Homologues of the Arabidopsis thaliana SHI/STY/LRP1 genes control auxin biosynthesis and affect growth and development in the moss Physcomitrella patens

D. Magnus Eklund1,*, Mattias Thelander1,*, Katarina Landberg1, Veronika Ståldal1, Anders Nilsson2, Monika Johansson1,2,#, Isabel Valsecchi1, Eric R. A. Pederson1, Mariusz Kowalczyk3, Karin Ljung3, Hans Ronne2 and Eva Sundberg1,‡

SUMMARY
The plant hormone auxin plays fundamental roles in vascular plants. Although exogenous auxin also stimulates developmental transitions and growth in non-vascular plants, the effects of manipulating endogenous auxin levels have thus far not been reported. Here, we have altered the levels and sites of auxin production and accumulation in the moss Physcomitrella patens by changing the expression level of homologues of the Arabidopsis SHI/STY family proteins, which are positive regulators of auxin biosynthesis genes. Constitutive expression of PpSHI1 resulted in elevated auxin levels, increased and ectopic expression of the auxin response reporter GmGH3pro:GUS, and in an increased caulonema/chloronema ratio, an effect also induced by exogenous auxin application. In addition, we observed premature ageing and necrosis in cells ectopically expressing PpSHI1. Knockout of either of the two PpSHI genes resulted in reduced auxin levels and auxin biosynthesis rates in leafy shoots, reduced internode elongation, delayed ageing, a decreased caulonema/chloronema ratio and an increased number of axillary hairs, which constitute potential auxin biosynthesis sites. Some of the identified auxin functions appear to be analogous in vascular and non-vascular plants. Furthermore, the spatiotemporal expression of the PpSHI genes and GmGH3pro:GUS strongly overlap, suggesting that local auxin biosynthesis is important for the regulation of auxin peak formation in non-vascular plants.

KEY WORDS: Physcomitrella patens, SHI, STY, LRP1, Auxin, Moss

INTRODUCTION
The most important naturally occurring auxin, indole-3-acetic acid (IAA), is essential throughout the life cycle of vascular plants and is required for embryo axis formation, root apical meristem patterning, organ positioning and initiation, tissue differentiation and phototropic and gravitropic responses (Dolan, 1998; Friml et al., 2003; Kaufman et al., 1995; Marchant et al., 1999; Mattsson et al., 1999; Sabatini et al., 1999; Went, 1974). Auxin is believed to mediate instructions through concentration gradients and local auxin maxima created by the combined forces of auxin synthesis and polar auxin transport (PAT) (Teale et al., 2006). Although Grieneisen and co-workers showed that transport of auxin is crucial for auxin maxima formation in root tips (Grieneisen et al., 2007), recent studies on auxin biosynthesis regulation have convincingly demonstrated the importance of local biosynthesis in establishing and maintaining auxin maxima throughout plant development (Cheng et al., 2006; Cheng et al., 2007; Ikeda et al., 2009; Stepanova et al., 2008; Tao et al., 2008). There are at least four pathways for biosynthesis of IAA in seed plants, one of which is tryptophan independent, the other three tryptophan dependent (for reviews see Chandler, 2009; Lau et al., 2008). The different pathways appear to function non-redundantly because they produce IAA in different spatiotemporal patterns, for different purposes and in response to different stimuli (Tao et al., 2008). This suggests that the spatiotemporal regulation of auxin biosynthesis plays an important role in auxin gradient or peak formation. We have previously demonstrated that SHI/STY family proteins in Arabidopsis induce the activity of auxin biosynthesis genes (e.g. YUC4), thus playing important roles in the positioning of local auxin maxima (Sohlborg et al., 2006; Ståldal et al., 2008; Eklund et al., 2010).

Auxin also functions as a hormone in bryophytes, and IAA application triggers physiological responses such as the chloronemato-caulonema transition (Johri and Desai, 1973), stem elongation (Fujita et al., 2008) and rhizoid emergence from the leafy shoot (Ashton et al., 1979). With the completion of the genome sequence of the moss Physcomitrella patens, it became clear that genes involved in auxin transport, perception and signalling in seed plants are present also in mosses (Rensing et al., 2008). This remarkable conservation between plants separated by 450 million years of evolution suggests that this machinery was already in place when early plants colonised land (Cooke et al., 2002).

The P. patens genome contains four putative PIN-type auxin efflux facilitator genes (Floyd and Bowman, 2007), which suggests the presence of active PAT in mosses. Whereas some studies have reported that PAT inhibitors have effects on gametophyte development (Geier et al., 1990; Rose and Bopp, 1983; Rose et al., 1983; Schwuchow et al., 2001), a recent study of five different mosses suggested that long-range PAT occurs in the subordinate.
sporophyte generation, but not in the gametophyte leafy shoot (Fujita et al., 2008). Consequently, it appears likely that tightly controlled local auxin biosynthesis is important in mosses, and that increased knowledge about these processes could shed light on the ancestral mechanisms for creation of auxin maxima in plants. We searched the P. patens genome sequence for auxin biosynthesis genes and found homologues of six Arabidopsis thaliana (At) YUCCA (AtYUC) (Gallavotti et al., 2008; Rensing et al., 2008) and four AtAIA1 genes, which encode enzymes proposed to act in two separate tryptophan-dependent pathways (Stepanova et al., 2008; Tao et al., 2008; Zhao et al., 2001). By contrast, although numerous cytochrome P450-related genes exist in moss (Rensing et al., 2008), we were unable to find a gene with a deduced protein sequence clustering within the CYP79 subgroup, which harbours enzymes that catalyze the conversion of tryptophan to indole-3-acetaldimine in Arabidopsis (Wercz-Reichhart et al., 2002). This suggests that at least two tryptophan-dependent pathways for auxin biosynthesis are likely to exist in P. patens.

Here, we report the functional characterisation of PpSHI1 and PpSHI2, two highly similar P. patens members of the SHI/STY gene family. Our data show that the PpSHI genes are co-expressed with an auxin response reporter construct, and that a knockout of either PpSHI gene results in reduced auxin biosynthesis rates and accumulation in the apical end of leafy shoots, suggesting that the PpSHI genes might induce auxin biosynthesis at their expression sites. The phenotypic effects of PpSHI knockouts are consistent with the expected effects of reduced endogenous auxin production. We further show that constitutive expression of PpSHI results in elevated IAA levels and some phenotypic abnormalities normally associated with high auxin levels. These experiments also revealed that PpSHI1 can induce necrosis when ectopically expressed. Finally, our results suggest a possible role for axillary hairs in auxin production or secretion.

MATERIALS AND METHODS

Growth conditions

Conditions for growth and culturing and BCD media were as described (Thelander et al., 2007).

Gene identification and cloning

The P. patens ssp. patens draft genome (v1.1) was screened for SHI/STY TAA1, YUCC4 and CYP79B2/3 homologues using TBLASTN with query sequences from Arabidopsis. SHI/STY loci were also searched for in the S. moellendorfii (v1.0), C. reinhardtii (v3.0), O. lucimarins (v2.0), O. tauri (v2.0) and V. carteri f. Nagariensis (v1.0) genomes deposited at the Joint Genome Institute (JGI). The PpSHI cDNA (Kuusk et al., 2006) was used to design primers for amplification of the PpSHI gene, which was cloned into pCR2.1-TOPO (Invitrogen, Lidingö, Sweden) and sequenced.

PpSHI1 in vitro mutagenesis

The BamHI site in exon 3 of PpSHI1 was mutated to AGATCC using primers 5’Gex3A and 3’Gex3A (see Table S1 in the supplementary material) together with gene-specific primers. The PCR products were combined in another PCR to fuse the two fragments. To mutate the BamHI site in intron 2 to GGATCT we used primers 5’Gin2A and 3’Gin2A. In a final PCR, the fragments containing the two mutations were fused. The resulting fragment was cloned into pCRII-TOPO T/A (Invitrogen) to produce plasmid pPpSHI1GexintA. The PpSHI1 cDNA was mutated using a similar strategy.

GUS staining and fluorescence microscopy

GUS staining was performed according to Jefferson (Jefferson, 1987) but with 2 mM ferri/ferrocyanide. Tissue was stained at room temperature for 2-24 hours before destaining in 70% ethanol and analysis using a stereo dissection microscope (Nikon SMZ1500) with a Nikon DS-Fi1 camera and NIS-Elements D2.30 imaging software. GFP fluorescence was analysed with a Zeiss Axioskop 2 Mot microscope with an AxioCam HR camera and AxioVision software. GFP was detected using a FITC filter, autofluorescence using a TRITC filter and Hoechst 33342 using a DAPI filter.

PpSHI1pro:PpSHI1-GFP and PpSHI2pro:PpSHI2-GUS lines

The vector pPPGUS was constructed by inserting a GUS-TNOS-1 BamHI/EcoRI fragment from plasmid pH101.3 (Clontech, Stockholm, Sweden) into the BamHI/EcoRI sites of pCR-BluntII-TOPO (Invitrogen), after which a P35S-NPTII-T35S neomycin resistance EcoRI cassette from plasmid pMT164 (Thelander et al., 2007) was inserted into the EcoRI site of the product. Plasmid pPPGUS was created using the same strategy with a GUS-TNOS-1 BamHI/EcoRI fragment from plasmid psmRS-GFP (Davis and Vierstra, 1996).

A PpSHI1 fragment amplified from pPpSHI1GexintA using primers 165BamHI1F and 165BamHI1R (see Table S1 in the supplementary material) was inserted into the BamHI site of pPPGUS. Another fragment directly downstream of the stop codon was amplified with primers 5’PpUTR and 3’PpUTR and inserted between the NotI and XhoI sites of the previous product, creating plasmid pPpSHI1-gFP. Similarly, a PpSHI2 genomic fragment terminating immediately prior to the stop codon was amplified with primers 1652BglIIIF and 1652BglIIR and inserted into the BamHI site of the vector pPPGUS. A NotI/XbaI fragment in the resulting product was exchanged for a genomic fragment amplified with primers 3UTR2NotF and 3UTRXbaIR and covering the 3’UTR of PpSHI2 to produce plasmid pPpSHI2-GUS.

The targeting constructs pPpSHI1-IGFP and pPpSHI2-IGUS (see Fig. S3A,B in the supplementary material) were released by XbaI digestion prior to transformation (Schafer et al., 1991). Transformants were selected on 50 µg/ml G418 (G9516, Sigma, Stockholm, Sweden). PCR genotyping of stable transformants was performed with primers shown in Fig. S3A,B and Table S1 in the supplementary material.

PpSHI1 overexpression

PpSHI1 was amplified from cDNA using primers PpSHIox5’ and PpSHIox3’ (see Table S1 in the supplementary material) and inserted into the Apal site of pCMK1 creating pPpSHI1ox1. pPpSHI1ox1 was NotI-linearised before transformation. Transformants were selected on 50 µg/ml zeocine (Invitrogen).

PpSHI1 and PpSHI2 gene disruption

Two PpSHI1 targeting constructs were used. For construct 1, a PpSHI1 fragment was amplified using primers PpSHI1-5A and PpSHI1-3A (see Table S1 in the supplementary material) and cloned into the Apal site of pCMK1 creating pPpSHI1ox1. pPpSHI1ox1 was NotI-linearised before transformation. Transformants were selected on 50 µg/ml hygromycin (pPpKOSHI1) or G418 (G9516, Sigma, Stockholm, Sweden). PCR genotyping of stable transformants was performed with primers shown in Fig. S3A,B and Table S1 in the supplementary material.

PpSHI2 overexpression

PpSHI2 was amplified from cDNA using primers PpSHIox5’ and PpSHIox3’ (see Table S1 in the supplementary material) and cloned into the Apal site of pCMK1 creating pPpSHI2ox1. pPpSHI2ox1 was NotI-linearised before transformation. Transformants were selected on 50 µg/ml hygromycin (pPpKOSHI1).

PpSHI1 and PpSHI2 gene disruption

Two PpSHI2 targeting constructs were used. For construct 1, a PpSHI2 fragment was amplified using primers PpSHI2-5A and PpSHI2-3A (see Table S1 in the supplementary material) and cloned into the Apal site of pCMK1 creating pPpSHI2ox1. pPpSHI2ox1 was NotI-linearised before transformation. Transformants were selected on 50 µg/ml hygromycin (pPpKOSHI1).

PpSHI2 overexpression

PpSHI2 was amplified from cDNA using primers PpSHIox5’ and PpSHIox3’ (see Table S1 in the supplementary material) and inserted into the Apal site of pCMK1 creating pPpSHI2ox1. pPpSHI2ox1 was NotI-linearised before transformation. Transformants were selected on 50 µg/ml zeocine (Invitrogen).

PpSHI1 and PpSHI2 gene disruption

Two PpSHI1 targeting constructs were used. For construct 1, a PpSHI1 fragment was amplified using primers PpSHI1-5A and PpSHI1-3A (see Table S1 in the supplementary material) and cloned into the Apal site of pCMK1 creating pPpSHI1ox1. pPpSHI1ox1 was NotI-linearised before transformation. Transformants were selected on 50 µg/ml hygromycin (pPpKOSHI1).
Auxin concentration and biosynthesis rate measurements
Moss colonies grown for 23 days on cellophane-overlayed BCD media (PpSHI1ox) or apices from 50 gametophores grown for 7 weeks on BCD media (Ppshi1-l, Ppshi2-l) were harvested from three independent biological replicates. For IAA synthesis measurements, gametophores were incubated with liquid BCD medium containing 30% $^3$H$_2$O for 24 hours before apices were harvested. $^{13}$C$_2$-IAA (500 pg, internal standard) was added to each sample before extraction. Extraction and purification of samples were performed as described (Andersen et al., 2008). IAA quantification and biosynthesis measurements were performed by combined gas chromatography-mass spectrometry (GC-MS) (Edlund et al., 1995; Edlund et al., 1999).

Phenotypic analysis
For phenotypic analysis, small protonemata pieces (~1 mm in diameter) were inoculated onto BCD plates and grown under standard conditions. Colonies or individual gametophores harvested with intact rhizoid bundles were photographed using the stereo dissection microscope described above. To visualise axillary hairs and rhizoid initials, detached gametophores were incubated in 0.05% Evan’s Blue (46160, Fluka, Stockholm, Sweden) dissolved in PBS (pH 8.0) for 1-2 hours and then washed in PBS.

Transgene copy number determinations
Real-time PCR was performed using the IQ5 Detection System (BioRad, Sundbyberg, Sweden). We used 10 min at 95°C followed by 35 cycles of 10 seconds at 95°C and 20 seconds at 60°C (amplification), or 71 cycles of 1 minute at 95°C, 1 minute at 60°C and 10 seconds at 60°C (dissociation). 1× DyNAamo Flash SYBR Green qPCR Kit solution (Finnzymes, Stockholcm, Sweden), each primer at 0.3 μM, 1 mM EDTA, 0.1 mg/ml BSA and 8 ng of genomic DNA were mixed in a final volume of 20 μl. PsSHI1 amplification with primers FgSHI1 and RegSHI1 (132 bp) was normalised against PpSHI1 (Thelander et al., 2007) amplification with primers FgSK1 and RgSK1 (150 bp) (see Table S1 in the supplementary material). Specificity was verified by melting curve and gel analysis. Standard curves based on a dilution series of genomic wild-type DNA confirmed that both targets were amplified with efficiencies close to 100%. For each line, three biological repeats with three technical repeats were assayed using both primer pairs. ΔCtPpSHI1–PpSHI1 was first calculated for technical replicates from both the PpSHI1ox and wild-type (WT) lines, after which ΔCtPpSHI1ox–WT values were calculated. The wild type contains one PsSHI1 copy and copy number in the PpSHI1ox lines therefore equals $2^{\Delta\Delta Ct(PpSHI1ox–WT)}$.

Southern blot analysis
DNA extractions, blotting and hybridisation were performed as described (Murén et al., 2009) using 0.4 g protonemata and subsequently 10 μg of DNA per restriction digest. Probe templates were PCR amplified with primers PpSH11-3′ probe and PpSH11-3′ probe and with PpSH2-5′ probe and PpSH11-3′ probe, respectively (see Table S1 in the supplementary material), and cloned into pCR-BluntII-TOPO, from which they were excised with EcoRI and isolated by gel purification prior to DIG labelling using the DIG High Prime DNA Labelling and Detection Kit (Roche).

RESULTS
The SHI/STY gene family may be unique to streptophytes
All members of the SHI/STY gene family possess two conserved regions that encode a zinc-finger domain (Fridborg et al., 1999) and the unique IGGH domain (Fridborg et al., 2001). The family has a comparatively high copy number in seed plants, as exemplified by the ten genes in Arabidopsis (Kuusk et al., 2006), whereas the lycophyte Selaginella moellendorffii, which belongs to a primitive group of vascular plants, has three genes, and the moss P. patens two, suggesting that the SHI/STY gene copy number in embryophytes correlates with their morphological complexity. A survey of the draft genome sequences of the algal species Chlamydomonas reinhardtii, Ostreococcus lucimarinus, Ostreococcus tauri and Volvox carteri failed to identify any SHI/STY genes, indicating that the family is not present in chlorophytes and is thus restricted to streptophytes, or even embryophytes, as it is still unclear whether they exist in chlorophyte algae. Hence, the presence of SHI/STY genes might correlate with the appearance of the TIR/AFB receptor and the ARF response genes that have so far only been found in embryophytes (Lau et al., 2009).

A phylogenetic analysis shows that the two moss SHI genes are highly similar and that they, like many of the Arabidopsis SHI/STY genes, are likely to result from a recent gene duplication (see Fig. S1A in the supplementary material) (Kuusk et al., 2006).

Characterisation of the two Physcomitrella SHI/STY genes
The two Physcomitrella SHI/STY genes share an identical organisation, with three exons, whereas the Arabidopsis homologues have two exons (Kuusk et al., 2006). PpSHI1 and PpSHI2 encode proteins of 289 and 291 amino acids, respectively, sharing 93% identity (see Fig. S1B in the supplementary material). The two proteins are 100% identical over the conserved zinc-finger and IGGH domains and share 79% sequence identity with the AtLRP1 (At5g12330) protein (see Fig. S1B in the supplementary material). The regions between the N-termini and the zinc-finger domains of the two Physcomitrella proteins are strikingly rich in glutamine (Q) residues and several Arabidopsis SHI/STY proteins also possess one or more Q-rich regions. Q-rich regions have been shown to act as transcriptional activation domains and experimental studies support such a function for Arabidopsis and Physcomitrella members of the SHI/STY family (Kuusk et al., 2006; Eklund et al., 2010).

PpSHI1 and PpSHI2 are mainly localised to the nucleus
PpSHI1 and PpSHI2 share a short region that is enriched for positively charged amino acids (see Fig. S1B in the supplementary material) with the majority of the Arabidopsis, rice and poplar SHI/STY gene products. This sequence resembles a monopartite nuclear localisation signal (Lange et al., 2007) and strong evidence suggests that AtSTY1 exerts its function in the nucleus (Sohlberg et al., 2006; Eklund et al., 2010). We therefore transiently expressed a PpSHI1-GFP fusion protein in Physcomitrella protoplasts and found, as expected, a mainly nuclear GFP signal (see Fig. S2A-D in the supplementary material). This result was confirmed using gene targeting to integrate GFP and GUS reporter genes, in frame, immediately upstream of the stop codons of the PpSHI genes, resulting in stable PpSHI1pro:PpSHI1-GFP and PpSHI2pro:PpSHI2-GUS transformants in which the fusion proteins are expressed from the native promoters, thus precluding overexpression artefacts (Fig. 1G-I; see Fig. S2E in the supplementary material). A nuclear GUS signal was only detected after a short staining time and when ethanol destaining was omitted. We conclude that PpSHI1 and PpSHI2 are most likely active in the nucleus.

Cell- and tissue-specific expression of PpSHI1 and PpSHI2
To determine the spatial and temporal expression patterns of the two moss SHI/STY genes, we utilised the targeting constructs described above (see Fig. S3A,B in the supplementary material) to generate seven independent PpSHI1pro:PpSHI1-GUS lines and a single PpSHI1pro:PpSHI1-GFP line, all of which were verified by PCR analysis (see Fig. S3C,D in the supplementary material). All PpSHI2pro:PpSHI2-GUS lines and the PpSHI1pro:PpSHI1-GFP line showed the same overall expression pattern.
**PpSHI2** was expressed in caulonemal cells, whereas its expression was extremely low or absent in chloronemal cells (Fig. 1A). Buds and juvenile and adult gametophores showed staining restricted to the emerging rhizoids and to distinct spots in the apex, which could represent axillary hair primordia (Fig. 1B,C). In rhizoids, **PpSHI2** expression was detected already in the primordia (not shown), and subsequently peaked in mitotically active apical cells of developing rhizoids (Fig. 1C,D). Expression was stronger in short compared with longer rhizoids, and staining appeared similar in basal and mid-stem rhizoids [rhizoid definition according to Sakakibara et al. (Sakakibara et al., 2003)]. We did not see any evidence of a corresponding pattern in the morphologically similar caulonemata, in which **PpSHI2** appeared to be relatively evenly expressed regardless of the position, age and mitotic activity of the cells.

Furthermore, one to four **PpSHI2**-expressing axillary hairs were found adaxially and basally on each side of the midrib of young leaves (Fig. 1E,F). At the gametophore apex, stained cells, which most likely represent young axillary hairs, were evident already in the mass of mitotically active cells, surrounded by leaf primordia (Fig. 1E). The abundance of **PpSHI2**-expressing axillary hairs appeared to decrease gradually as the more basal parts of a gametophore were examined. Juvenile adventitious gametophores that occasionally grow out from the epidermal cell layer of an adult gametophore also expressed **PpSHI2** (not shown). Very little or no GUS staining was detected in the stems and leaves of the gametophores.

The **PpSHI1-GFP** expression pattern of the single **PpSHI1-GFP** line was very similar to that of **PpSHI2-GUS**: signals were detected in caulonema, rhizoids and axillary hairs, whereas chloronemal cells showed no or very low-level signals (Fig. 1G-I). In all cases, the **PpSHI1-GFP** product was mainly localised to the cell nuclei. Together, our results suggest that **PpSHI1** and **PpSHI2** have identical expression patterns in the vegetative gametophyte.

**PpSHI1/2 expression coincides with sites of auxin response**

The **PpSHI1/2** expression pattern in the adult gametophore, with maxima in rhizoids and axillary hairs, is almost identical to that of a *GmGH3pro:GUS* reporter (*Gm*, Glycine max (soybean)) used to detect sites of auxin response (Bierfreund et al., 2003; Fujita et al., 2008). If not coincidental, this correlation could indicate that the **PpSHI** genes are induced by auxin, or that their expression is a prerequisite for the generation of auxin peaks or auxin responsiveness. In order to discriminate between these alternatives, we compared the responses of *GmGH3pro:GUS* and **PpSHI2pro:GUS** lines to exogenous auxin. As previously reported (Bierfreund et al., 2003; Fujita et al., 2008), the *GmGH3* promoter was induced by auxin treatment in a concentration-dependent manner, which resulted in an almost uniform staining of the entire gametophore, suggesting that all cell types can respond to auxin (Decker et al., 2006) (Fig. 2). The fact that wild-type cells and tissues not expressing the **PpSHI** genes still exhibited auxin-induced *GmGH3pro:GUS* expression indicates that their products are not required for auxin sensing or signalling, but could be important for the generation of local auxin peaks.

**Phenotypic effects of constitutive **PpSHI1** expression**

In order to alter the strength and tissue specificity of **PpSHI1** activity, we expressed it from a modified 35S (E7113) promoter (Mitsuhara et al., 1996). Gene targeting was used to integrate the 35S-**PpSHI1** expression cassette into the nuclear BS213 locus, which has been shown not to affect moss growth and development per se when disrupted (Schaefer and Zryd, 1997). The background line used for these experiments carried the *GmGH3pro:GUS* reporter (Bierfreund et al., 2003), enabling monitoring of the spatial distribution of auxin activity.
Ten stable transformants were generated, and although the PpSHI1 expression level varied, it was enhanced in all five lines examined (see Fig. S4 in the supplementary material). Gene targeting in P. patens frequently results in the integration of multiple transgenes into the targeted locus (Kamisugi et al., 2006) and using a genomic real-time PCR approach we detected different transgene copy numbers in the five lines. Surprisingly, the correlation between transgene copy number and expression level was negative, indicating co-suppression activity (see Fig. S4 in the supplementary material). The expression levels in high-copy-number lines never dropped below that of the control line, though, suggesting that co-suppression might be transgene specific and that the native PpSHI1 copy escapes downregulation. We also found a negative correlation between the expression of PpSHI2 and PpSHI1 (see Fig. S4 in the supplementary material), suggesting that PpSHI2 could be feedback inhibited by elevated PpSHI1 expression. Given the overlap in expression patterns, it appears likely that in wild-type moss, feedback inhibition may be mediated by both PpSHI1/2 proteins and that the PpSHI protein level, directly or indirectly (but not via auxin), affects the activity of both genes. Alternatively, the endogenous genes might be co-suppressed by the transgenes.

The transgenic lines exhibited a distinct set of phenotypes that correlated roughly with the degree of PpSHI1 expression. Hence, the phenotypic differences to the background line were subtle in PpSHI1ox-3, moderate in PpSHI1ox-5, and striking in PpSHI1ox-1, PpSHI1ox-2 and PpSHI1ox-4. At the protonemal stage, caulonemata growth was stimulated while chloronemata growth was suppressed, resulting in low-density star-like colonies (Fig. 3). The stimulation of caulonema and suppression of chloronema formation are well known effects of external auxin treatment (Ashton et al., 1979), suggesting that the PpSHI1ox lines might exhibit increased auxin levels or auxin hypersensitivity. The PpSHI1ox caulonemata was hyperpigmented (Fig. 3A,B,G,H), which is another known effect of auxin treatment (Ashton et al., 1979). Interestingly, the formation of chloronemata from subapical caulonemal side branch initials at the periphery of the colony appeared to be normal. Instead, the reduction in chloronemata resulted from growth suppression and even regression in the central older part of the colony. Thus, aged chloronemal cells in the PpSHI1ox lines appeared to undergo necrosis: they lost chloroplasts, accumulated a water-soluble pigment and became increasingly vacuolated, resulting in what appeared to be empty shells (Fig. 3G,H).

Bud formation and subsequent transition into juvenile gametophores were induced earlier in PpSHI1ox lines than in the control (Fig. 3A,B). Whereas apical young leaves appeared normal,
mature leaves in the basal part of the transgenic gametophores wilted (Fig. 3E,F), resulting in smaller and less developed shoots (Fig. 3A-F). As described for aged chloronemata above, the majority of mature leaf cells showed a reduced number of chloroplasts, pigment accumulation and appeared to be dead or dying (Fig. 3L). The latter impression was reinforced by our finding that leaves from the older, basal part of the *PpSHI1*-overexpressing gametophores failed to regenerate when excised and placed on ammonium tartrate-enriched BCD media.

**PpSHI1 overexpression causes elevated auxin levels and activity**

In order to test whether *PpSHI1* overexpression could be linked to changes in auxin homeostasis, we measured IAA levels in 23-day-old colonies of the control line and of *PpSHI1ox-2, PpSHI1ox-3, PpSHI1ox-4* and *PpSHI1ox-5*. Three transgenic lines exhibited dramatically elevated IAA levels, with an average 14.5-fold increase compared with the control (Fig. 4A). The fourth line, *PpSHI1ox-3*, which did not show a significant increase, had only moderately elevated *PpSHI1* expression (see Fig. S4 in the supplementary material) and a very subtle phenotype.

We also assayed *GmGH3pro:GUS* activity in the *PpSHI1ox* lines to identify changes in auxin activity (Fig. 4B,C). In agreement with previous reports (Bierfreund et al., 2003; Fujita et al., 2008), *GmGH3pro:GUS* was expressed primarily in the base of the stem, in rhizoids and in axillary hairs in the wild type. *PpSHI1* overexpression resulted in increased stem and rhizoid staining, in terms of signal strength, penetration and distribution. Furthermore, a weak but clear staining of some leaf cells was also detected in *PpSHI1ox* lines, but never in the control line. However, we did not observe any increase in axillary hair staining. The differences observed generally correlated with *PpSHI1* expression levels (see Fig. S4 in the supplementary material), being more striking in the highly expressing lines.

Taken together, the auxin and *GmGH3pro:GUS* expression levels strongly suggest that *PpSHI1* overexpression causes an overproduction of auxin.

**Developmental abnormalities and reduced auxin biosynthesis in *Ppsi1* and *Ppsi2* knockout mutants**

We next produced two *PpSHI1* (*Ppsi1-1, Ppsi1-2*) and two *PpSHI2* (*Ppsi2-1, Ppsi2-2*) gene disruption lines by homologous recombination using the targeting constructs shown in Fig. S5A,B in the supplementary material. We used PCR to confirm that the wild-type loci had been properly targeted (see Fig. S5C,D in the supplementary material) and verified the loss of the corresponding transcripts by RT-PCR (see Fig. S5E in the supplementary material). Southern blots confirmed that the correct locus had been targeted in

---

**Table 1. Phenotypic data for knockout lines**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Wild type</th>
<th>Ppsi1-1</th>
<th>Ppsi1-2</th>
<th>Ppsi2-1</th>
<th>Ppsi2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Colony diameter (mm)</td>
<td>10.6±1.0</td>
<td>8.7±0.7</td>
<td>9.2±0.8</td>
<td>8.6±0.6</td>
<td>8.0±0.4</td>
</tr>
<tr>
<td>(B) Gametophores/colony</td>
<td>48.1±9.4</td>
<td>31.0±2.9</td>
<td>36.0±4.5</td>
<td>34.7±5.4</td>
<td>27.7±3.2</td>
</tr>
<tr>
<td>(C) Shoot length (mm)</td>
<td>6.7±0.7</td>
<td>3.1±1.1</td>
<td>3.2±1.0</td>
<td>2.5±0.2</td>
<td>3.2±0.5</td>
</tr>
<tr>
<td>(D) Leaves</td>
<td>28.0±4.6</td>
<td>28.0±3.0</td>
<td>27.8±3.3</td>
<td>28.2±2.6</td>
<td>25.6±3.1</td>
</tr>
<tr>
<td>(E) Internode length (mm)</td>
<td>0.24±0.04</td>
<td>0.11±0.04</td>
<td>0.12±0.03</td>
<td>0.09±0.01</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>(F) Rhizoids</td>
<td>126±27</td>
<td>152±38</td>
<td>107±22</td>
<td>108±19</td>
<td>125±30</td>
</tr>
<tr>
<td>(G) Most apical rhizoid</td>
<td>6.8±1.3</td>
<td>13.2±2.9</td>
<td>13.7±2.2</td>
<td>17.7±2.2</td>
<td>12.5±1.7</td>
</tr>
<tr>
<td>(H) Rhizoid length, 3w (mm)</td>
<td>5.3±1.2</td>
<td>5.6±1.0</td>
<td>n.d.</td>
<td>5.7±0.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>(I) Rhizoid length, 5w (mm)</td>
<td>5.1±0.5</td>
<td>7.3±0.8</td>
<td>n.d.</td>
<td>7.6±1.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>(J) Rhizoid length, 7w (mm)</td>
<td>6.2±0.9</td>
<td>9.8±1.1</td>
<td>n.d.</td>
<td>9.9±0.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>(K) Thick brown rhizoids</td>
<td>19.8±6.6</td>
<td>39.4±12.9</td>
<td>36.2±10.2</td>
<td>31.5±6.9</td>
<td>47.9±8.9</td>
</tr>
<tr>
<td>(L) Axillary hairs</td>
<td>2.7±0.9</td>
<td>4.6±1.0</td>
<td>4.7±0.9</td>
<td>4.5±1.3</td>
<td>5.2±1.0</td>
</tr>
</tbody>
</table>

Two- (A,B) and nine- (C-G,KL) week-old colonies. Means and s.d. are based on the analysis of 10-12 colonies or gametophores. (A) Colony diameter calculated as the mean of three measurements made at 45°, 90° and 135° angles. (B) Average number of gametophores per colony. (C) Average length of the shoot stem (measured after leaves had been detached). (D) Average number of juvenile and adult leaves per gametophore. (E) Internode length calculated using the values in rows C and D. (F) Average number of basal and mid-stem rhizoids per gametophore. (G) Average number of leaves above the most apical rhizoid. (H-J) Average length of the rhizoid bundle. Colony age is indicated as number of weeks (w). (K) Average number of thick brown rhizoids per gametophore. (L) Average number of axillary hairs associated with each of the ten apical-most leaves.

*P<0.001 (by Student’s t-test, as compared with wild type).
each line and showed that \textit{Ppshi}-1 lacked additional integration sites, whereas the other three lines also carried a small number of line-specific random insertions (see Fig. S6 in the supplementary material). Thus, the essentially identical phenotypes displayed by these four lines apparently depend on the one genetic alteration they have in common: the targeting of one of two redundant \( PpSHI \) loci. This indicates that the two genes provide similar functions and that one gene alone is insufficient to support wild-type growth and development. We also found three lines that appeared to be \( PpSHI \) knockouts based on both PCR and RT-PCR data (not shown), but which were phenotypically indistinguishable from the wild type, a fact for which we currently lack an explanation.

We were unable to PCR amplify the targeted locus in any of the four knockout lines using flanking primers (see Fig. SS5 in the supplementary material). This was probably due to the presence of tandem repeats of the transforming DNA at the insertion site, a common outcome after gene targeting in \( P. patens \) owing to in vivo concatenation prior to integration (Kamisugi et al., 2006). Southern blots confirmed this, but also revealed the inclusion of vector DNA in some of the repeats. Concatenation can be the result of either recombination between microhomologies, preferentially those longer than 12 bp, or of ligation of compatible sticky ends (Murén et al., 2009). None of our targeting fragments possesses direct repeats longer than 12 bp, and as we mainly used different restriction enzymes for their release from the vector, the vector was most likely used as a linker to produce the tandem repeats.

The colony diameter and emerging gametophore number were slightly reduced in young protonemal colonies of the knockout lines (Table 1; Fig. 5A). Both these effects appeared to result from a less efficient protrusion of caulonema filaments, which promote the radial spread of colonies (Cove et al., 2006) and serve as precursors for gametophore emergence (Ashton et al., 1979; Lehner and Bopp, 1983). The reduced size and compact appearance of \( Ppshi \) and \( Ppshi2 \) colonies were retained in older colonies.

The gametophore stem length was reduced at least 2-fold in all four mutants, whereas the leaf number remained unaffected, revealing that internode lengths were significantly reduced (Table 1). This phenotype, and the apparent inhibition of caulonema proliferation, are consistent with decreased auxin levels, as treatment with exogenous auxin produces the opposite phenotypes. Indeed, we found that the IAA level and biosynthesis rate were both reduced in mutants, whereas the leaf number remained unaffected, suggesting that the auxin level might not be reduced below the threshold level for rhizoid initiation in the basal stem. Instead, a successive increase in the difference in rhizoid length between mutants and wild type suggested that mutant rhizoids have an increased growth rate (Table 1) and most likely also a delayed elongation arrest (Fig. 5A). The mutants also displayed an unusually high proportion of thick and dark brown rhizoids (Table 1).

The occurrence of axillary hairs and emerging mid-stem rhizoids in leaf axils of Evans Blue-stained gametophores was examined (Table 1). Whereas in wild type the most apical mid-stem rhizoids are found associated with a leaf axil that has an average of 6.8 discernible leaves above it, the corresponding number was between 12.5 and 17.7 for the mutants, revealing a more basal mid-stem rhizoid localisation. We also found an unexpected increase in the number of axillary hairs in the mutant lines. The average number of hairs per axil for the ten apical-most leaves varied between 4.5 and 5.2 in the mutants, whereas there were only 2.7 hairs per axil in the wild type.

**Fig. 5.** \( Ppshi1 \) and \( Ppshi2 \) single-knockout phenotypes and IAA levels. (A) Colony morphology. The left and middle columns show 2- and 6-week-old colonies, respectively, whereas the right-hand column shows vertical slices through 7-week-old colonies of mutants and wild type. (B) Adult gametophores with intact rhizoid bundles. The same magnifications were used when comparing wild-type and knockout lines. (C, D) IAA concentration (C) and IAA biosynthesis rate (D) in apices from \( Ppshi1 \), \( Ppshi2 \) and the wild type as determined by GC-MS analysis. The IAA biosynthesis rate was calculated as the ratio between labelled IAA \([m/z (203+204)]\), measured as the incorporation of one to two deuterium atoms into the IAA molecule, and unlabelled IAA \([m/z 203]\), and corrections were made for the contribution of natural isotopic abundances to \( m/z \) 203 and \( m/z \) 204 as described (Ljung et al., 2005). Each data point is the average of three independent samples; error bars represent s.d.; *, \( P<0.05 \) (by Student’s \( t \)-test, as compared with the wild type).
Finally, we noticed a putative delay of ageing in the $Ppshi1$ and $Ppshi2$ knockout lines. Thus, gametophores in the central part of old wild-type colonies had a wilty phenotype with brownish stems and leaves. By contrast, $Ppshi1$ or $Ppshi2$ mutant colonies of the same age had mainly green and healthy gametophores, regardless of whether a central or peripheral part of the colony was examined.

**DISCUSSION**

Here, we have for the first time actively modified the endogenous auxin levels in $P. patens$, providing new tools for studies of the role of auxin in bryophytes. The changes in auxin levels were achieved by altering the expression of the two $PpSHI$ genes, and our data strongly suggest that these genes cooperatively induce IAA biosynthesis, at least in the gametophyte.

**The $PpSHI$ genes act as positive regulators of auxin biosynthesis**

The IAA level was increased more than 10-fold in lines substantially overexpressing $PpSHI1$. This is consistent with previous results from AtSTY1 overexpression (Sohlberg et al., 2006), suggesting a conserved role of $SHI/STY$ genes in auxin homeostasis. In accordance, GmGH3pro:GUS expression was significantly increased in $PpSHI1ox$ rhizoids and gametophore stems, and expanded to cells outside the normal GmGH3 expression domain, causing a uniform staining of both rhizoids and gametophore stems, as well as the staining of some leaf cells. Reduced IAA levels and biosynthesis rates in gametophore shoots of $PpSHI1$ and $PpSHI2$ knockout lines provide additional support for the hypothesis that $SHI/STY$ proteins act as positive spatiotemporal regulators of auxin biosynthesis not only in Arabidopsis, but also in moss. It would be most interesting to investigate whether the $PpSHI$ genes and AtSTY1 act through homologous auxin biosynthesis target genes, but our experience from Arabidopsis (Sohlberg et al., 2006) suggests that we need inducible overexpressor lines for such studies in order to avoid looking at secondary effects, and we do not have access to such moss lines yet.

**Overlapping $PpSHI1/2$ and GmGH3 expression suggests that local auxin biosynthesis contributes to the auxin response maxima**

The $PpSHI1pro:PpSHI1-GFP$ and $PpSHI2pro:PpSHI2-GUS$ reporters were expressed in caulonema, at rhizoid initiation sites, in rhizoids with a specific preference for apical cells, and in axillary hairs, suggesting that these are the sites of auxin biosynthesis. They also represent sites of high auxin activity in moss (Bierfreund et al., 2003; Fujita et al., 2008), strongly implying that auxin response positions are at least partially determined by spatiotemporal control of auxin biosynthesis, in which the $PpSHI$ genes most likely play an important role. The one exception to the overlap between $PpSHI$ and auxin response activity was in developing rhizoids, where GmGH3pro:GUS had a basal and $PpSHI$ an apical expression maximum. Interestingly, this apparent discrepancy could support the existence of basipetal auxin transport in rhizoids, as suggested by Rose and Bopp (Rose and Bopp, 1983).

**Phenotypic effects of reduced $PpSHI$ expression are consistent with a reduction in endogenous auxin levels**

Since moss lines with verified reductions in the endogenous auxin levels have not been described previously, it is not known what phenotypic effects such alterations would cause. However, one might expect effects, i.e. reduced caulonema formation and gametophore internode elongation, opposite to those resulting from exogenous IAA application (Ashton et al., 1979; Fujita et al., 2008; Johri and Desai, 1973). Indeed, the $Ppshi1$ and $Ppshi2$ single-knockout mutants possessed these expected traits. In addition, they produced fewer gametophores per colony, supporting previous assumptions that gametophore number is dependent on auxin levels (Ashton et al., 1979). Interestingly, the mutant colonies and gametophores stayed green and healthy much longer than in the wild type and the rhizoid growth period appeared significantly extended, suggesting a delay in growth arrest and perhaps also ageing. Rhizoids are tip-growing filaments that have rooting functions similar to the root hairs of vascular plants (Menand et al., 2007). Rhizoid initiation is stimulated by exogenous auxin (Sakakibara et al., 2003), but to our surprise we found that the number of rhizoids in the $Ppshi1$ and $Ppshi2$ mutants was unaffected, suggesting that the auxin levels in the rhizoid-forming stem epidermal cells remained above the threshold level required for rhizoid formation. However, the more basal position of mid-stem rhizoids in the mutant lines might be a consequence of reduced auxin levels at least in the apical axillary hair-dense part of the leafy shoot, as exogenous auxin induces a more apical positioning of mid-stem rhizoids in wild-type gametophores (Sakakibara et al., 2003). Furthermore, the number of axillary hairs was significantly increased in the knockout mutants. This suggests that the reduced $PpSHI$ expression might induce a compensatory programme that strives to provide the shoot with more auxin-producing units. These data are intriguing, and further studies of the regulatory pathways for axillary hair formation would be very interesting.

No $Ppshi1$ $Ppshi2$ double-knockout mutants were recovered, despite several independent attempts to make such lines, suggesting that the $PpSHI$ genes together perform an essential function during the early stages of protoplast regeneration and/or colony formation. This, together with the fact that the single-knockout lines have identical phenotypes, strongly suggests that the two $PpSHI$ genes act in concert in a dose-dependent manner. The activity of the $SHI/STY$ proteins in Arabidopsis is also very dose dependent, and a dominant-negative suppressor construct results in seedling lethality (Kuusk et al., 2006; Ekland et al., 2010), suggesting that the Arabidopsis SHI/STY proteins together perform essential functions during early development.

**Ectopic PpSHI activity promotes premature cell death**

Apart from affecting the caulonema-to-chloronema ratio positively, $PpSHI1$ overexpression also appears to induce premature cell death in chloronema and leafy shoots, most likely through elevated auxin levels. Necrotic cells, exhibiting pigment accumulation, a reduced number of chloroplasts and a loss of regeneration capacity, were identified in older chloronema and leaves. Since we were unable to detect $PpSHI1pro:PpSHI1-GFP$, $PpSHI2pro:PpSHI2-GUS$ or GmGH3pro:GUS expression in leaf cells of wild-type control lines, we conclude that the necrotic effects are likely to be due to ectopic misexpression of the $PpSHI1$ protein, suggesting that leaf cells normally represent low auxin zones, and that the ectopic $PpSHI1$ expression might induce premature cell death in leaf cells. This might well apply also to chloronema, which are morphologically very similar to leaf cells. Accordingly, the observed excess of caulonema in the $PpSHI1$-overexpression lines could, at least in part, be due to selective loss of chloronema.
Auxin appears to regulate similar developmental decisions in vascular plants and bryophytes

Analogous to its function in vascular plants, auxin plays a role in aerial tissue elongation in moss, as exemplified by its role in the induction of gametophore stem elongation (our data) (Fujita et al., 2008). In vascular plants, auxin accumulation also occurs at organ initiation sites, both during aerial organ development and the initiation of lateral root primordia (Benkova et al., 2003). Our data and studies using auxin reporter constructs (Bierfreund et al., 2003; Fujita et al., 2008) suggest that auxin in moss accumulates in rhizoid initial cells, but not in leaf initials. Instead, the PtySHI genes and auxin response reporter genes are expressed in axillary hairs, which are positioned close to the lateral meristem-like sites from which mid-stem rhizoids and gene expression of axillary hairs, which are positioned close to the lateral meristem-like sites from which mid-stem rhizoids and adventitious shoots emerge. Their structural complexity varies between moss species, but in P. patens we have found them to normally consist of one large protruding cell anchored to the epidermal cell layer via one or two small connector cells (Fig. 1F, inset). Axillary hairs from some species secrete mucilage (Ligrone, 1986), but little else is known about their function. The mucilage has been proposed to protect young leaves from desiccation (Schofield and Hebant, 1984), but the hairs might also have additional functions. It is tempting to speculate that axillary hairs contribute to the regulation of lateral organ formation. A possible mechanism is the secretion of auxin into the mucilage, from where it could be transported to, and thus affect, nearby cells. We found numerous axillary hairs associated with very young leaf primordia close to both apical and lateral meristem-like regions, and Harrison et al. (Harrison et al., 2009) have shown that an axillary hair is initiated on the young bud, prior to the formation of the first leaf primordia, suggesting the possibility that axillary hairs contribute to the regulation of organ initiation, growth, development and differentiation. The organ in flowering plants, which occupies a position seemingly analogous to that of axillary hairs in mosses, is the stipule. Although future studies will be needed to show whether stipules and axillary hairs are functionally analogous, or even homologous, we note that stipules in Arabidopsis represent potential sites of auxin production and accumulation, based on the expression of auxin response markers (Aloni et al., 2003) as well as of SHI/STY gene family members (Fredborg et al., 2001) and their proposed targets (Cheng et al., 2007). In conclusion, our results show that manipulations of endogenous auxin levels are useful tools for elucidating the functional roles of auxin in moss. We were able to test hypotheses from previous work that used exogenous application of auxin, and were able to identify new phenotypes that are most likely caused by ectopic auxin production as well as by the reduction in auxin production in specific tissues.

Acknowledgements

We thank Mitsuyasu Hasebe for the pCMK1 plasmid, Eva Decker for the GmGH3pro::GUS strains and Sofia Eitem and Roger Granborn for technical assistance. This work was supported by grants from the Carl Tryggers Foundation to M.T., from the Swedish Research Councils Formas and VR to E.S. and from Formas to H.R.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.orglookup/suppl?doi=10.1242/dev.039594/DC1

References


