self-ligated to generate *Ppetr7-1* in pGEM® T easy. Both *PpETR7* and *Ppetr7-1* sequences were then cloned into pBRACT211 (http://www.bract.org/bract.html) downstream of a maize ubiquitin promoter (PUbi; Figure S5a). About 10 μg of the plasmid containing the construct PUbi:*PpETR7* or PUbi:*Ppetr7-1*, linearized using *Not*I and *Hae*II, was used for each transformation experiment. *Physcomitrella patens* was transformed after polyethylene glycol-mediated DNA uptake into protoplasts, regenerated and screened for stable transformants essentially according to Schaefer *et al.* (1991). Transformants were screened by PCR to check for construct integration, followed by DNA gel blot analysis to determine insert copy number (Figure S5b).

DNA and RNA analysis

The DNA and RNA were extracted using a guanidine-based buffer essentially according to Langdale *et al.* (1988). The detailed protocol is available on http://dps.plants.ox.ac.uk/langdalelab/protocols/RNA/RNA.html. Gel blots (Figure S5b,c) were prepared and hybridized as described by Langdale *et al.* (1988) using a 466-bp fragment of *PpETR7* corresponding to positions 1648–2113 of accession number DS545065.1 sequence as a probe.

Semi-quantitative RT-PCR

Complementary DNA was generated using Superscript II reverse transcriptase (Life Technologies, http://www.lifetechnologies.com/) from 4 μg of DNaseI-treated (amplification grade; Life Technologies) total RNA with oligo dT-Anchor (ODTA; see Table S1). Taq polymerase (Life Technologies) was used for PCR, according to the manufacturer's instructions. Primers used are listed in Table S1. The PCR conditions were: 94°C for 120 sec, 17–28 cycles of 94°C for 30 sec, 54–57°C for 30 sec and 72°C for 70 sec, and then 72°C for 10 min. The PCR products were visualized using SYBR Green (Life Technologies), and the cycle number was adjusted to obtain data during the log phase of PCR amplification (confirmed by checking the amplification status at three different cycle numbers; ±2 cycles of the cycle at which the data were taken; Figure S9). Experiments were repeated using two biological replicates.

Accession numbers

Sequence data can be found in GenBank under the accession numbers listed in Tables S6–S9 and under the following accession numbers: *PpPIP2;2*, AY494192.1; *PpPIP2;3*, DQ018113.1; *PpTUB*, AB096719.1; *PpRSL4*, EF156396; *PpRSL6*, EF156398; putative *PpPYR1*, XP_001762113.1; *PpABI1A*, AB369256.1; *PpLEA1*, AW145397.

ACKNOWLEDGEMENTS

We thank Yasuko Kamisugi (University of Leeds, UK) for *P. patens*; Rob Welschen (Utrecht University, Netherlands) and John Baker (Oxford, UK) for technical assistance; Kimitsune Ishizaki and Takayuki Kohchi (Kyoto, Japan), and Katsuyuki Yamato (Kinki University, Japan) for help with BLAST search and sharing of *Marchantia* EST data; Ruth Timmes, Jay Thierer and Charles Delwiche (University of Maryland, USA) for help with BLAST search and sharing of charophytic EST data; Mark Smedley (Norwich, UK) for plasmid pBract211, Nuno D. Pires (Zurich, Switzerland) for *PpRSL* gene primers and Jeremy Solly (Cambridge, UK) for ABA signalling gene primers. This work was supported in part by award no. KUK-I1-002-03 (to NPH) made by King Abdullah University of Science and Technology (KAUST), and by British Council Partnership Programme in Science made by the British Council and Platform Bèta Techniek.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Phylogenetic analysis of candidate ethylene receptors from land plants and charophytic algae.

Figure S2. Phylogenetic analysis of CTR1 proteins and related kinases from land plants and charophytic algae.

Figure S3. Phylogenetic analysis of EIN3 and EIN3-like proteins from land plants and charophytic algae.

Figure S4. Phylogenetic analysis of Arabidopsis group VII-IX ERF proteins and *Physcomitrella patens* ERF-like proteins.

Figure S5. PUbi:*PpETR7* and PUbi:*Ppetr7-1* transgenes were integrated in the genome and expressed in PUbi:*PpETR7* and PUbi:*Ppetr7-1* lines.

Figure S6. Filamentous cell size of wild-type, PUbi:*PpETR7* and PUbi:*Ppetr7-1* lines.

Figure S7. Increased osmotic stress tolerance of PUbi:*Ppetr7-1* cells.

Figure S8. Examples of *Physcomitrella patens* plant pictured with radial sectors.

Figure S9. Full result of semi-quantitative RT-PCR analysis with amplification status at ± two cycles.

Table S1. Sequences of oligos used in this work.

Table S2. Unedited sequence alignment of ethylene receptor family proteins.

Table S3. Unedited sequence alignment of CTR1 proteins and related kinases.

Table S4. Unedited sequence alignment of EIN3 and EIN3-like proteins.

Table S5. Unedited sequence alignment of Arabidopsis group VII-IX ERF proteins and *Physcomitrella patens* ERF-like proteins.

Table S6. Accession numbers and descriptions of the sequences used in the phylogenetic analysis of ethylene receptor family shown in Figures 2 and S1.

Table S7. Accession numbers and descriptions of the sequences used in the phylogenetic analysis of CTR1 proteins and related kinases shown in Figure S2.

Table S8. Accession numbers and descriptions of the sequences used in the phylogenetic analysis of EIN3 and EIN3-like proteins shown in Figure S3.

Table S9. Accession numbers and descriptions of the sequences used in the phylogenetic analysis of Arabidopsis group VII-IX ERF proteins and *Physcomitrella patens* ERF-like proteins shown in Figure S4.

Table S10. Sequence alignment generated for the phylogenetic analysis of ethylene receptor family shown in Figures 2 and S1.

Table S11. Sequence alignment generated for the phylogenetic analysis of CTR1 proteins and related kinases shown in Figure S2.
Table S12. Sequence alignment generated for the phylogenetic analysis of EIN3 and EIN3-like proteins shown in Figure S3.
Table S13. Sequence alignment generated for the phylogenetic analysis of Arabidopsis group VII-IX ERF proteins and *Physcomitrella patens* ERF-like proteins shown in Figure S4.
Methods S1. Experimental procedures (phylogenetic ananlysis).
Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.

Bailey-Serres, J. and Voesenek, L.A.C.J. (2008) Flooding stress: Acclimations and genetic diversity. *Annu. Rev. Plant Biol.* **59**, 313–339.

Banks, J.A., Nishiyama, T., Hasebe, M. *et al.* (2011) The Selaginella genome identifies genetic changes associated with the evolution of vascular plants. *Science*, **332**, 960–963.

Bleecker, A.B., Estelle, M.A., Somerville, C. and Kende, H. (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science*, **241**, 1086–1089.

Chater, C., Kamisugi, Y., Movahedi, M., Fleming, A., Cuming, A.C., Gray, J.E. and Beerling, D.J. (2011) Regulatory mechanism controlling stomatal behavior conserved across 400 million years of land plant evolution. *Curr. Biol.* **21**, 1025–1029.

Cuming, A.C., Cho, S.H., Kamisugi, Y., Graham, H. and Quatrano, R.S. (2007) Microarray analysis of transcriptional responses to abscisic acid and osmotic, salt, and drought stress in the moss, *Physcomitrella patens*. *New Phytol.* **176**, 275–287.

English, P.J., Lycett, G.W., Roberts, J.A. and Jackson, M.B. (1995) Increased 1-aminocyclopropane-1-carboxylic acid oxidase activity in shoots of flooded tomato plants raises ethylene production to physiologically active levels. *Plant Physiol.* **109**, 1435–1440.

Finet, C., Timme, R.E., Delwiche, C.F. and Marlétaz, F. (2010) Multigene phylogeny of the green lineage reveals the origin and diversification of land plants. *Curr. Biol.* **20**, 2217–2222.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell*, **12**, 393–404.

Fukao, T. and Bailey-Serres, J. (2008) Submergence tolerance conferred by *Sub1A* is mediated by SLR1 and SLRL1 restriction of gibberellin responses in rice. *Proc. Natl. Acad. Sci. USA*, **105**, 16814–16819.

Fukao, T., Xu, K., Ronald, P.C. and Bailey-Serres, J. (2006) A variable cluster of ethylene response factor-like genes regulates metabolic and developmental acclimation responses to submergence in rice. *Plant Cell*, **18**, 2021–2034.

Fukao, T., Yeung, E. and Bailey-Serres, J. (2011) The submergence tolerance regulator SUB1A mediates crosstalk between submergence and drought tolerance in rice. *Plant Cell*, **23**, 412–427.

Grimsley, N.H., Ashton, N.W. and Cove, D.J. (1977) Production of somatic hybrids by protoplast fusion in moss, *Physcomitrella-Patens*. *Mol. Gen. Genet.* **154**, 97–100.

Hachez, C., Zelazny, E. and Chaumont, F. (2006) Modulating the expression of aquaporin genes in planta: a key to understand their physiological functions? *Biochim. Biophys. Acta*, **1758**, 1142–1156.

Hall, A.E., Chen, Q.G., Findell, J.L., Schaller, G.E. and Bleecker, A.B. (1999) The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the ETR1 ethylene receptor. *Plant Physiol.* **121**, 291–300.

Harwood, W.A., Bartlett, J.G., Alves, S.C., Smedley, M. and Snape, J.W. (2008) High-throughput *Agrobacterium*-mediated barley transformation. *Plant Methods*, **4**, 22.

Hattori, Y., Nagai, K., Furukawa, S. *et al.* (2009) The ethylene response factors *SNORKEL1* and *SNORKEL2* allow rice to adapt to deep water. *Nature*, **460**, 1026–1030.

Hirano, K., Nakajima, M., Asano, K. *et al.* (2007) The GID1-mediated gibberellin perception mechanism is conserved in the Lycophyte *Selaginella moellendorffii* but not in the Bryophyte *Physcomitrella patens*. *Plant Cell*, **19**, 3058–3079.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.H.G., Bleecker, A.B., Ecker, J.R. and Meyerowitz, E.M. (1998) *EIN4* and *ERS2* are members of the putative ethylene receptor gene family in Arabidopsis. *Plant Cell*, **10**, 1321–1332.

Hussain, A., Black, C.R., Taylor, I.B. and Roberts, J.A. (2000) Does an antagonistic relationship between ABA and ethylene mediate shoot growth when tomato (*Lycopersicon esculentum* Mill.) plants encounter compacted soil? *Plant, Cell Environ.* **23**, 1217–1226.

Ishida, K., Yamashino, T., Nakanishi, H. and Mizuno, T. (2010) Classification of the genes involved in the two-component system of the moss *Physcomitrella patens*. *Biosci. Biotechnol. Biochem.* **74**, 2542–2545.

Jackson, M.B. (1985) Ethylene and responses of plants to soil waterlogging and submergence. *Annu. Rev. Plant Physiol.* **36**, 145–174.

Jackson, M.B. (2008) Ethylene-promoted elongation: an adaptation to submergence stress. *Ann. Bot.* **101**, 229–248.

Kamisugi, Y. and Cuming, A.C. (2005) The evolution of the abscisic acid-response in land plants: comparative analysis of group 1 LEA gene expression in moss and cereals. *Plant Mol. Biol.* **59**, 723–737.

Katoh, K., Misawa, K., Kuma, K. and Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **30**, 3059–3066.

Kenrick, P. and Crane, P.R. (1997) The origin and early evolution of plants on land. *Nature*, **389**, 33–39.

Khandelwal, A., Cho, S.H., Marella, H., Sakata, Y., Perroud, P.F., Pan, A. and Quatrano, R.S. (2010) Role of ABA and ABI3 in desiccation tolerance. *Science*, **327**, 546.

Komatsu, K., Nishikawa, Y., Ohtsuka, T., Taji, T., Quatrano, R.S., Tanaka, S. and Sakata, Y. (2009) Functional analyses of the ABI1-related protein phosphatase type 2C reveal evolutionarily conserved regulation of abscisic acid signalling between Arabidopsis and the moss *Physcomitrella patens*. *Plant Mol. Biol.* **70**, 327–340.

Ku, H.S., Suge, H., Rappaport, L. and Pratt, H.K. (1970) Stimulation of rice coleoptile growth by ethylene. *Planta*, **90**, 333–339.

Langdale, J.A., Rothermel, B.A. and Nelson, T. (1988) Cellular-Pattern of Photosynthetic Gene-Expression in Developing Maize Leaves. *Gene Dev.* **2**, 106–115.

Lienard, D., Durambur, G., Kiefer-Meyer, M.C., Nogue, F., Menu-Bouaouiche, L., Charlot, F., Gomord, V. and Lassalles, J.P. (2008) Water transport by aquaporins in the extant plant *Physcomitrella patens*. *Plant Physiol.* **146**, 1207–1218.

Maurel, C., Verdoucq, L., Luu, D.T. and Santoni, V. (2008) Plant aquaporins: membrane channels with multiple integrated functions. *Annu. Rev. Plant Biol.* **59**, 595–624.

Mayaba, N., Beckett, R.P., Csintalan, Z. and Tuba, Z. (2001) ABA increases the desiccation tolerance of photosynthesis in the afromontane understorey moss *Atrichum androgynum*. *Ann. Bot.* **88**, 1093–1100.

Menand, B., Yi, K., Jouannic, S., Hoffmann, L., Ryan, E., Linstead, P., Schaefer, D.G. and Dolan, L. (2007) An ancient mechanism controls the development of cells with a rooting function in land plants. *Science*, **316**, 1477–1480.

Mount, S.M. and Chang, C. (2002) Evidence for a plastid origin of plant ethylene receptor genes. *Plant Physiol.* **130**, 10–14.

Musgrave, A. and Walters, J. (1974) Ethylene and buoyancy control rachis elongation of the semi-aquatic fern *Regnillidium diphyllum*. *Planta*, **121**, 51–56.

Musgrave, A., Jackson, M.B. and Ling, E. (1972) Callitriche stem elongation is controlled by ethylene and gibberellin. *Nature-New Biol.* **238**, 93–96.

Osborne, D.J., Walters, J., Milborrow, B.V., Norville, A. and Stange, L.M.C. (1995) Evidence for a non-ACC ethylene biosynthesis pathway in lower plants. *Phytochemistry*, **42**, 51–60.

Otsu, N. (1979) Threshold selection method from gray-level histograms. *IEEE Trans. Syst. Man Cybern.* **9**, 62–66.

Prigge, M.J., Lavy, M., Ashton, N.W. and Estelle, M. (2010) *Physcomitrella patens* auxin-resistant mutants affect conserved elements of an auxin-signalling pathway. *Curr. Biol.* **20**, 1907–1912.

Rensing, S.A., Lang, D., Zimmer, A.D. *et al.* (2008) The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science*, **319**, 64–69.

Rohwer, F. and Bopp, M. (1985) Ethylene synthesis in moss protonema. *J. Plant Physiol.* **117**, 331–338.

Schaefer, D.G. and Zryd, J.P. (1997) Efficient gene targeting in the moss *Physcomitrella patens*. *Plant J.* **11**, 1195–1206.

Schaefer, D., Zryd, J.P., Knight, C.D. and Cove, D.J. (1991) Stable transformation of the moss *Physcomitrella-Patens*. *Mol. Gen. Genet.* **226**, 418–424.

Schaller, G.E. and Bleecker, A.B. (1995) Ethylene-binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. *Science*, **270**, 1809–1811.

Sisler, E.C. and Serek, M. (1997) Inhibitors of ethylene responses in plants at the receptor level: recent developments. *Physiol. Plant.* **100**, 577–582.

Solano, R., Stepanova, A., Chao, Q. and Ecker, J.R. (1998) Nuclear events in ethylene signalling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev.* **12**, 3703–3714.

Spollen, W.G., LeNoble, M.E., Samuels, T.D., Bernstein, N. and Sharp, R.E. (2000) Abscisic acid accumulation maintains maize primary root elongation at low water potentials by restricting ethylene production. *Plant Physiol.* **122**, 967–976.

Stange, L. and Osborne, D.J. (1988) Cell specificity in auxin-induced and ethylene-induced supergrowth in *Riella helicophylla*. *Planta*, **175**, 341–347.

Stepanova, A.N. and Alonso, J.M. (2009) Ethylene signalling and response: where different regulatory modules meet. *Curr. Opin. Plant Biol.* **12**, 548–555.

Timme, R.E. and Delwiche, C.F. (2010) Uncovering the evolutionary origin of plant molecular processes: comparison of *Coleochaete* (Coleochaetales) and *Spirogyra* (Zygnematales) transcriptomes. *BMC Plant Biol.* **10**, 96.

Turmel, M., Otis, C. and Lemieux, C. (2006) The chloroplast genome sequence of *Chara vulgaris* sheds new light into the closest green algal relatives of land plants. *Mol. Biol. Evol.* **23**, 1324–1338.

Umezawa, T., Nakashima, K., Miyakawa, T., Kuromori, T., Tanokura, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2010) Molecular basis of the core regulatory network in ABA responses: sensing, signalling and transport. *Plant Cell Physiol.* **51**, 1821–1839.

Van der Straeten, D., Zhou, Z.Y., Prinsen, E., Van Onckelen, H.A. and Van Montagu, M.C. (2001) A comparative molecular-physiological study of submergence response in lowland and deepwater rice. *Plant Physiol.* **125**, 955–968.

Voesenek, L.A.C.J., Jackson, M.B., Toebes, A.H.W., Huibers, W., Vriezen, W.H. and Colmer, T.D. (2003) De-submergence-induced ethylene production in *Rumex palustris*: regulation and ecophysiological significance. *Plant J.* **33**, 341–352.

Voesenek, L.A.C.J., Rijnders, J.H.G.M., Peeters, A.J.M., Van de Steeg, H.M.V. and De Kroon, H. (2004) Plant hormones regulate fast shoot elongation under water: from genes to communities. *Ecology*, **85**, 16–27.

Wang, W., Esch, J.J., Shiu, S.H., Agula, H., Binder, B.M., Chang, C., Patterson, S.E. and Bleecker, A.B. (2006) Identification of important regions for ethylene binding and signalling in the transmembrane domain of the ETR1 ethylene receptor of Arabidopsis. *Plant Cell*, **18**, 3429–3442.

Wilkinson, S. and Davies, W.J. (2010) Drought, ozone, ABA and ethylene: new insights from cell to plant to community. *Plant, Cell Environ.* **33**, 510–525.

Xu, K., Xu, X., Fukao, T., Canlas, P., Maghirang-Rodriguez, R., Heuer, S., Ismail, A.M., Bailey-Serres, J., Ronald, P.C. and Mackill, D.J. (2006) *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature*, **442**, 705–708.

Yasumura, Y., Crumpton-Taylor, M., Fuentes, S. and Harberd, N.P. (2007) Step-by-step acquisition of the gibberellin-DELLA growth-regulatory mechanism during land-plant evolution. *Curr. Biol.* **17**, 1225–1230.

Yoo, S.D., Cho, Y. and Sheen, J. (2009) Emerging connections in the ethylene signalling network. *Trends Plant Sci.* **14**, 270–279.

Zuiderveld, K. (1994) Contrast limited adaptive histograph equalization. In *Graphics Gems IV* (Heckbert, P.S., ed.). San Diego: Academic Press Professional, pp. 474–485.

