# Moss (*Physcomitrella patens*) GH3 proteins act in auxin homeostasis

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## Summary

• Auxins are hormones involved in many cellular, physiological and developmental processes in seed plants and in mosses such as *Physcomitrella patens*. Control of auxin levels is achieved in higher plants via synthesis of auxin conjugates by members of the GH3 family. The role of the two GH3-like proteins from *P. patens* for growth and auxin homeostasis was therefore analysed.

• The *in vivo*-function of the two *P. patens* GH3 genes was investigated using single and double knockout mutants. The two *P. patens* GH3 proteins were also heterologously expressed to determine their enzymatic activity.

• Both *P. patens* GH3 enzymes accepted the auxin indole acetic acid (IAA) as substrate, but with different preferences for the amino acid to which it is attached. Cytoplasmic localization was shown for PpGH3-1 tagged with green fluorescent protein (GFP). Targeted knock-out of either gene exhibited an increased sensitivity to auxin, resulting in growth inhibition. On plain mineral media mutants had higher levels of free IAA and less conjugated IAA than the wild type, and this effect was enhanced when auxin was supplied. The *ΔPpGH3-1/ΔPpGH3-2* double knockout had almost no IAA amide conjugates but still synthesized ester conjugates.

• Taken together, these data suggest a developmentally controlled involvement of *P. patens* GH3 proteins in auxin homeostasis by conjugating excess of physiologically active free auxin to inactive IAA-amide conjugates.

## Introduction

Auxins regulate or influence diverse aspects of plant growth and development, like tropisms, apical dominance and root initiation. On the cellular level, responses such as cell elongation, division and differentiation are triggered by auxins (Davies, 2004). While indole-3-acetic acid (IAA) influences the growth of plants positively at low concentrations, higher concentrations may be toxic (Bandurski *et al.*, 1995). Therefore, an IAA homeostasis is required for coordinated plant development. Several mechanisms to achieve such a control are possible: biosynthesis, degradation, conjugation and transport (Normanly & Bartel, 1999). Indoleacetic acid is presumably stored as physiologically inactive conjugates. These may be formed with amino acids or sugars (Cohen & Bandurski, 1982), which appear to be present at varying concentrations in the diverse tissues of angiosperms (Domagalski *et al.*, 1987). In addition, IAA can be conjugated to proteins in different plant species (Walz *et al.*, 2002; Seidel *et al.*, 2006). In seed plants up to 90% of the total IAA can be stored as conjugates in different tissues, while in nonseed plants this percentage could drop to 30% (Sztein *et al.*, 2000).

The hydrolysis of amino acid-type IAA conjugates has been studied in great detail, revealing gene families from various plant species with distinct, yet overlapping substrate specificities for various conjugates of IAA and of indole-3-butyric acid (IBA) (LeClere *et al.*, 2002; Campanella *et al.*, 2003, 2004, 2008; Schuller & Ludwig-Müller, 2006). Genes for the synthesis of IAA amide conjugates were not uncovered until recently (Staswick *et al.*, 2002, 2005). In *Arabidopsis thaliana* several members of the GH3-protein family activate IAA by

adenylation (Staswick *et al.*, 2002), which subsequently leads to the addition of the amino acid (Staswick *et al.*, 2005). *GH3* was initially isolated by differential hybridization screening as an auxin-induced cDNA clone from etiolated soybean hypocotyls (Hagen *et al.*, 1984). It was classified as a primary response gene because induction starts just 5 min after auxin application (Hagen & Guilfoyle, 1985). *GH3*-like homologs were also isolated and characterized from tobacco (Roux & Perrot-Rechenmann, 1997), *A. thaliana* (Hsieh *et al.*, 2000; Nakazawa *et al.*, 2001; Tanaka *et al.*, 2002; Takase *et al.*, 2003, 2004), rice (Jain *et al.*, 2006; Terol *et al.*, 2006) and the moss *Physcomitrella patens* (Imaizumi *et al.*, 2002; Bierfreund *et al.*, 2004). The function of GH3 proteins is still not clear for all members. In *A. thaliana*, mutant analysis has indicated a possible function in light signaling (Nakazawa *et al.*, 2001; Tanaka *et al.*, 2002; Takase *et al.*, 2003, 2004; Park *et al.*, 2007a), but an involvement in stress responses was also reported (Park *et al.*, 2007b). Another member AtGH3-11 (JAR1) is able to synthesize jasmonic acid amino acid conjugates (Staswick & Tiryaki, 2004). *AtGH3-9* seems to be specifically active in roots, controlling development by maintaining auxin homeostasis (Khan & Stone, 2007). Also, *AtGH3-13*, which does not adenylate IAA, is involved in defence responses against bacterial pathogens (Jagadeeswaran *et al.*, 2007). In rice it was shown that activation of *GH3*-gene expression increased tolerance to bacterial pathogens, possibly via an influence on downregulation of expansin gene expression (Ding *et al.*, 2008).

The moss *P. patens* has become a model organism for studying plant differentiation and developmental processes under an evolutionary perspective (Cove *et al.*, 2006). In the bryophytes, including mosses, liverworts and hornworts, auxin conjugation was already developed, although conjugate formation was slower than in angiosperms (Sztein *et al.*, 2000; Cooke *et al.*, 2002) indicating that conjugates may play a different role in homeostasis or other mechanisms are still active to detoxify excess hormone. Thus, *P. patens* could be a valuable organism to investigate evolutionary aspects of plant development and plant hormone homeostasis. In addition, the average size of gene families is reduced compared, for example, with *A. thaliana* (Rensing *et al.*, 2002), thus allowing a more comprehensive analysis of individual members with less functional redundancy.

In mosses, auxin induces the transition from the chloronema to the caulonema cell type within the juvenile protonema stage (Johri & Desai, 1973) and a shift in cell-cycle control (Schween *et al.*, 2003). The protonema is a branched filamentous tissue that grows by tip cell divisions (Reski, 1998). An auxin-sensitive beta-glucuronidase (GUS) reporter system indicated differential auxin responsiveness within the protonema and adult gametophore tissues, respectively, with highest levels in actively growing cells (Bierfreund *et al.*, 2004). Two *GH3*-like homologs, named *PpGH3-1* and *PpGH3-2*, were isolated from the *P. patens* genome (Bierfreund *et al.*, 2004), indicating that auxin conjugation may also take place in this plant. Both genes are expressed in gametophytic tissues, with expression starting very early in moss development. However, no obvious differences were found between wild type and *ΔPpGH3* single knockout mutants under various light conditions (white-light, red-light, far-red light), indicating at least partially overlapping functions of the two GH3 proteins (Bierfreund *et al.*, 2003). We extended this study and investigated hormone sensitivity and hormone content in these *ΔPpGH3* mutants. We concentrated on IAA and jasmonic acid (JA) because in *A. thaliana* these two hormones are substrates for several members of the GH3-protein family (Staswick *et al.*, 2002). In addition, we analysed the role of the two GH3 homologs from *P. patens* by *in vivo* metabolism studies as well as by determination of possible adenylating enzyme activity of recombinantly expressed PpGH3 proteins with IAA and JA. Finally, to overcome possible functional redundancy, we generated *ΔPpGH3-1/ΔPpGH3-2* double mutants by targeted gene disruption which were also analysed for free and conjugated IAA levels. Our data reveal a role for moss GH3 proteins in auxin homeostasis and indicate an additional mechanism for detoxification of auxin in moss.

## Materials and Methods

### Plant material and growth conditions

*Physcomitrella patens* (Hedw.) B.S.G. were cultivated as previously described (Frank *et al.*, 2005). Creation of PpGH3-1 and PpGH3-2 single knockout lines was described earlier (Bierfreund *et al.*, 2004). Plants in suspension cultures were subcultivated weekly. For protoplast isolation, protonema was grown for 1 wk in Knop medium (Reski & Abel, 1985) with a reduced pH (4.5). All knockout lines described in this study are deposited in the International Moss Stock Center with the accessions IMSC 40206-40212 (GH3-1/A31, GH3-1/B34, GH3-2/22, GH3-doKO-A96, GH3-doKO-A129, GH3-doKO-B2 and GH3-doKO-B12).

### Generation of *ΔPpGH3-1/ΔPpGH3-2* double mutants

For preparation of *ΔPpGH3-1/ΔPpGH3-2* double knockout mutants, protoplasts from *ΔPpGH3-1* mutants were co-transfected with the *PpGH3-2* knockout construct (Bierfreund *et al.*, 2004) and a hygromycin selection cassette. After 2 wk of regeneration the plants were grown under selective conditions on Knop medium containing 30 mg $l^{-1}$ hygromycin for 3 wk. Direct polymerase chain reaction (PCR) analysis from plant material (Schween *et al.*, 2002) was performed to check homologous integration of the PpGH3-2 knockout construct in the *GH3-2* gene locus with the primer combinations described before (Bierfreund *et al.*, 2004). The loss of the *GH3-2* transcript in the double mutants was confirmed with the

primers: 5′-CTGAGCTTCGCTCAAGTGTTCT-3′ and 5′-GACATACGAAGGCCACCTTGG-3′.

### Construction of GH3-1::GFP and detection by confocal laser scanning microscopy (CLSM)

To generate a GH3-1::GFP fusion construct the complete open reading frame of *PpGH3-1* (accession no. AJ428956) was inserted upstream of the green fluorescent protein (GFP) coding sequence into the plasmid mAV4mcs. Localization of the GH3-1::GFP fusion was analyzed in transfected moss protoplasts by CLSM (TCS 4D; Leica Microsystems, Heidelberg, Germany) using 488 nm excitation and measurement of emission from 510 to 580 nm. As a control for cytoplasmic and nuclear localization, protoplasts were transfected with the 'empty' mAV4mcs plasmid.

### Hormone sensitivity assay

Single gametophores were harvested from agar cultures and placed on fresh agar plates with and without either 10 μM IAA or JA. For the concentration dependence the plates contained 0.1, 0.5, 1, 5, 10, 50 and 100 μM IAA or JA. For each concentration and line five plates were set up containing five individual gametophores each. Light conditions were as described above. Plants were inspected for the first time after 2 wk and then every week the diameter of each culture was recorded at the widest point between borders of the culture. At the end of the experiment (4 or 5 wk after transplanting) the number of protonema-forming cultures was also recorded, and in the case of 100 μM IAA the number of plants that survived this concentration was counted.

### Gravitropism assay

Gametophores were placed on Petri dishes with and without 10 μM IAA. The Petri dishes were cultivated for 1 wk to ensure plant growth and then turned vertically. The growing protonema was evaluated after 15 d and after 25 d.

### Auxin determinations

The gametophore cultures were harvested 5 wk after transplanting on agar containing hormone (10 μM IAA) or control agar. Protonema was grown as described above under control conditions and with 10 μM IAA in the liquid medium. One experiment was conducted with 10 μM IAA in the medium for the whole culture period and in a second set IAA was added 24 h before harvest. The plant material was extracted with isopropanol–acetic acid (95 : 5, v : v). To each sample 100 ng $^{13}C_6$-IAA (Cambridge Isotope Laboratories, Andover, MS, USA) was added. For each line three independent extractions were performed. The samples were incubated under continuous shaking (500 rpm) for 2 h at 4°C. The samples were then centrifuged for 10 min at 10 000 ***g***, the supernatant removed and evaporated to dryness under a stream of $N_2$. For determination of free IAA the residue was suspended in methanol, centrifuged again for 10 min at 10 000 ***g***, the supernatant removed and placed in a glass vial. The methanol was evaporated under a stream of $N_2$ and the sample was suspended in 50 μl ethyl acetate. Hydrolysis of ester-conjugated IAA was performed with 1 M NaOH for 1 h at 25°C and for amide conjugated IAA with 7 M NaOH at 100°C under $N_2$ for 3 h. The hydrolysate was filtered, the pH brought to 2.5 and the auxins were extracted twice with equal volumes of ethyl acetate. The organic phase was evaporated and the residue dissolved in 50 μl ethyl acetate. Methylation of all samples was carried out with diazomethane (Cohen, 1984). For gas chromatography–mass spectrometry (GC-MS) analysis the sample was suspended in 20 μl ethyl acetate. The GC-MS analysis was carried out on a Varian Saturn 2100 ion-trap mass spectrometer using electron impact ionization at 70 eV, connected to a Varian CP-3900 gas chromatograph equipped with a CP-8400 autosampler (Varian, Walnut Creek, CA, USA). For the analysis 2.5 μl of the methylated sample was injected in the splitless mode (splitter opening 1 : 100 after 1 min) onto a Phenomenex ZB-5 column, 30 m × 0.25 mm × 0.25 μm (Phenomenex, Darmstadt, Germany) using He carrier gas at 1 ml min$^{-1}$. The injector temperature was 250°C and the temperature program was 70°C for 1 min, followed by an increase of 20°C min$^{-1}$ to 280°C, then 5 min isothermically at 280°C. For higher sensitivity, the μSIS mode (Wells & Huston, 1995) was used. The settings of the mass spectrometer were as described in (Campanella *et al.*, 2003). The endogenous concentrations of IAA were calculated according to the principles of isotope dilution (Cohen *et al.*, 1986) monitoring the quinolinium ions at *m/z* 130/136 (ions deriving from endogenous and $^{13}C_6$-IAA, respectively). Conjugated IAA was calculated by subtraction of the amount of free IAA from the amount obtained after hydrolysis with 7 M NaOH. Ester conjugates were calculated by subtracting free IAA levels and amide conjugates obtained after subtracting the ester-bound fraction from total conjugates.

### Metabolism of IAA

For the metabolite analysis, methylated extracts of gametophores and protonema cultures grown both, in the absence and presence of 10 μM IAA as described above were evaporated, dissolved in methanol and analysed by high-pressure liquid chromatography (HPLC) using Jasco BT8100 pumps coupled to an AS1550 autosampler and a MD-915 photo diode array detector (Jasco GmbH, Groß-Umstadt, Germany). Separation was on a Luna® Reverse phase $C_{18}$ column (Phenomenex) using a gradient system with 1% aqueous acetic acid and 100% methanol as solvent. The gradient started with 20% B, then increased in 20 min to 50% B, increased further within 10 min to 70% B, and finally within 10 min to 100% B,

followed by an equilibration step to the initial run conditions. Metabolites were quantified based on the HPLC peak area at 280 nm and methylated standards of IAA and the amide conjugates IAA-Ala, IAA-Asp, IAA-Gly, IAA-Leu, IAA-Ile, IAA-Phe and IAA-Val. Alkaline hydrolysis was carried out as described above with 7 N NaOH for 3 h at 100°C and the extract was chromatographed after extraction with ethyl acetate at pH 3.5 under the same conditions. For identification of the IAA conjugates formed on auxin containing medium approx. 10 g of wild-type gametophore tissue grown for 4 wk on 10 μM IAA was extracted and methylated as described above and fractions corresponding to the putative IAA conjugate peaks were collected after HPLC separation. The fractions were evaporated to dryness, suspended in 10 μl ethyl acetate and analyzed by GC-MS (see earlier).

### Identification of IAA amino acid conjugates

The HPLC-purified fractions corresponding to the putative IAA conjugate peaks were analysed by GC-MS. The settings were the same as described above. The following temperature program was used for the separation of IAA and its conjugates. The program started at 70°C for 2 min, followed by an increase of 10°C $min^{-1}$ to 300°C, then 10 min isothermically at 300°C. The methyl esters of IAA and the conjugates with Ala, Asp, Leu, Ile, Phe and Val eluted under these conditions at 16.8, 22.2, 24.2, 23.8, 27.2 and 23.1 min, respectively. Spectra were taken in the full scan mode and compared with the spectra of respective standards.

### Expression of PpGH3-1 and PpGH3-2 cDNA in *Escherichia coli*

The full length *PpGH3*-cDNA sequences (accession numbers AJ428956 and AJ429070 for *PpGH3-1* and *PpGH3-2*, respectively) were cloned into an expression vector (pGEX 4T3, Amersham Bioscience) for expression as glutathione S-transferase (GST) fusion proteins in *E. coli* BL21 codon plus cells (Stratagene, La Jolla, CA, USA). The cDNAs were amplified from the original plasmids (Bierfreund *et al.*, 2004) using gene-specific primers (*PpGH3-1* forward: 5′-ATGCGGATCCATGGCATCTGAGGTCGCGAAGC-3′; reverse: 5′-GTACGAATTCGGACCCGGTGAGGGCGATTGG-3′; *PpGH3-2* forward: 5′-ATGCGGATCCATGGCGCTTAGAATAGAGAACC-3′; reverse: 5′-CGGCGAATTCTCCAACGATTTTTGTAGATGGT-3′) including restriction sites for *Bam*H1 (forward primers) and *Eco*R1 (reverse primers), which have been used to clone the GH3 genes into pGEX. For comparison of the enzymatic activity *A. thaliana GH3-3* (At2g23170) amplified from leaf cDNA was cloned into the same expression vector (*AtGH3-3* forward: 5′-TAGGATCCATGACCGTTGATTCAGCTCTGC-3′; reverse: 5′-TAGAATTCACGACGACGTTCTGGTGACCAATG-3′). All amplifications were performed with AccuPrime Pfx polymerase (Invitrogen, Carlsbad, CA, USA) using standard amplification conditions. Annealing temperatures and cycle numbers were 68°C for 30 cycles, 66°C for 35 cycles and 68°C for 35 cycles for *PpGH3-1*, *PpGH3-2* and *AtGH3-3*, respectively. The correct orientation and reading frame of the sequence was confirmed by cycle sequencing (Beckman Coulter, Fullerton, CA, USA). Expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 3 h at 22°C. Cells were harvested, washed in buffer (9.1 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 55 mM $MgCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7) and dissolved for cell lysis in 1 ml lysis buffer (140 mM $NaCl_2$, 2.7 mM KCl, 10 mM $NaHPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4) containing freshly added lysozyme (1 mg $ml^{-1}$), 10 mM $MgCl_2$, and 10 U $ml^{-1}$ DNase 1. The suspended cells were subjected to three freeze–thaw cycles (30 s in liquid $N_2$, then 5 min at 37°C), centrifuged for 20 min at 4°C and 13 000 ***g***. Protein purification of the supernatant was performed with Glutathione Sepharose 4B (Bulk GST Purification Module; Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturer's protocol. After elution from the matrix the supernatant was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% SDS-PAGE minigels; Bio-Rad, Hercules, CA, USA), prepared and run according to the manufacturer's protocols) to analyse the purity and determine the approximate amount of protein in the sample.

### *Enzyme* assay for conjugate synthetase activity

Elution of bound fusion proteins for the enzyme assay was performed as described earlier. Activity assays with IAA, IBA or JA as substrates were performed according to Staswick & Tiryaki (2004) and Staswick *et al.* (2005). Assays for conjugate formation were done at room temperature for 1 h or 12 h in a total volume of 20 μl 50 mM Tris-HCl, pH 8.6 (for the pH dependence pH values ranging from 7 to 9 were adjusted), 3 mM $MgCl_2$, 3 mM ATP, 1 mM dithiothreitol (DTT), 1 mM IAA, IBA or JA, and 1 mM of the respective amino acid. The reaction was started with 10 μg of purified GH3-GST fusion protein per assay. The complete reactions were analyzed on silica gel 60 $F_{260}$ plates (Merck, Darmstadt, Germany) developed in chloroform–ethyl acetate–formic acid (35 : 55 : 10, v : v), except that 2-propanol–ammonium hydroxide–$H_2O$ (8 : 1 : 1, v : v) was used for reactions with amino acids His, Ser, Thr, Arg, Lys, and Cys. Staining for indoles was done with van Urk–Salkowski reagent (Staswick *et al.*, 2005) and for jasmonates with vanillin reagent (Staswick & Tiryaki, 2004).

## Results

### PpGH3-1 and PpGH3-2 convert IAA, IBA and JA to amide conjugates

The two GH3-like cDNAs from *P. patens* (Bierfreund *et al.*, 2004) are homologous to *A. thaliana* GH3 proteins that are

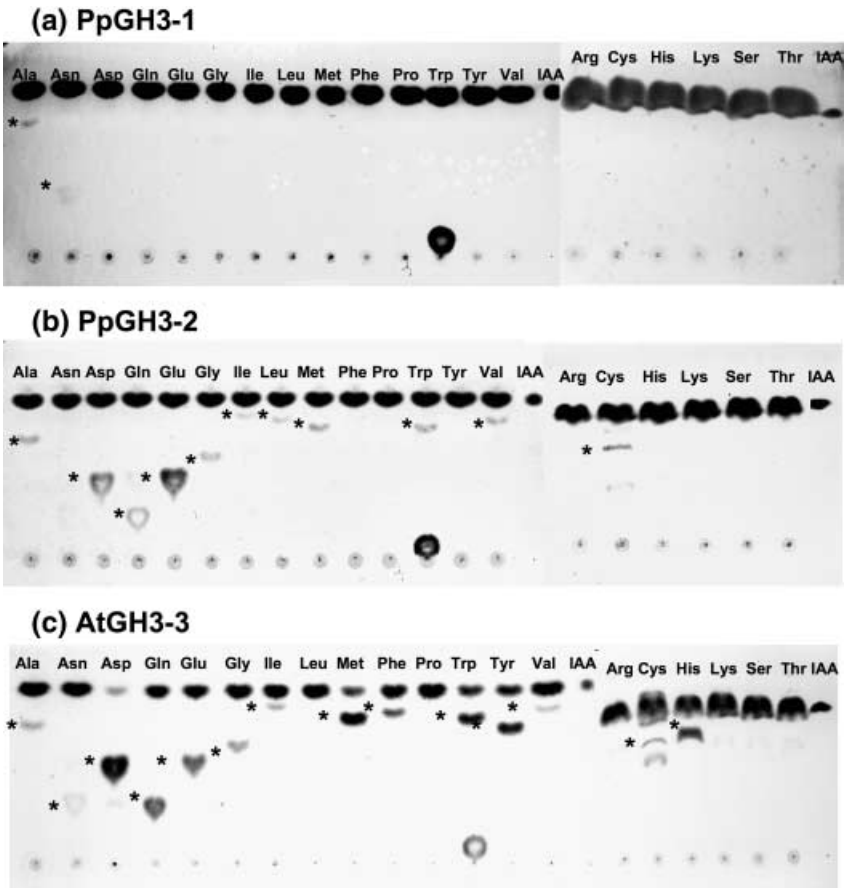


**Fig. 1** Conjugation of auxin indole acetic acid (IAA) to various amino acids by GH3 proteins from *Physcomitrella patens* (a, PpGH3-1; b, PpGH3-2) and *Arabidopsis thaliana* (c, AtGH3-3). Incubation time was 12 h for all purified enzymes expressed in *Escherichia coli*. The separation was done on thin-layer chromatography (TLC) plates as described in the Materials and Methods section. Note that for the amino acids Arg, Cys, His, Lys, Ser and Thr a different solvent system was used. Detection of indole derivatives has been performed with Ehmann's reagent. Amino acids used are given above the respective lane in three-letter codes. The asterisk indicates the band for the respective conjugate. For the IAA amino acid conjugates available standards were also chromatographed which showed the same retention time as the conjugates formed by the PpGH3 enzymes (data not shown).

able to catalyse the formation of amino acid conjugates with IAA and JA (Staswick & Tiryaki, 2004; Staswick *et al.*, 2005). Genome analysis confirmed that the two GH3 genes previously described are the only homologs present in *P. patens* (Rensing *et al.*, 2008; www.cosmoss.org). Both *P. patens GH3* genes are more closely related to JAR1, the protein that forms JA conjugates, than to the group of IAA adenylating enzymes (Bierfreund *et al.*, 2004; Terol *et al.*, 2006). However, to date there is no direct evidence for the involvement of jasmonates in the development of *P. patens*, whereas there are indications for various roles of auxin (Rensing *et al.*, 2008). In addition, there is evidence for the conjugation of IAA to regulate auxin homeostasis in bryophytes (Sztein *et al.*, 1999, 2000). In order to analyse their substrate specificity, the two *P. patens GH3* cDNAs were cloned and expressed in *E. coli* as GST fusions. We have concentrated on the two possible hormone substrates auxin and JA with 20 amino acids. Both *P. patens* GH3 proteins showed activity as amino acid conjugate synthetases, as shown by thin-layer chromatography (TLC) analysis (Figs 1–3). We tested two different auxins, IAA and indole-3-butyric acid (IBA) which have been described to be substrates for the adenylating enzymes of *A. thaliana* (Staswick *et al.*, 2005). On TLC the PpGH3-1 protein produced a band of IAA-Ala and in very small amounts also formed the IAA-Asn conjugate (Fig. 1a). In one experiment a reaction product was also observed with IAA and Gly (data not shown). The PpGH3-2 enzyme formed several IAA conjugates *in vitro* including conjugates with Asp, Ala, Gln, Glu, Gly, Met, Trp, Cys, Leu, Ile and Val, the last three being potential *in vivo* metabolites (Fig. 1b). However, the enzyme was less active than AtGH3-3 from *A. thaliana*, which was assayed for comparison (Fig. 1c). We chose reaction conditions described previously for *A. thaliana* (Staswick *et al.*, 2005), with a prolonged incubation time for the *P. patens* GH3 enzymes. Quantification of IAA conjugates on HPLC showed that compared with AtGH3-3 the PpGH3-2 protein had only *c.* 20% of the activity with Asp and IAA (data not shown). PpGH3-2 had a pH optimum at high pH values (8.5–9.0) when assayed with IAA which was similar to that of the *A. thaliana* GH3 proteins (data not shown) (Staswick *et al.*, 2005).

Indole-3-butyric acid was also a good substrate when it was tested with a subset of amino acids (see the Supporting Information, Fig. S1 and Table S1), but in case of PpGH3-2 fewer amino acids were accepted as substrates. Amino acid conjugates were formed by PpGH3-1with Ala, Asn and

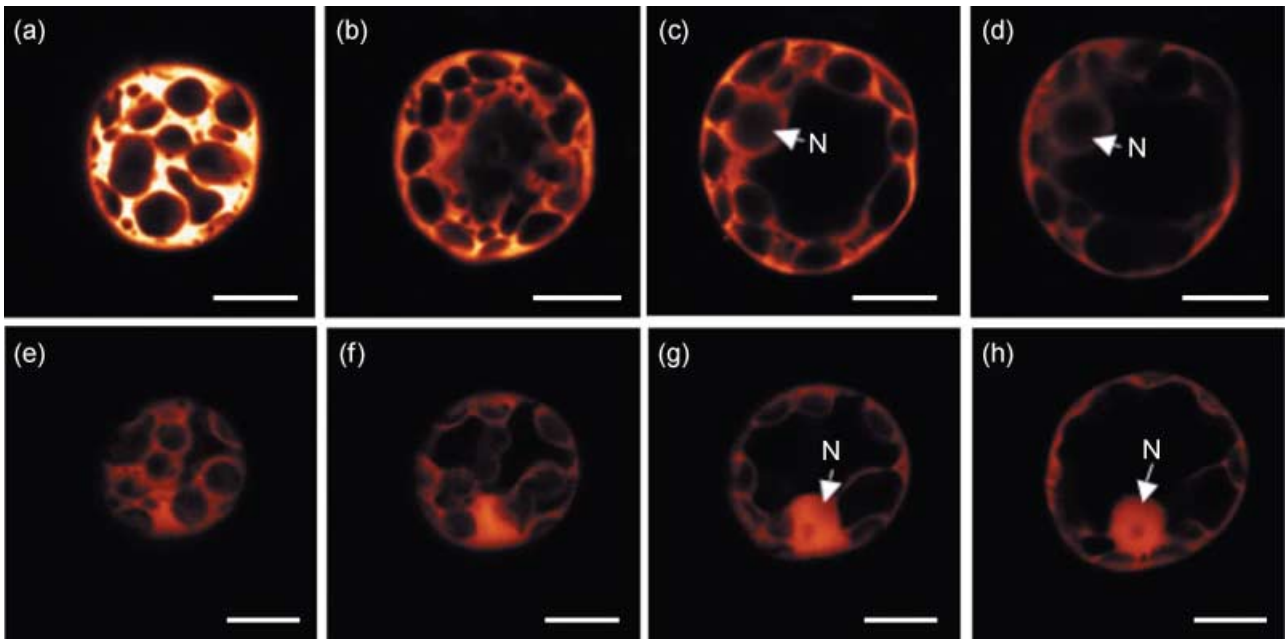


**Fig. 2** PpGH3-1 is localized within the cytoplasm. Optical sections of a *Physcomitrella* protoplast transfected with a PpGH3-1::GFP *C*-terminal fusion construct (a–d) as well as unfused GFP (e–h), which can be detected in cytoplasm as well as in the nucleus (N). The GH3-1 fusion prevents GFP from passive transport into the nucleus. Bars, 10 μm.

Gly, and by PpGH3-2 with Asp, Glu, Trp and Val. The activity of PpGH3-2 with IBA and Glu was remarkably higher than any of the tested combinations with IAA. The physiological relevance of IBA and Glu in mosses has yet to be determined.

When the two *P. patens* proteins were assayed with JA as substrate, both enzymes were able to form a range of amino acid conjugates with JA, but almost did not overlap in substrate specificity (Fig. S2, Table S1). PpGH3-1 showed enzymatic activity with JA and the amino acids Ala, Asn, Gly, Ser and Thr, as shown by the conjugate formation on TLC, whereas PpGH3-2 was active with the amino acids Asp, Gln, Glu, Gly, Ile, Leu, Trp, Tyr and Val. Interestingly, only PpGH3-1 was able to conjugate the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) to JA as described for the *A. thaliana* JAR1 protein (Staswick & Tiryaki, 2004). Jasmonic acid-Ile, the major JA conjugate in *A. thaliana* (Staswick & Tiryaki, 2004) was formed only in very low amounts by PpGH3-2. It is interesting to note that the range of amino acids used by PpGH3-2 for IAA and JA conjugate formation almost completely overlapped (Table S1).

## Subcellular localization of PpGH3-1

To reveal the subcellular localization of PpGH3 proteins, the PpGH3-1 open reading frame was fused to that of GFP under the control of the CaMV 35S promoter. Using CLSM GFP fluorescence was detected to be evenly distributed within the cytoplasm (Fig. 2a–d). Optical sections of *Physcomitrella* protoplasts transfected with unfused GFP (Fig. 2e–h) showed GFP in the cytoplasm as well as in the nucleus. The tendency to accumulate in the nucleus is characteristic of cytosolic unfused GFP (Haseloff *et al.*, 1997). The GH3-1 fusion prevents GFP passively transporting into the nucleus. The observed pattern for *PpGH3* was identical to other GH3 proteins analysed (Roux & Perrot-Rechenmann, 1997; Wright *et al.*, 1987). 1-Naphthaleneacetic acid (NAA) application did not change the cytoplasmic distribution (data not shown). Since the sequence of *PpGH3-2* does not possess a different localization signal and is predicted to be most likely cytosolic by PSORT (http://psort.ims.u-tokyo.ac.jp/form.html) we do not expect differences in the subcellular localization of the two proteins.

## *Physcomitrella patens* GH3-knockout mutants are more sensitive to IAA

Loss-of-function mutants for *PpGH3-1* or *PpGH3-2* did not show an apparent aberrant phenotype under different light qualities and during development (Bierfreund *et al.*, 2004). However, we have shown that both enzymes are capable of the synthesis of either auxin or JA conjugates with a range of amino acids (Figs 1, S1, S2, Table S1). Therefore, if IAA or JA conjugate formation is hampered in *ΔPpGH3* mutants, these should show altered sensitivity to IAA or JA if the respective hormone is growth inhibiting. We therefore tested moss

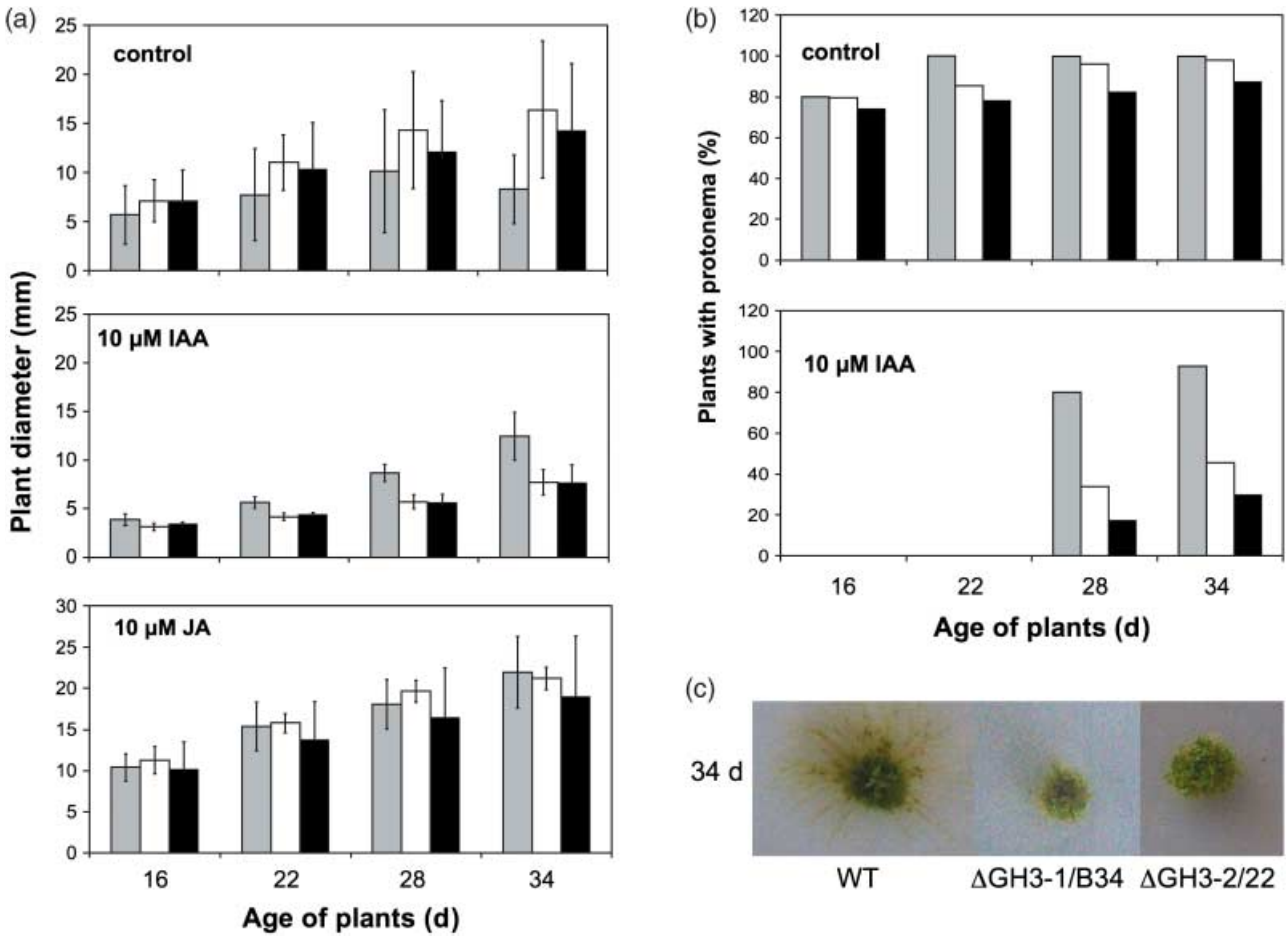


**Fig. 3** Hormone sensitivity of *Physcomitrella patens* gametophores. (a) Hormone sensitivity measured as size (diameter) of single moss plants placed on agar containing either auxin indole acetic acid (IAA) or jasmonic acid (JA) at 10 μM concentration in comparison to control conditions. (b) Percentage of plants which showed protonema growth on 10 μM IAA. (c) Individual colonies for a representative GH3-1 and GH3-2 knockout line, respectively, showing the typical phenotype 34 d after transplanting on IAA containing medium in comparison to the wild type. Wild type (tinted bars), ΔPpGH3-1 (open bars), ΔPpGH3-2 (closed bars).

wild-type and four *ΔPpGH3-1* as well as three *ΔPpGH3-2* mutant lines for developmental defects on medium supplemented with IAA or JA.

Growth of the moss plants was inhibited by 10 μM IAA but not JA (Figs 3 and 4) and this effect was more pronounced in all knockout lines tested than in the wild type. All mutant colonies formed less protonema on IAA than the wild type after 4 wk and 5 wk of culture on agar (Fig. 3b,c). Quantification of this effect shows that the reduction of plant size on IAA is at least partly caused by the lack of protonema formation. However, protonema cultures supplemented with 10 μM IAA showed no visible growth inhibition (data not shown). The time-course clearly shows that the inhibition of gametophore culture growth persisted over the whole experimental period. Gametophores with and without 10 μM IAA or NAA treatment showed elongation of the shoot apex over a short period of 1–3 d. Both the natural and the artificial auxin had similar effects. A long-term experiment with IAA showed that concentrations above 2 μM inhibited colony growth (data not shown). Concentrations of 5 μM IAA and greater inhibited growth of the wild type, while growth of *ΔPpGH3* plants decreased at 1 μM IAA (Fig. 4a). Graviresponse of the mutants was not altered under control conditions or on medium supplemented with 10 μM IAA (data not shown). Two representative of *ΔPpGH3-1* and *ΔPpGH3-2* mutants were analysed and proved to be more sensitive to IAA between concentrations of 0.1–10 μM than were the wild-type plants (Fig. 4). However, all lines were strongly inhibited in growth on 50 μM IAA and on 100 μM IAA growth was almost completely inhibited.

By contrast, JA did not inhibit growth of wild type or of the *ΔPpGH3-1* mutant while growth of the *ΔPpGH3-2* mutant was inhibited on high JA concentrations (50 μM and 100 μM; Fig. 4). Jasmonic acid slightly stimulated growth at a concentration

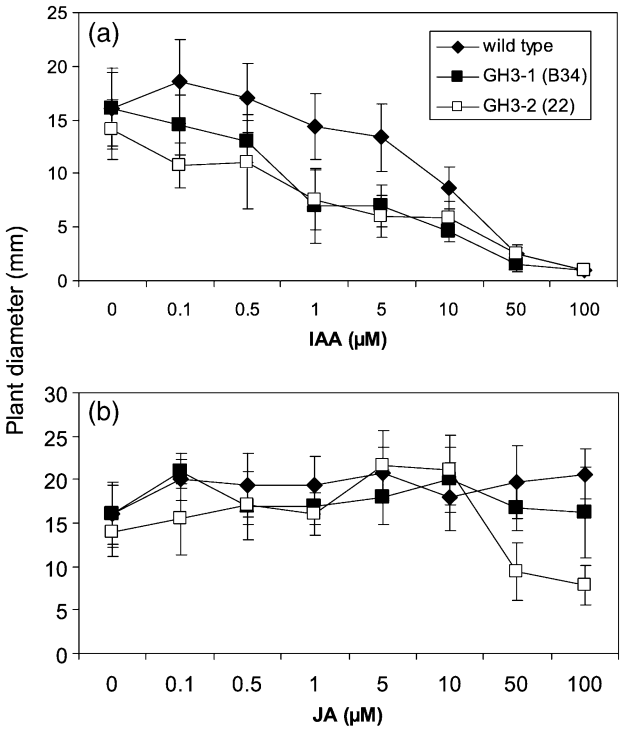


**Fig. 4** Hormone sensitivity of two selected lines of the two respective GH3 knockouts (ΔGH3-1/B34, closed squares; ΔGH3-2/22, open squares) and wild type (diamonds) on different concentrations of (a) auxin indole acetic acid (IAA) and (b) jasmonic acid (JA).

of 10 μM (Fig. 3a), but this effect was GH3-independent, because the mutants were stimulated in the same way as the wild type.

## IAA conjugate formation is altered in GH3 mutant lines

The content of free and conjugated IAA was determined in gametophore cultures grown under control conditions and on 10 μM IAA. Under control conditions the mutants showed increased free IAA levels (Fig. 5a,b) compared with wild type, whereas no differences were found for conjugated IAA. On 10 μM IAA the free IAA content increased in all lines and was highest in the *ΔPpGH3-2* mutants, followed by *ΔPpGH3-1* mutants (Fig. 5a). On IAA medium the amount of conjugated IAA was slightly lower in the mutants than in the wild type, but no differences between *ΔPpGH3-1* and *ΔPpGH3-2* lines were detected (Fig. 5b). A clear induction of conjugated IAA levels after IAA treatment was found in the wild type but not in the mutants.

Protonema cultures were incubated for two periods (24 h and 7 d) with 10 μM IAA to study short- and long-term effects. Little difference was seen in the IAA content between the two treatments (Fig. 5c,d). Free IAA content was not altered in the mutants compared with the wild type (Fig. 5c) and growth in medium supplemented with IAA did not change this result. No visible difference between wild type and mutant lines in the levels of conjugated IAA were observed

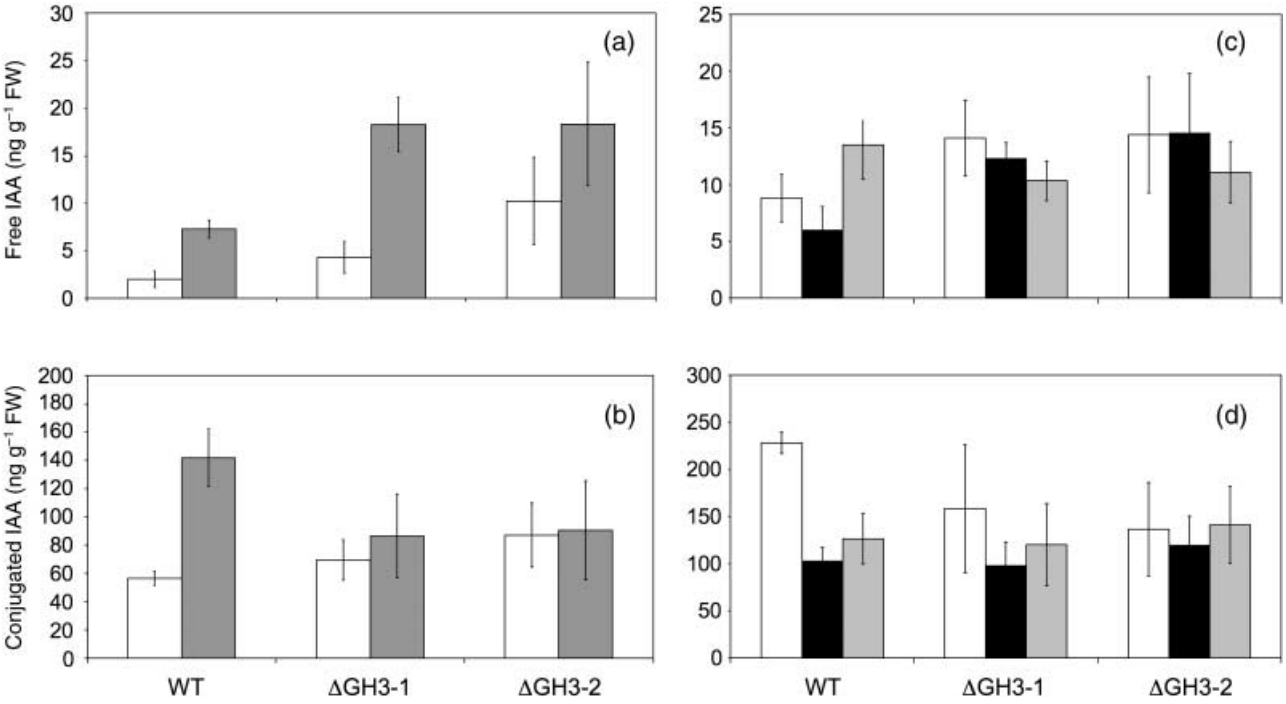


**Fig. 5** Levels of free and conjugated auxin indole acetic acid (IAA). Free (a,c) and conjugated IAA (b,d) determined in gametophores (a,b) and protonema (c,d) of GH3 knockout lines and wild type. Gametophores were analysed under control conditions (open bars) and grown continuously for 28 d on 10 μM IAA (closed bars) and protonema under control conditions (open bars) and grown for 24 h (closed bars) or 7 d (tinted bars) on 10 μM IAA.

(Fig. 5d). Whereas IAA conjugate formation was inducible in gametophores (i.e. the adult moss plants), this was not the case in protonema cultures which represent the early stages of moss development.

### Tissue-specific differences in IAA metabolism

The IAA metabolites were profiled via HPLC after growth on medium containing IAA and under control conditions without exogenous auxin, as previously described for other plants (Venis, 1972; Barratt *et al.*, 1999). The extracts were separated into two parts. One was subjected directly to HPLC analysis, the other to alkaline hydrolysis to determine if the metabolites formed could be auxin conjugates. After application of 10 μM IAA we expected to see metabolites that were not present under control conditions. Such metabolites would indicate the fate of excess IAA. Gametophore cultures metabolized IAA to three major compounds with retention times after HPLC analysis of 25 min (metabolite 2), 32 min (metabolite 3) and 37 min (metabolite 4), and free IAA (metabolite 1) was also still present (Fig. 6a). The three metabolites (2–4) may be IAA conjugates because they were no longer visible after strong alkaline hydrolysis (dotted line). Semiquantitative evaluation of metabolites formed on 10 μM IAA showed that the amount of metabolite 3 was greater in wild type gametophores than in the other two metabolites and it was strongly reduced in all mutant lines whereas these same two metabolites were less affected (Fig. 6a,b). The same metabolite profile was found in protonema cultures, but there were no prominent differences between wild type and mutant lines (Fig. 6c,d). After 24 h on 10 μM IAA the protonema cultures formed predominantly metabolite 4, whereas metabolite 3, the main metabolite in gametophores was hardly detectable (Fig. 6c). When cultures were grown on 10 μM IAA for 7 d, amounts of metabolite 4 were very low, with no change in the other metabolites (Fig. 6d).

Metabolite 2 was identified by GC-MS as IAA-Val and metabolite 3 as IAA-Leu or IAA-Ile (those two conjugates did not separate either on HPLC or on GC and produce the same molecular ion and fragmentation pattern). Methylated IAA-Val showed the molecular ion of *m*/*z* 288 and the quinolinium ion at *m*/*z* 130, and the methyl ester of IAA-Leu was identified with its molecular ion at *m*/*z* 302 and the quinolinium ion at *m*/*z* 130 (Fig. 7). In the GC chromatograms we found an additional small peak which was identified as IAA-Ala (data not shown), but this peak was not visible in the HPLC traces. Metabolite 4 did not co-chromatograph with any of the standards for IAA amino acid conjugates tested and will therefore be a subject for future analysis.

Val, Leu and Ile were substrates for the PpGH3-2 conjugate synthetase *in vitro*, whereas Ala was accepted by both PpGH3 proteins as substrate (Fig. 1). The *in vitro* usage of substrates might not reflect the *in vivo* situation because of possible availability of the amino acid substrates.

### Generation and molecular characterization of double mutants

ΔPpGH3-1/ΔPpGH3-2 double knockouts were created by targeted gene disruption to overcome a possible functional complementation of the PpGH3 proteins. Protoplasts from ΔGH3-1 lines (GH3-1/A31 and GH3-1/B34, respectively; Bierfreund *et al.*, 2004) were co-transfected with the *PpGH3-2* knockout construct and a hygromycin selection cassette. After regeneration and growth on selective medium, resistant plants were screened for the disruption of the *GH3-2* gene via homologous recombination of the knockout construct (Fig. 8a–c). Polymerase chain reaction was performed with primer combinations verifying the homologous integration into the *GH3-2* gene locus at the 5′- and 3′-end, respectively (Fig. 8b, 'KO'). To validate the generation of loss-of-function mutants, four putative ΔGH3-1/2 lines were checked for the absence of a *GH3-2* transcript (Fig. 8c). The four ΔGH3-1/2 ('doKO') lines as well as a ΔGH3-2 control failed to give any PCR product while with the ΔGH3-1 controls, and with wild type moss RNA a GH3-2 transcript could be amplified, confirming the successful creation of GH3-1/2 double knockout lines.

The ΔGH3-1/2 double mutants showed no differences in free IAA levels on control medium compared with wild type (Fig. 8d), while on 10 μM IAA the free IAA increased above the values obtained for a single KO mutant (see Fig. 5). Conjugated IAA was much more reduced in the ΔGH3-1/2 double mutants, but did not drop to zero as expected (data not shown). This might be caused by the existence of additional conjugates (except for amide conjugates). Therefore, the occurrence of ester conjugates was analysed. However, ester conjugate formation was not inducible by IAA; on the contrary, they seemed to be reduced in wild-type plants on IAA (Fig. 8d). The fraction of amide bound IAA in the ΔGH3-1/2 double mutants was almost zero on control medium, but there was still amide-conjugated IAA present when the mutant plants were grown on medium containing IAA. This indicates that an additional mechanism for IAA amide conjugate formation must be present in *P. patens*.

## Discussion

The maintenance of auxin homeostasis in plants is important for all aspects of development (Bandurski *et al.*, 1995). However, the strategies to achieve this goal may be different during evolution. Possibilities to control auxin levels include biosynthesis, degradation, transport and the reversible conjugation to small molecules such as amino acids or sugars but also to proteins (Seidel *et al.*, 2006). Low molecular weight IAA conjugates have been detected in many different higher plant species and consequently, the genes for members of the GH3 family of IAA amino acid conjugate synthases are well conserved in a variety of plant species (Terol *et al.*, 2006).

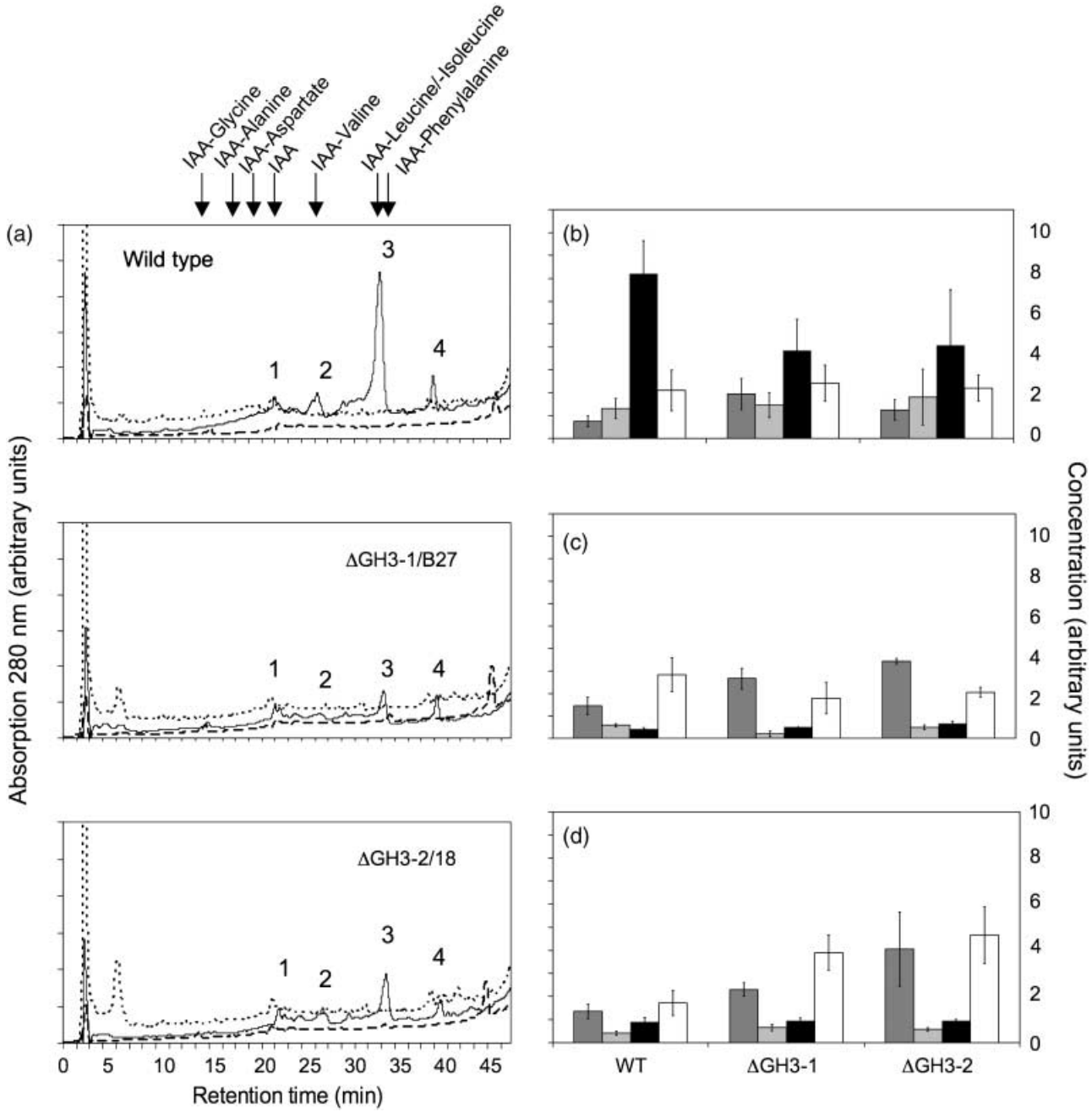


**Fig. 6** Metabolism of auxin indole acetic acid (IAA) in moss cultures. (a) High-pressure liquid chromatography (HPLC) of gametophores of wild type and selected *GH3-1* and *GH3-2* knockout lines after growth on 10 μM IAA. The arrows indicate the position of standards of IAA and a selection of IAA amino acid conjugates. Dashed line, extract without IAA addition; solid line, extract plus 10 μM IAA; dotted line, extract plus IAA after alkaline hydrolysis. Putative conjugate peaks with IAA should be eliminated under these conditions. For better comparison all HPLC chromatograms are the same scale. (b–d) Semiquantitative analysis of the metabolites 1–4 in gametophores grown on 10 μM IAA (b), protonema cultures which were treated for 24 h with 10 μM IAA (c) and protonema cultures treated for 7 d with 10 μM IAA (d). IAA (dark tinted bars), metabolite 2 (light tinted bars), metabolite 3 (closed bars), metabolite 4 (open bars).

Two genes have been described which could be involved in the regulation of auxin conjugate biosynthesis in *P. patens* (Bierfreund *et al.*, 2004). Mosses/bryophytes separated from seed plants > 450 million yr ago (Theissen *et al.*, 2001), suggesting that the evolution of protein families linked to auxin homeostasis is very ancient (Cooke *et al.*, 2002). Experimental evidence indicated that different possibilities to regulate auxin content have evolved. While in charophytes and liverworts the auxin conjugation rates are very slow and therefore biosynthesis is a major contributor to free auxin, the

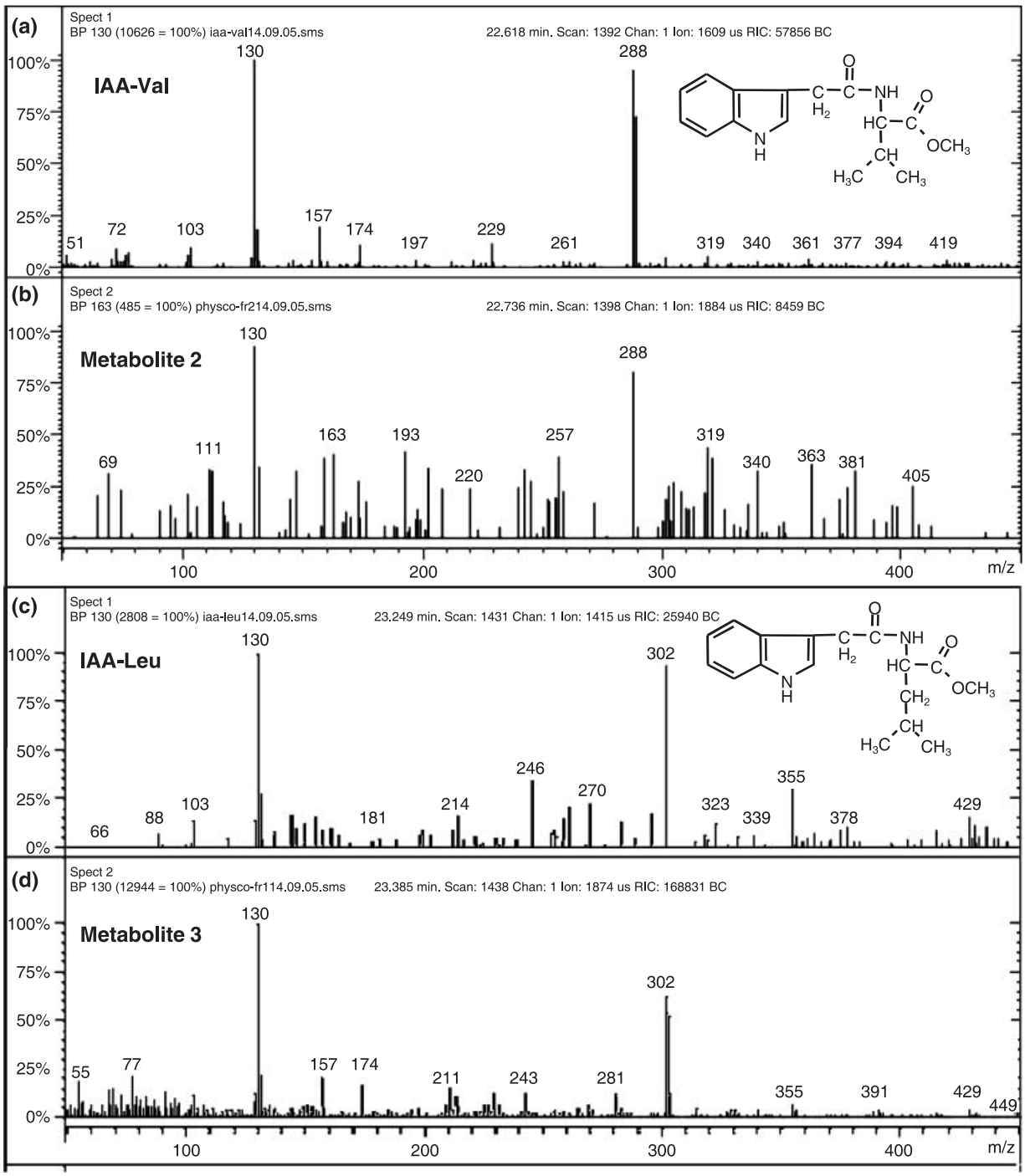


**Fig. 7** Gas chromatography–mass spectrometry identification of auxin indole acetic acid (IAA) metabolites. (a) Mass spectrum of IAA-Val standard, (b) mass spectrum of metabolite 2; (c) Mass spectrum of IAA-Leu standard; (d) mass spectrum of metabolite 3. The chemical structures of the IAA conjugates are also shown. The characteristic ions for the methyl ester of IAA-Val are the molecular ion at *m/z* 288 and the quinolinium ion at *m/z* 130 and for the methyl ester of IAA-Leu the molecular ion at *m/z* 302 and the quinolinium ion at *m/z* 130. Note that IAA-Leu and IAA-Ile cannot be distinguished under these conditions.

conjugation rate increases from hornworts and mosses to vascular plants and thus also its importance in auxin homeostasis (Cooke *et al.*, 2002). The first land plants where auxin conjugation could play a larger role were identified within the mosses by feeding experiments (Sztein *et al.*, 2000). While in higher plants approx. 90% of the total IAA can be found in conjugated forms, in lower land plants the percentage of conjugated IAA is lower and reaches an average

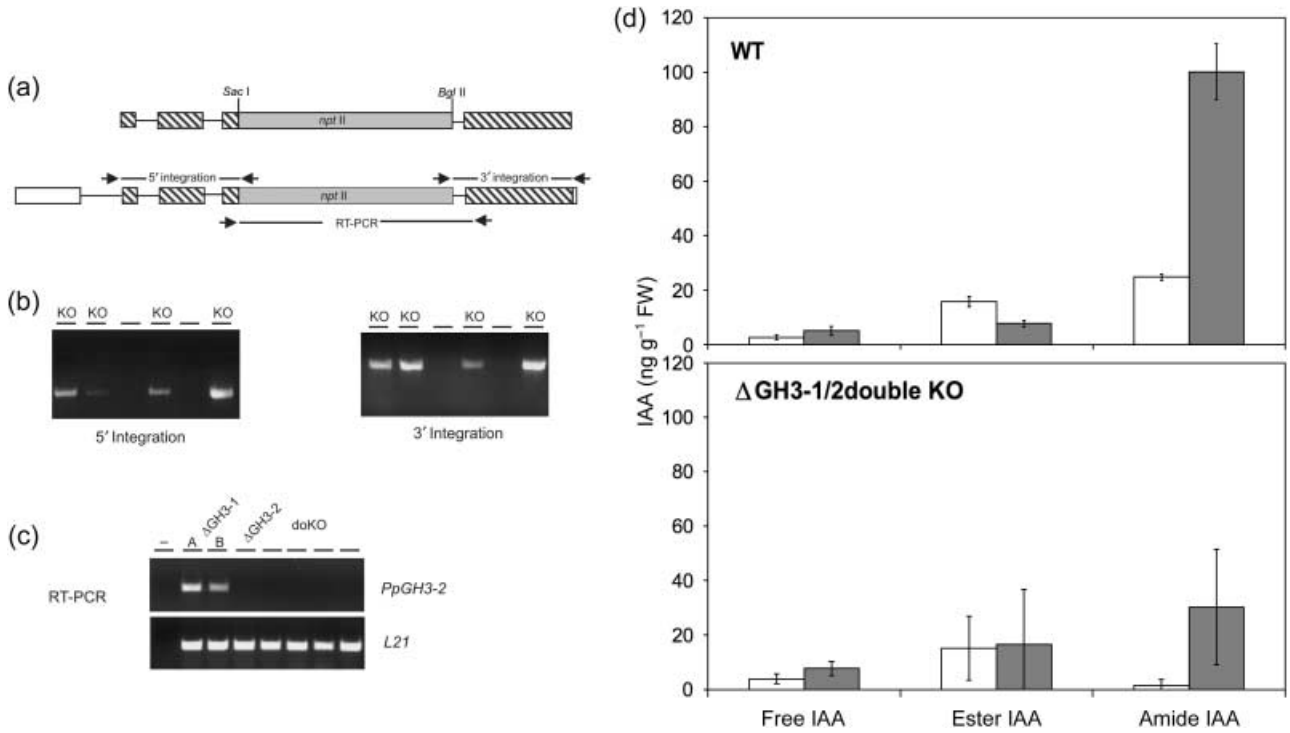


**Fig. 8** Generation of GH3-1/2 double mutant lines based on a genetic GH3-1 single KO background. (a) Illustration of the GH3-2 knockout construct and the GH3-2 gene locus after homologous integration of the constructs. The primer combinations and resulting polymerase chain reaction (PCR) products used for the molecular analysis are indicated. Exons included in the KO constructs are shown by hatched bars, additional *GH3-2* exons by clear boxes and introns by lines; the nptII selection cassette is indicated as a tinted box. (b) Representative gels showing PCR analysis for proper 5′- and 3′-integration (KO), respectively. (c) Reverse transcriptase-PCR analysis of the GH3-2 transcript in representative GH3-1 KO and GH3-2 KO lines and four putative GH3-1/2 double KO lines (doKO). –, negative control. Lower panel: internal control PCR of the constitutively expressed gene for the ribosomal protein L21. (d) Auxin indole acetic acid (IAA) levels in double knockout mutant lines. Levels of free ester-, and amide-bound IAA were determined in gametophores of *GH3-1/GH3-2* knockout lines and wild type (WT) under control conditions (open bars) and grown continuously for 28 d on 10 μM IAA (closed bars).

of approx. 70% in mosses (Sztein *et al.*, 1999, 2000). This is consistent with the values found in this study (Figs 5 and 8).

Auxins play an important role during moss development. Increased endogenous auxin levels result in the formation of caulonema and auxins are probably also involved in bud formation together with cytokinins (Schumaker & Dietrich, 1998). In addition, auxin can induce rhizoids from epidermal cells (Sakakibara *et al.*, 2003). The previously isolated *P. patens GH3*-like homologs *PpGH3-1* and *PpGH3-2* display considerable sequence similarities to those described in seed plants (Bierfreund *et al.*, 2004; Staswick *et al.*, 2005). The smaller number of GH3-like homologs in *P. patens* compared with *A. thaliana* suggests further analysis to determine their function in plant development. In phylogenetic analyses the two *P. patens* GH3 homologues clustered with AtGH3-11 (JAR1, FIN219) (Bierfreund *et al.*, 2004). Especially important for a possible enzymatic activity are three weakly conserved motifs in PpGH3-1 and PpGH3-2 which were also detected in JAR1 and mediate adenylation activity (Staswick *et al.*, 2002). This is interesting because JAR1 encodes an amino acid conjugate synthetase which couples JA to several amino acids (Staswick & Tiryaki, 2004). In addition, the GH3 family of *A. thaliana* contains several other members which can adenylate IAA in a similar fashion to JAR1, with a broad substrate specificity towards the amino acids used (Staswick *et al.*, 2002, 2005). However, there are several members for which no substrate has been found and whose functions are far from clear. We have therefore investigated possible substrates for the two PpGH3 proteins (Fig. 1, Figs S1–S2). PpGH3-2 acts as an IAA-synthetase in the same way as described for seven members of the *A. thaliana* GH3 family (Staswick *et al.*, 2005). The *P. patens* enzyme converted Asp, Ala, Leu, Val, Trp and Cys preferentially, which was partly supported by the *in vivo* data. However, it has not yet been shown which IAA conjugates are present in *P. patens.* The *A. thaliana* GH3 enzymes formed several IAA conjugates *in vitro* that have not been reported to occur in plants (Kowalczyk & Sandberg, 2001; Staswick *et al.*, 2005). In contrast to PpGH3-2, PpGH3-1 had only a weak *in vitro* activity with IAA. Only two IAA amino acid conjugates were detected in the enzymatic assay. Interestingly, both *P. patens* GH3 proteins were able to use IBA as substrate. This is a naturally

occurring auxin that has so far only been detected in higher plants (Ludwig-Müller, 2000). Finally, both PpGH3 proteins accepted JA with a variety of amino acids as substrates and formed the respective conjugates. Here, we found an overlap but still a distinct preference for various amino acids. No aberrant phenotype could be linked to the knockout of any of the PpGH3s and JA, however, in contrast to the inactive IAA conjugates, JA conjugates may have biological activity in some plant species (Kramell *et al.*, 1997; Staswick & Tiryaki, 2004; Thines *et al.*, 2007). Obviously, the regulatory networks of phytohormone action have evolved from *P. patens* to seed plants such as *A. thaliana* (Vandenbussche *et al.*, 2007; Rensing *et al.*, 2008). This may explain why, *in vitro*, both PpGH3 proteins are able to conjugate JA to amino acids and homologs to genes involved in JA biosynthesis are present in the moss genome, although no JA effect was discernible for *P. patens*. However, we cannot rule out the possibility of a preference for an additional substrate which has yet to be identified. Crosstalk between hormones, including a GH3 protein, has been shown for *A. thaliana* WES1/AtGH3-5. This gene can be induced by abscisic acid (ABA) and salicylic acid (SA) (Park *et al.*, 2007b). AtGH3-9 mutants have a jasmonate-related phenotype (Khan & Stone, 2007). The mutant is slightly more resistant to JA than *jar1*. In addition, auxin and ethylene crosstalk has been determined for a GH3 homolog from *Capsicum chinense* (Liu *et al.*, 2005). Therefore, the *ΔPpGH3* mutants described here are a promising tool for analysing hormone interactions in *P. patens*.

Further evidence for the possible role of PpGH3 proteins in the development of *P. patens* comes from hormone sensitivity assays on JA and IAA with several lines of *ΔPpGH3-1* and *ΔPpGH3-2* (Figs 3 and 4). All knockout mutants tested for the two different *GH3* genes showed similar growth inhibition on IAA, but not on JA, indicating similar or overlapping functions of the two proteins. The growth inhibition on IAA was mainly caused by inhibition of protonema growth deriving from adult moss colonies (gametophores), although protonema in liquid culture did not show any sign of growth inhibition with the same IAA concentration. Bierfreund *et al.* (2004) have shown that both *GH3* genes are expressed in protoplasts, in protonema, and in gametophores. Transcript levels of both genes were highest in gametophores, which might explain the tissue-specific effects we found with the mutants. In addition, investigations on the distribution of endogenous IAA using the soybean *GH3* promoter indicated auxin responsiveness in dividing cells such as protonema filaments and buds but also in the apex of gametophores (Bierfreund *et al.*, 2003).

The patterns of free and total IAA in gametophores revealed that IAA conjugation is disturbed in the mutants (Fig. 5). Free IAA was present in higher amounts in the mutants compared with wild type, while the levels of conjugates were reduced. This effect was even more pronounced when the moss plants were grown on 10 μM IAA. This was different in protonema culture, indicating that other mechanisms may be used for detoxification. An important function of auxin efflux has to be assumed during the early development of *P. patens* as a high proportion of the auxin produced by the juvenile filamentous protonema is released into the surrounding medium (Reutter *et al.*, 1998). Conjugation of IAA was acquired in evolution between liverworts and mosses (Cooke *et al.*, 2004), although it can not be ruled out that different developmental stages may cope with excess IAA differently. By growing gametophores and protonema on IAA we tested the existence of an auxin-inducible IAA conjugation system as described for seed plants (Venis, 1972). In accordance to findings with seed plants, the amounts of IAA metabolites were increased after growing gametophores on higher IAA concentrations. In seed plants mainly the conjugate of IAA with Asp is formed after addition of high amounts of exogenous IAA (Barratt *et al.*, 1999). However, in *P. patens* wild type grown on IAA no conjugate of IAA with Asp was detectable by GC-MS. Probably other amino acids are conjugated predominately to IAA, such as Val and Leu/Ile. These amino acids were found as conjugates with IAA in *in vivo* metabolism studies (Figs 1 and 6). These amino acids were also accepted by the PpGH3-2 enzyme and attached to IAA. Interestingly, Sztein *et al.* (2000) found IAA-Val as a major metabolite after feeding of IAA in the hornwort *Phaeroceros*, whereas no IAA-Asp was detected in this plant. The IAA-Asp conjugate was shown to be one substrate for a degradation route in seed plants (Östin *et al.*, 1992, 1998), whereas other conjugates such as IAA-Ala and IAA-Leu are hydrolysed to free IAA (LeClere *et al.*, 2002). A possible explanation for the discrepancies found in the use of amino acids *in vivo* and *in vitro* by PpGH3-1 could be the availability of amino acid pools, either by *de novo* synthesis or compartmentalization, for IAA conjugate formation, as previously demonstrated for *A. thaliana* (Barratt *et al.*, 1999). In a mutant that accumulated glutamine because of a defect in glutamate synthase IAA-Gln, and not IAA-Asp was the abundant IAA amide conjugate after feeding excess IAA. In this context, it is also interesting to note that differences in the metabolite pattern between gametophores and protonema cultures occurred.

From work with *A. thaliana* it can be assumed that GH3-like proteins have partially redundant functions (Hsieh *et al.*, 2000; Nakazawa *et al.*, 2001; Tanaka *et al.*, 2002), but also possibly nonredundant roles in auxin and/or light signaling. We have generated double knockout mutants (*ΔPpGH3-1/2,* Fig. 8), which would be expected to have no auxin amino acid conjugates because the *P. patens* genome does not encode any other *GH3* homolog. A third PpGH3 cDNA clone that has been reported previously (Bierfreund *et al.*, 2004) emerged as a sequencing artifact. However, the *ΔPpGH3-1/2* double mutants displayed normal growth as protonema as well as gametophores. Only the IAA levels in gametophores were altered compared with wild type and with *GH3* single knockout mutants. In addition, the double mutants had

reduced conjugated IAA levels when grown on exogenously supplied IAA, albeit these levels were not zero, as has to be expected in a plant devoid of any GH3 activity.

Therefore, we tested the different fractions of conjugates that were identified in seed plants (i.e. ester and amide conjugate; Fig. 8). In the double knockout lines we found levels of amide conjugates close to zero, which was to be expected, but there was a considerable amount of IAA conjugated via ester bonds present. However, when grown on IAA, there were still amide conjugates present. This might indicate that amino acid conjugate formation is only part of the detoxification strategy in *P. patens* and that other regulatory mechanisms must be present, such as ester conjugate formation, degradation of IAA or transport/export for the fine tuning of this hormone. In addition, there could be other, as yet unidentified enzymes present that are capable of synthesizing auxin amide conjugates. The existence of only two *GH3* genes in *P. patens* compared with 19 genes in *A. thaliana* may indicate additional ways for control of auxin homeostasis, a strategy that has already been demonstrated in sulfate assimilation of *P. patens* (Kopriva *et al.*, 2007a,b; Wiedemann *et al.*, 2007). In addition, putative *ZmIaglu*/*AtUGT* homologs are present in *P. patens* which, according to their homology to the IAA-glucose synthase from maize (Szerszen *et al.*, 1994) and *A. thaliana* (Jackson *et al.*, 2001), are good candidates for the ester conjugate formation.

The different ways of controlling auxin homeostasis in *P. patens*, however, may depend on the developmental stage with conjugation playing a role in the adult gametophyte and being less important in the early stages. Here we characterized enzymes that demonstrate the evolution of GH3 function within one plant: the moss GH3s did not show any obvious function in the 'alga-like' juvenile protonema stage. By contrast, they conjugate auxin in the adult gametophore stage which resembles vascular plants in structure. Therefore, from an evolutionary point of view *P. patens* GH3 proteins potentially adopt an intermediate position, suggesting that the establishment of GH3 function goes along with the further development of organs and auxin function.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Conjugation of indole-3-butyric acid (IBA) to various amino acids by the two GH3 proteins from *Physcomitrella patens*. The asterisk indicates the band for the respective conjugate.

**Fig. S2** Conjugation of jasmonic acid (JA) to various amino acids and ACC (amino-cyclopropane carboxylic acid) by the two GH3 proteins from *Physcomitrella patens*. The asterisk indicates the band for the respective conjugate.

**Table S1** Semiquantitative comparison of enzymatic activities of the two PpGH3 proteins with different hormones and amino acids as substrates

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