Published in Science 316, no 5830, 1477-1480, 2007
which should be used for any reference to this work

An Ancient Mechanism Controls the Development of Cells with a Rooting Function in Land Plants

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Root hairs and rhizoids are cells with rooting functions in land plants. We describe two basic helix-loop-helix transcription factors that control root hair development in the sporophyte (2*n*) of the angiosperm *Arabidopsis thaliana* and rhizoid development in the gametophytes (*n*) of the bryophyte *Physcomitrella patens*. The phylogeny of land plants supports the hypothesis that early land plants were bryophyte-like and possessed a dominant gametophyte and later the sporophyte rose to dominance. If this hypothesis is correct, our data suggest that the increase in morphological complexity of the sporophyte body in the Paleozoic resulted at least in part from the recruitment of regulatory genes from gametophyte to sporophyte.

The invasion of land by plants in the Paleozoic was accompanied by marked changes in plant structure and life cycle and resulted in diversification of terrestrial ecosystems and pronounced climate change (*1*–*3*). One of the most important transformations that occurred during the first 100 million years after plants colonized the land was the rise to dominance of the diploid phase (sporophyte) of the life cycle (the land-plant life cycle comprises independent haploid and diploid organisms). The phylogenetic relationship among green algae and land plants suggests that the haploid phase (gametophyte) was morphologically more complex than the smaller diploid phase (sporophyte) in the earliest land plants (*4*). This changed over a period of ~100 million years to a situation in which the diploid phase became larger and more morphologically complex (*4*). This rise to dominance of the diploid phase of the life cycle was accompanied by an enormous increase in morphological diversity evident in Devonian floras and has persisted to the present day, when the land floras are largely dominated by diploid plants (*3*). To date, we have little understanding of the genetic basis of such a metamorphosis of the land plant body. The characterization of the function of regulatory genes such as *LEAFY* (*LFY*) in both bryophytes and angiosperms suggests that the increase in sporophyte diversity was brought about through the modification of the activities of sporophyte-specific genes with sporophyte-specific functions (*5*). Here we show that genes that specifically promote the development of root hairs in diploid sporophytes of angiosperms also control the development of cells with similar functions in the haploid gametophytes of mosses. This suggests that genes with gametophyte functions in ancestral land plants were recruited to function in the sporophyte during the metamorphosis of the land plant body.

Root hairs are highly polarized cells that increase the surface area of the plant that is in contact with the growth substrate. They play important roles in nutrient acquisition and anchorage in those land plants that have roots (*6*, *7*). The *Arabidopsis thaliana* root epidermis is organized in alternate rows of hair-forming cells (H cells) that produce a tip-growing protuberance (root hairs) and rows of non–hair cells (N cells) that remain hairless. *AtRHD6* (*ROOT HAIR DEFECTIVE 6*) positively regulates the development of H cells–*Atrhd6* mutants develop few root hairs (Fig. 1A) (*8*). We cloned *AtRHD6* using an enhancer trap line (*Atrhd6-2*) in which the *GUS* reporter gene is expressed in H cells but not in N cells (Fig. 1, C and D, and fig. S1). *AtRHD6* encodes the basic-helix-loop-helix (bHLH) transcription factor At1g66470 (*9*). The identification of another independent allele (*Atrhd6-3*) with a similar phenotype and the complementation of the *Atrhd6-3* mutation with a whole gene *AtRHD6p::GFP:AtRHD6* translational fusion with the GREEN FLUORESCENT PROTEIN (GFP) confirmed that the defect in root hair development observed in this mutant is due to mutation of *At1g66470* (Fig. 1A). This complementing *AtRHD6p::GFP:AtRHD6* fusion indicates that AtRHD6 protein accumulates in H-cell nuclei in the meristem and elongation zones (Fig. 1B) but disappears before the emergence of the root hair (data not shown). The spatial pattern of N cells and H cells in the *A. thaliana* root epidermis is controlled by a transcriptional network including the posi-

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tive regulator of H-cell identity *CPC* and the negative regulators of H-cell identity *WER*, *TTG*, and *GL2* (*10*). To determine if *AtRHD6* is regulated by these genes, we analyzed the promoter activity of the *Atrhd6*-2 enhancer trap in different mutant backgrounds. While the *Atrhd6-2* enhancer trap expresses GUS in cells in the H position, this expression spreads to the cells in the N position in the *wer*, *ttg*, and *gl2* mutant backgrounds, indicating that *WER*, *TTG*, and *GL2* negatively regulate transcription of *AtRHD6* in the N position (Fig. 1D). No expression was observed in the *cpc* mutant, indicating that *CPC* positively regulates *AtRHD6* expression (Fig. 1D). Thus, *AtRHD6* controls the development of root hair cells and acts downstream of the genes involved in epidermal pattern formation.

AtRHD6 is a member of subfamily VIIIc of bHLH transcription factors that comprises five other members (*9*, *11*). One of these genes, *At5g37800*, hereafter named *RHD SIX-LIKE1* (*AtRSL1*), is very similar to *AtRHD6*, suggesting that these two genes derive from a relatively recent duplication event (*9*). This suggests that *AtRHD6* and *AtRSL1* might have redundant functions. To determine if *AtRSL1* is also required for root hair development, we identified a line (*Atrsl1-1*) carrying a complete loss-of-function mutation in the *AtRSL1* gene and created the *Atrhd6-3 Atrsl1-1* double mutant (fig. S1). Because no new phenotypes were observed when these mutants were grown in our standard growth conditions, we grew them on the surface of cellophane disks, where small numbers of root hairs develop in the *Atrhd6-3* single mutant (Fig. 2A). Plants homozygous for the *Atrsl1-1* mutation had wild-type root hair morphology when grown on cellophane disks (Fig. 2A). However, the *Atrhd6-3 Atrsl1-1* double mutant did not develop root hairs, indicating that *AtRHD6* and *AtRSL1* have partially redundant functions in root hair development (Fig. 2A). *Atrhd6-3 Atrsl1-1* double-mutant plants carrying the genomic construct *AtRSL1p::GFP:AtRSL1* displayed the AtRhd6-3–mutant phenotype, confirming that the extreme hairless phenotype of the *Atrhd6-3 Atrsl1-1* double mutant is the result of a loss of function of both *AtRHD6* and *AtRSL1* genes (Fig. 2A). The complementing GFP::AtRSL1 fusion protein accumulates in hair cell nuclei in the meristem and elongation zones, indicating that *AtRHD6* and *AtRSL1* have similar expression patterns (Fig. 2B). These data indicate that *AtRSL1* and *AtRHD6* act together to positively regulate root hair development. To determine if *AtRHD6* and *AtRSL1* are required for the development of the only other tip-growing cell in flowering plants, the pollen tube, we characterized the phenotypes of pollen tubes in *Atrhd6-3*, *Atrsl1-1*, and *Atrhd6-3 Atrsl1-1* mutants both in vitro and in vivo. We detected neither a defect in pollen tube growth nor in the segregation of mutant alleles in the F_2 progeny of backcrosses to wild type (fig. S2). No other defective phenotype was detected in any other part of *Atrhd6-3*, *Atrsl1-1*, or *Atrhd6-3 Atrsl1-1* mutants. Together these data indicate that AtRHD6 and AtRSL1 are bHLH transcription factors that are specifically required for the

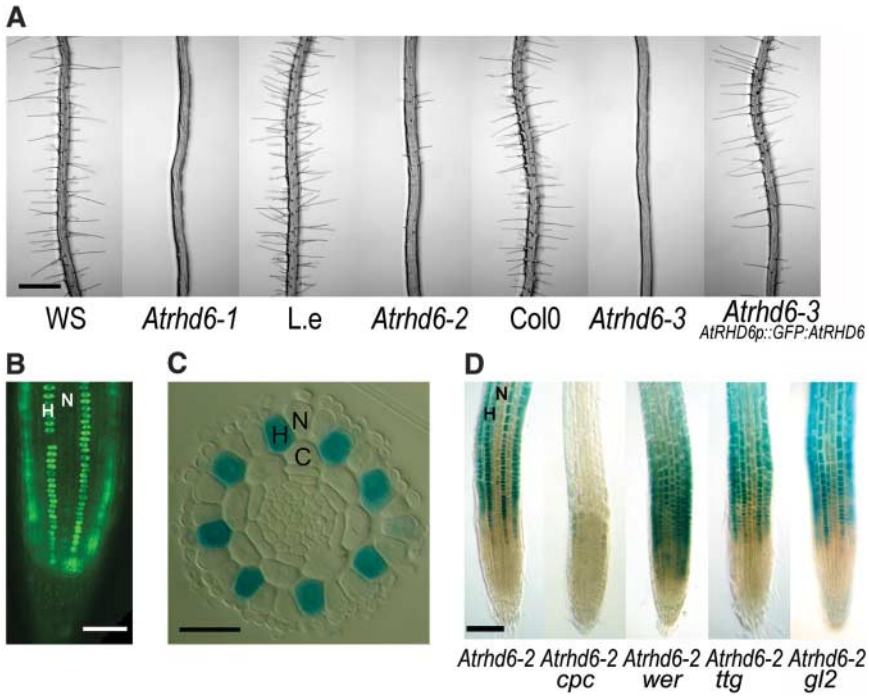

Fig. 1. *AtRHD6* is a positive regulator of root hair development in *A. thaliana*. (**A**) Roots of *Atrhd6-1*, *Atrhd6-2*, and *Atrhd6-3* mutants with their respective wild-type ecotype (WS, Wassìlewskìja; Col0, Columbia 0; L.e., *Lansburg erecta*) and complementation of the *Atrhd6-3* mutant with a genomic *AtRHD6p::GFP:AtRHD6* fusion. (**B**) Fluorescent image of the genomic *AtRHD6p::GFP:AtRHD6* fusion in the *Atrhd6-3* background showing AtRHD6 protein in hair cells nuclei. (**C**) Expression of the *Atrhd6-2* enhancer trap *GUS* gene in root cross section. (**D**) Whole-mount longitudinal view of the expression of the enhancer trap *GUS* gene in *Atrhd6-2* and in different backgrounds (*cpc*, *wer*, *ttg1*, and *gl2*). H, hair cell; N, non–hair cells; C, cortex. Scales bars, 500 μm (A), 50 μm (B), 25 μm (C), and 100 μm (D).

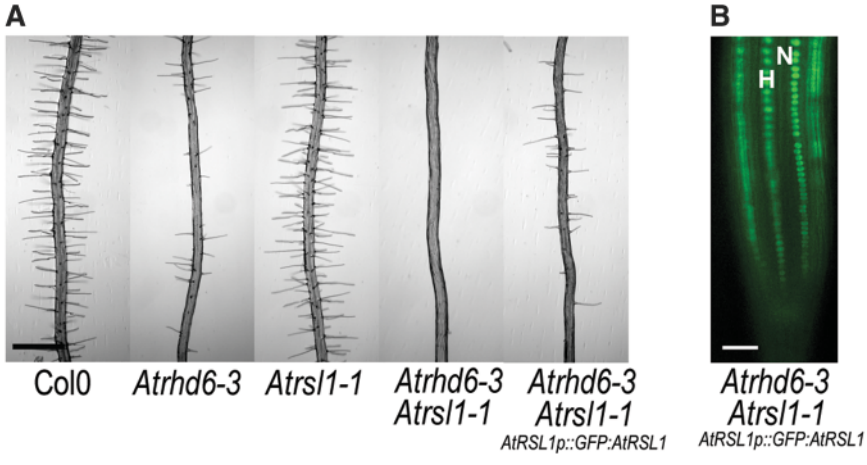

Fig. 2. *AtRSL1* positively regulates root hairs development in *A. thaliana*. (**A**) Roots of WT, *Atrhd6-3* single mutant, *Atrsl1-1* single mutant, *Atrhd6-3 Atrsl1-1* double mutant, and *Atrhd6-3 Atrsl1-1* double mutant bearing the *AtRSL1p::GFP:AtRSL1* transgene. Plants were grown on MS media with sucrose overlaid with a cellophane disk to increase root hair production in the *Atrhd6-3* mutant. (**B**) Fluorescent image of the genomic *AtRSL1p::GFP:AtRSL1* fusion in the *Atrhd6-3 Atrsl1-1* background showing AtRSL1 protein in hair cells nuclei. H, hair cell; N, non–hair cells. Scale bars, 500 μm (A) and 50 μm (B).

development of root hairs and act downstream of the genes that regulate epidermal pattern formation in the flowering plant *A. thaliana*.

The most ancestral grade of land plants are the bryophytes—the earliest microfossils of land plants from the middle Ordovician (~475 million years ago) have bryophyte characteristics (*12*). Bryophytes do not have roots but possess tip-growing cells that are morphologically similar to root hairs and fulfill rooting functions. In mosses, caulonemal cells increase the surface area of the filamentous protonema tissue in contact with the substrate and rhizoids anchor the leafy gametophore to their growth substrate (*13*, *14*); both cell types are hypothesized to be involved in nutrient acquisition (*13*). However, rhizoids and caulonema develop from the gametophyte of mosses, whereas root hairs develop from the sporophyte of modern vascular plants. Thus, according to the current view that land plants evolved by the intercalation of a sporophytic generation from a haplontic algal ancestor followed by the progressive increase of size and complexity of the sporophyte in parallel to a reduction of the gametophyte (*4*, *15*), neither rhizoids nor caulonema are homologous to root hairs. To determine if the developmental mechanism that controls the development of root hairs in angiosperms also controls the development of nonhomologous tip-growing cells with a rooting function in bryophytes, we identified *RHD6-LIKE* genes from the moss *Physcomitrella patens*. We identified seven members of the *AtRHD6* subfamily of bHLH genes from the publicly available *P. patens* genomic sequence (http://moss.nibb.ac.jp/), suggesting that these genes have been conserved through the land plant evolution. These were designated *Physcomitrella patens RHD SIX-LIKE 1* to *7* (*PpRSL1* to *PpRSL7*). To analyze the relationship between *P. patens* and *A. thaliana RSL* genes, we constructed trees by maximum parsimony. A strict consensus tree shows that *AtRHD6*, *AtRSL1*, and the two *P. patens* genes *PpRSL1* and *PpRSL2* are closely related and together form a monophyletic clade (AtRHD6 clade) that is sister to the clade comprising all the other members of the subfamily (sister clade) (Fig. 3 and fig. S3). This indicates that the AtRHD6 clade evolved before the separation of the bryophytes and the vascular plants from a common ancestor.

To characterize the function of the *RHD6-LIKE* genes in moss, we constructed deletion mutants that lacked the function of *PpRSL1* and *PpRSL2* genes and determined whether they developed morphological defects. Three independent RNA null mutants with single insertions into the *PpRSL1* and *PpRSL2* genes were made. Double mutants with single insertions into both genes were also generated (fig. S4). The phenotypes of each of these mutants were then analyzed. A haploid protonema develops upon germination of a wild-type *P.*

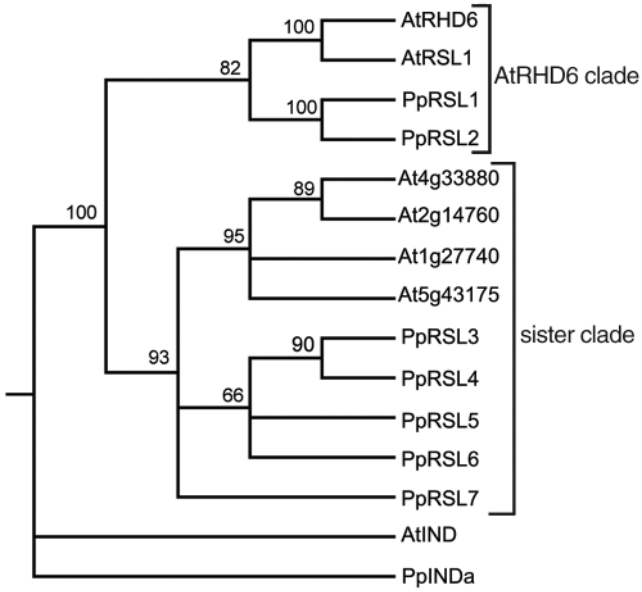

Fig. 3. Relationship between RHD6-LIKE proteins from *A. thaliana* and *P. patens*. The tree is a strict consensus tree of 12 most parsimonious trees generated with the alignment of bHLH domains amino acids sequences shown in fig. S3. The *A. thaliana* genes used are the members of bHLH subfamily VIIIc, except AtIND (INDEHISCENT)/At4g00120, which was used as out-group and belongs to the bHLH subfamily VIIIb (*9*, *11*, *21*). *P. patens* PpRSL 1 to 7 sequences were obtained by BLAST of the *P. patens* genomic sequence. PpIND1 is a *P. patens* sequence similar to AtIND and a putative member of family VIIIb in *P. patens*. Numbers are bootstrap values and indicates an 82% level of confidence for the occurrence of the AtRHD6 clade. The brackets indicate the AtRHD6 clade and the sister clade.

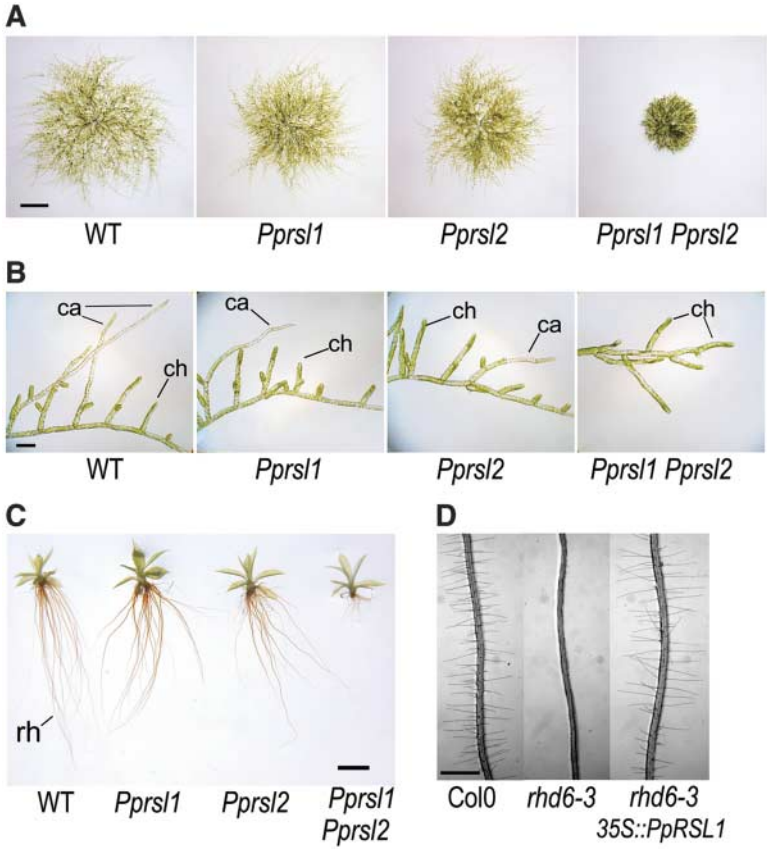

Fig. 4. *PpRSL1* and *PpRSL2* positively control the development of caulonemal cells and rhizoids in *P. patens*, and PpRSL1 and AtRHD6 have a conserved molecular function. (**A** and **B**) Eighteen-day-old protonema from WT, *Pprsl1*, and *Pprsl2* single mutants, and *Pprsl1 Pprsl2* double mutant, were grown from spores on 0.8% agar. (A) Whole protonema growing from a single spore. (B) Dissected filaments from protonema shown in (A). (**C**) Isolated 1-month-old gametophores. (**D**) Roots of the *A. thaliana Atrhd6-3* mutant carrying the *35S::PpRSL1* transgene compared to WT and *Atrhd6-3* roots. ca, caulonemal cell; ch, chloronemal cell; rh, rhizoid. Scale bars, 1 mm (A), 100 μm (B), 1 mm (C), and 500 μm (D).

patens spore (*13*). This filamentous tissue comprises two cell types, the chloronema and the caulonema (Fig. 4, A and B). Chloronemal cells contain large chloroplasts and grow by a slow tip-growth mechanism (*16*). Caulonemal cells are more elongated, contain few smaller chloroplasts, grow by rapid tip growth, and are involved in the colonization of the substrate. Leafy gametophores usually develop from caulonema and are anchored to their substrate by tip-growing multicellular rhizoids that are morphologically similar to caulonema (Fig. 4C). The *Pprsl1* and *Pprsl2* single mutants have slightly smaller and greener protonema cultures than the wild type (WT), and this phenotype is much stronger in the *Pprsl1 Pprsl2* double mutant, which produces small dark-green protonema (Fig. 4A). *Pprsl1* and *Pprsl2* single mutants produce fewer caulonemal cells than the WT, indicating that the greener protonema phenotype is the result of a defect in the development of caulonemal cells (Fig. 4B). No caulonemal cells develop in the *Pprsl1 Pprsl2* double mutant, and the protonema of this mutant consists of chloronemal cells only (Fig. 4B). In wild-type plants gametophores develop from caulonema, but in the *Pprsl1 Pprsl2* double mutants the gametophores develop from chloronema, as previously observed in another caulonema-defective mutant (*17*). The gametophores of the *Pprsl1 Pprsl2* double mutant develop few very short rhizoids (Fig. 4C). No other defective phenotypes were detected in the chloronema, in the leafy part of the gametophore, or in the sporophyte in the single or double mutants. This indicates that *PpRSL1* and *PpRSL2* together regulate the development of caulonemal cells and rhizoids in the moss gametophyte. The lack of a defect in chloronemal cells, which are the other tip-growing cells that develop in moss (*16*), in the *Pprsl1 Pprsl2* double mutant shows that, as in *A. thaliana*, these genes are not general regulators of tip growth. Instead it suggests that they function specifically to regulate the development of cells with rooting functions such as caulonemal cells and rhizoids. To determine if protein function is conserved across the land plants, we performed a cross-species complementation experiment. Expression of *PpRSL1* under the cauliflower mosaic virus (CaMV) 35*S* promoter in the *Atrhd6-3* mutant resulted in the formation of wild-type root hairs (Fig. 4D). Thus, the moss *PpRSL1* gene can substitute for loss of *AtRHD6* function in *A. thaliana*. This indicates that the molecular function of *PpRSL1* and *AtRHD6* has been conserved since the divergence of seed plants and mosses from a common ancestor and suggests that the same molecular mechanism controls the development of *A. thaliana* root hairs and *P. patens* caulonema and rhizoids.

We have shown that closely related transcription factors control the development of root hairs and rhizoids in the seed plant sporophyte and the bryophyte gametophyte, respectively. The demonstration of the existence and function of these genes in plants derived from the earliest colonizers of the land (bryophytes) indicates that an ancient common mechanism controls the development of these two nonhomologous cell types. The *RHD6-LIKE* genes will have been important for the invasion of land by plants because they control the development of structures required for anchorage to the terrestrial substrate and nutrient acquisition. The observation that rhizoids have been found on some of the oldest land-plant fossils is consistent with this view (*18–20*).

Our demonstration that *RHD6*-related genes function in both bryophytes and angiosperms in the development of rhizoids and root hairs, respectively, suggests a mechanism to explain the increased morphological and cellular diversity of the sporophyte in the land plants derived from bryophyte ancestors. Our results suggest that *RHD6-LIKE* genes functioned in the haploid generation (gametophyte) of these early land plants that had a bryophyte-like life cycle (*18*), where they controlled the formation of cells with a rooting function. Then, during the subsequent radiation of the land plants, these genes were deployed in the development of the diploid generation (sporophyte) of the nonbryophyte land plants, where they controlled the development of rhizoids and root hairs. Here we propose a general model for the increase in morphological diversity of the land-plant sporophyte based on these findings. We suggest that some of the genes that controlled the development of the bryophyte haploid body were recruited by the diploid phase in their descendants, where they provided part of the genetic mechanism for the increased morphological and cellular diversity of the sporophyte. Thus, the recruitment of genes from haploid to diploid phases of the life cycle, in concert with the modification of function of sporophyte-specific genes, such as *LFY* (*5*), is a mechanism that may account for the explosion in morphological diversity of the diploid stage of the life cycle (sporophyte) that occurred in the middle Palaeozoic when green plants colonized the continental surfaces of the planet (*3*). The full extent of the recruitment of genes from the haploid to the diploid phases during the colonization of the land will be quantified through future comparative analysis of gene function in bryophytes and angiosperms. The discovery and description of more bryophyte fossils from the middle Paleozoic is necessary to unequivocally define the nature of early land-plant life histories. This is important because although it is likely that the earliest land plants had bryophyte-like life cycles, there is still a possibility that their life cycles were unlike those of modern bryophytes. Only through the combination of paleobotanical and developmental genetic approaches will we understand the mechanism by which the land plant body developed over 400 million years ago.

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22. This research is funded by a grant from the Natural Environment Research Council (Ne/c510732/1) and Human Frontier Science Program Organization (HFSPO) (to L.D.), and a grant-in-aid from the Biotechnology and Biological Sciences Research Council to the John Innes Centre. B.M. and S.J. were funded by the European Molecular Biology Organization (EMBO) and Marie Curie Fellowships (EMBO ALTF 89-2002 and Marie Curie HPMF-CT-2002-01935 to B.M.). E.R. was supported by a Marie Curie Fellowship. L.H. is funded by the Marie Curie TIPNET network. K.Y. is partially supported by a Joint Scholarship between the University of East Anglia and China Scholarship Council and HFSPO (RGP0012/2005-C). We are grateful to E. Moylan and J. Harrison for advice in using PAUP*. We also thank J. Doonan, N. Harberd, M. Pernas-Ochoa, S. Takeda, and Y. Yasumura for critical comments on the manuscript and N. Pires for help with BLAST of the *P. patens* genome. We thank J. Langdale and P. Kenrick for invaluable discussions and J. Duckett for teaching us moss morphology.