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Loss of *GH3* function does not affect phytochrome-mediated development in a moss, *Physcomitrella patens*

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Summary

Auxin-induced gene expression is described for a variety of different genes including the *SAUR*-, *Aux/IAA*- and *GH3*-families, members of which have been found in seed plants. The precise function of *GH3*-like proteins in plant development is not well characterised yet. Mutant analysis in *Arabidopsis thaliana* indicates a possible role for *GH3*-like proteins in connecting auxin and light signal transduction. Here, we report the isolation of three different *GH3*-like homologues from a lower land plant, the moss *Physcomitrella patens*. Two of the *GH3*-like homologues were chosen for further characterisation. Both genes are expressed in gametophytic tissues, with expression starting very early in moss development. Knockout plants were generated and analysed. In comparison to white-light growth, cultivation of the wild type and knockout plants under red-light conditions resulted in a delay in gametophytic tissue development. The leafy moss plants displayed an elongated phenotype. Growth delay and elongation were even stronger under far-red light conditions. No obvious differences between wild type and knockout plants could be detected under the examined conditions, indicating functional redundancy of the two genes.

Introduction

The phytohormone auxin is involved in various aspects of plant growth and development. Auxins are thought to regulate or influence diverse responses on the whole-plant level, like tropisms, apical dominance, and root initiation. On the cellular level, responses such as cell extension, division, and differentiation, are influenced by auxin (Abel and Theologis, 1996). Another

Abbreviations: aa, amino acid; CDS, coding sequence; EST, expressed sequence tag; NAA α-, naphthalene acetic acid; *npt*II, neomycinphosphotransferase

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well-described effect is auxin-inducible gene expression. The corresponding genes were isolated from different plants after application of auxin (Guilfoyle, 1999). They build up gene-families, which include the *SAUR*-, *Aux*/*IAA*-, and *GH3*-family whose members are widely distributed among seed plants (Hagen and Guilfoyle, 2002).

GH3 was initially isolated by differential hybridisation screening as an auxin-induced cDNA clone from etiolated soybean hypocotyls (Hagen et al., 1984). It is described as a primary response gene because induction already starts 5 min after auxin application (Hagen and Guilfoyle, 1985) and is unaffected by the protein synthesis inhibitor cycloheximide (Franco et al., 1990). *GH3*-like homologues were also characterised from tobacco (Roux and Perrot-Rechenmann, 1997) and *Arabidopsis* (Hsieh et al., 2000; Nakazawa et al., 2001; Tanaka et al., 2002). Two *GH3*-like homologues from a lower land plant, the moss *Physcomitrella patens*, are represented in public databases as a complete mRNA and as an expressed sequence tag (EST). In *Arabidopsis*, *GH3*-like proteins build up a large family consisting of 19 members. An additional partial sequence contains only the amino-terminal third of the protein (Hagen and Guilfoyle, 2002). Database searches revealed the presence of *GH3*-like homologues in other dicots and monocots, as well as in the cyanobacterium *Synechocystis*, mouse, man, and other vertebrates (Hagen and Guilfoyle, 2002). Most of the characterised plant *GH3*-like genes are described as auxin-inducible (Hagen and Guilfoyle, 2002). The function of *GH3*-like proteins is still unclear. They display no special protein domains correlated to a specific function except putative coiled-coil domains within some *Arabidopsis GH3* homologues (Hagen and Guilfoyle, 2002; Hsieh et al., 2000). A possible connection of *GH3*-like proteins to light was coming up after analysis of three *Arabidopsis* plants carrying a mutation in different *GH3*-like genes. *FIN*219 exhibited a far-red-specific long hypocotyl phenotype (Hsieh et al., 2000), *dfl*1-*D*, an overexpressor, displayed a shorter hypocotyl under different light conditions (Nakazawa et al., 2001), and the expression of the *AtGH3a* gene was under light control (Tanaka et al., 2002). An *in vitro* adenylation activity of auxin, jasmonic acid, or salicylic acid was demonstrated for different *Arabidopsis GH3*-like proteins (Staswick et al., 2002).

The moss *P. patens* combines some features that make it quite suitable as a model organism for studying plant differentiation and developmental processes. The moss has a phylogenetic position near the base of extant land plants along with the last common ancestor of mosses and vascular plants existing more than 400 million years ago (Theissen et al., 2001). Thus, *Physcomitrella* could be of great value to investigate evolutionary aspects of plant development. In addition, it is unique among land plants as gene targeting by homologous recombination works efficiently in this taxon (Reski, 1998a). There is EST data available covering more than 95% of the *Physcomitrella* transcriptome (Rensing et al., 2002) and two different approaches of targeted mutagenesis via transposon tagging were performed (Egener et al., 2002; Nishiyama et al., 2000). Gene content, expression, and regulation as well as codon usage are comparable to that of higher plants (Reski, 1998b), but the average size of gene-families is reduced compared to *Arabidopsis* (Rensing et al., 2002).

Here, we describe the identification and characterisation of two *GH3*-like homologues from the moss *P. patens*, named *PpGH3-1* and *PpGH3-2*. A third homologue was isolated as a partial cDNA. The expression of *PpGH3-1* and *PpGH3-2* was analysed, and finally, knockout plants of both genes were produced and analysed. The existence of a small family of *GH3*-like genes in *Physcomitrella* in combination with the advantages of the moss system should further elucidate the function of different members of this gene family in plant development.

Materials and methods

Plant material and growth conditions

P. patens (Hedw.) B.S.G. has been characterised previously (Reski et al., 1994). Plants were grown axenically under standard conditions with a light–dark regime of 16:8 h as described (Bierfreund et al., 2003). Plants were subcultured in 7 d-intervals. For protoplast isolation, protonema was grown in semi-continuous bioreactor cultures under a controlled pH of 4.5 (Hohe et al., 2002a). For knockout analysis, the moss plants were cultivated under different light conditions including white light, which was provided continuously by four Osram L18W/25 and three Lfluora L18W/77 fluorescent tubes; light flux of 38 μmol s^{-1} m^{-2}, red light with 658 nm, energy fluence rate of 6.5 W m^{-2} and far-red light with 730 nm, and energy fluence rate of 3.5 W m^{-2} (Schäfer, 1977). In addition, knockouts and wild type were grown in stacks of five plates under red light with an energy fluence ranging from 0.1 to 5 W m^{-2} and under far-red light from 0.05 to 2.7 W m^{-2}.

Protoplast isolation, transfection and regeneration

Protoplasts were isolated and 3×10^5 cells were transfected with 30 μg of linearised plasmid DNA and regenerated in regeneration medium (Knop medium, 3% mannitol, 5% glucose, pH 5.8) as described previously (Strepp et al., 1998). Three selection rounds of transgenic plants were performed on solidified Knop media supplemented with 50 μg ml^{-1} G418.

Isolation of GH3-like genomic and cDNA clones

Partial cDNA clones for *PpGH3-1* and *PpGH3-2* were obtained upon a BLAST search of the *P. patens* EST library generated in a joint project of the University of Freiburg and BASF Plant Science GmbH (Rensing et al., 2002). Amino acid (aa) sequences of different *GH3*-like homologues from soybean (accession no. CAA42636), tobacco (accession no. AAD32141), and *Arabidopsis thaliana* (accession nos. AAF86349, AAD14468, BAB08663, BAB17304) were used as a query. The missing 5′ ends of the cDNAs were obtained by 5′RACE-PCR with the FirstChoice™RLM-RACE Kit (Ambion, Austin, USA) according to the manufacturer's instructions starting from total RNA. The corresponding genomic DNA for *PpGH3-2* was obtained by PCR and sequenced by primer walking. The genomic *PpGH3-1* sequence was isolated by screening a lambda FIX DNA library (Stratagene, La Jolla, USA) of *Physcomitrella* according to the manufacturer's protocol. A genomic fragment of approximately 1 kb was generated by PCR with primers derived from the *PpGH3-1* EST sequence used as a probe. ECL-labelling and detection of the probe was performed according to the manufacturer's instructions (Amersham Biosciences, Freiburg, Germany). Lambda DNA of positive phages was isolated by using the Lambda Midi Kit (Qiagen, Hilden, Germany) and *Sac*I digested Lambda DNA was cloned into the pUC18 vector. A clone giving a positive result after a PCR approach with primers derived from the *PpGH3-1* EST sequence was sequenced by primer walking and contained the full-length *PpGH3-1* genomic sequence.

Construction of PpGH3-1 and PpGH3-2 knockout constructs

For the *PpGH3-1* knockout constructs A and B, genomic DNA fragments (1.2 and 1.6 kb, respectively) were amplified using the primers 5′-TGGAATCCATCCCAAACTTGAGCAG-3′ (forward) and 5′-GCCCTCCCAATTATCCTTCGAGCACT-3′ (reverse) for *PpGH3-1* A, and the same reverse primer and 5′-TTGGTCACACTTCTCGTCTGAACCC-3′ for *PpGH3-1* B. The genomic DNA fragment (1.6 kb) for the *PpGH3-2* knockout construct was amplified with primers 5′-GCTCGGGGACTACTGGAGGG-3′ and 5′-CGACTGTTGTACTCAGGAAC-3′. DNA fragments were cloned into the pCRII-TOPO vector (Invitrogen GmbH, Karlsruhe, Germany). The neomycinphosphotransferase (*npt*II) selection cassette was amplified from pRT101neo (Girke et al., 1998) introducing *Nde*I restriction sites for *PpGH3-1* and *Sac*I, *Bgl*II sites for *PpGH3-2*, respectively, and cloned into pCRII-TOPO. The plasmids containing the *PpGH3-1* fragments A and B were digested with *Nde*I and then the *Nde*I excised selection cassette was inserted in order to yield the *PpGH3-1* knockout constructs (Fig. 4A). For the *PpGH3-2* knockout construct, the plasmid containing the genomic fragment was digested with *Sac*I/*Bgl*II to cut out a 453 bp fragment containing part of the fourth exon and the fourth intron (Fig. 5B) and to replace it with the *Sac*I/*Bgl*II excised selection cassette. For transfection, 30 μg of the knockout constructs were cut with *Eco*RI, producing a linear fragment that contained the *npt*II selection cassette flanked by *PpGH3-1* and *PpGH3-2* genomic sequences.

Screening of Physcomitrella transformants

To confirm integration of the knockout construct into the *PpGH3-1* and *PpGH3-2* gene, respectively, stable transgenic plants were analysed after three rounds of selection by direct PCR from gametophores as described previously (Schween et al., 2002) with minor modifications. The incubation time was increased to 30 min, PCR volume was reduced to 25 μl, and polyvinylpyrrolidone was removed. The presence or absence of a wild-type PCR product was controlled with primer pairs spanning the *npt*II selection cassette. In addition, 5′ and 3′ integration of the knockout construct was checked with gene-specific primers outside of the construct combined with primers specific for the selection cassette (see Figs. 4B and 5B). Plants without any wild-type PCR product and with correct 5′ and 3′ integration were considered as putative knockouts.

RNA isolation and RT-PCR

RNA from moss protonema was prepared as described previously (Bierfreund et al., 2003). For analysis of tissue-specific expression, first strand

synthesis was performed with 500 ng of total RNA from wild-type protonema, long-day gametophores, short-day 1 gametophores, and short-day 2 gametophores (Hohe et al., 2002b), and 5 μg of total RNA from regenerating protoplasts 1, 4 and 7 days after protoplast isolation. SuperscriptII Reverse Transcriptase (Invitrogen GmbH) was used for first-strand synthesis according to the manufacturer's protocol. For the expression analysis of Δ*PpGH3-2* plants, 1 μg of total RNA from wild type and Δ*PpGH3-2* protonema was used. Reverse transcription was done according to the manufacturer's protocol with the following modifications: temperature and incubation time were changed to 65°C for 10 min, followed by 1 h at 37°C. The PCR reaction was performed according to standard protocols (Sambrook and Russell, 2001).

Northern blot analysis

Total RNA was separated on a formaldehyd–agarose gel, transferred onto a Hybond-N^+ nylon membrane (Amersham Biosciences) and hybridised with a ^{32}P-labelled DNA probe. The Rediprime™II kit (Amersham Biosciences) was used for generation of the probe following the manufacturer's instructions. The membrane was washed twice with 2 × SSC, 0.1% SDS at 64°C and exposed to X-ray film (BioMax™ MS, Kodak, New York, USA) using a BioMax Transcreen HE Intensifying Screen (Kodak) at −80°C overnight.

Sequence and phylogenetic analysis

Nucleic and aa sequence analysis was done with Accelrys USA Wisconsin Package Version 10.3. For phylogenetic analysis, sequences were aligned with CLUSTAL W (Thompson et al., 1994). TreeCon (Van de Peer and De Wachter, 1997) was used for constructing the neighbour-joining tree (Saitou and Nei, 1987) based on the distance estimation by Tajima and Nei (1984). Bootstrap analysis with 500 replicates was performed to test the significance of the nodes.

Nucleotide sequence accession numbers

The *PpGH3-1* and *PpGH3-2* cDNA sequences reported in this paper have been deposited in the EMBL database under the accession nos. AJ428956 and AJ429070, respectively. The genomic sequences were also deposited with accession no. AJ496302 for the *PpGH3-1* and AJ496301 for the *PpGH3-2* gene. *PpGH3-3* partial cDNA (EST) was deposited in the EMBL database under the accession no. AJ536598.

Results

Isolation and characterisation of two GH3-like homologues from *Physcomitrella*

A search of our clustered EST database (Rensing et al., 2002) revealed three different partial cDNAs representing possible *GH3*-like homologues in the moss *P. patens*, termed *PpGH3-1*, *PpGH3-2* and *PpGH3-3*.

Full-length cDNA of the *PpGH3-1* and *PpGH3-2* homologues were obtained by 5′ RACE-PCR and also by screening a *Physcomitrella* cDNA library, which was derived from protonema in the case of *PpGH3-1*. Sequencing revealed that the *PpGH3-1* cDNA is about 2321 bp long, including the coding sequence (CDS) of 1911 bp, and codes for a protein of 636 aa with a calculated mass of 71 kDa. The cDNA of *PpGH3-2* is shorter with a length of 2078 bp (CDS 1800 bp). The protein comprises 599 aa with a predicted mass of about 67 kDa. A homology search with BLAST was performed with the derived aa sequences of both genes to estimate the similarity of the two *Physcomitrella GH3*s to homologues from other plant species. Both proteins share about 40% identity and 60% similarity with soybean and *Arabidopsis GH3*-like homologues. A pairwise comparison between the deduced aa sequences of the two *Physcomitrella GH3*-like proteins revealed that they share 44% identity and 53% similarity to each other. Surprisingly, the identity between the two *Physcomitrella* sequences is not much higher than that of homologues from other plants, revealing that the two *Physcomitrella* homologues are quite divergent on the aa level. Specific domains located in the *PpGH3-1* and *PpGH3-2* proteins were searched by a motif and profile scan. Both proteins display no special protein features or domains matching currently recognised protein motifs in the databases. However, JA adenylation activity was described for *JAR1*, which is identical to *FIN219*, *AtGH3-11* (Staswick et al., 2002). Three aa motifs are described that are moderately conserved among various enzymes with adenylation activity (Chang et al., 1997). These motifs were also found in both *Physcomitrella GH3* homologues.

The *PpGH3-1* genomic sequence was obtained by screening a *Physcomitrella* genomic library and is about 2921 bp including the 5′ and 3′ untranslated region. The *PpGH3-2* gene was obtained by PCR with genomic *Physcomitrella* DNA and gene-specific

primers derived from the 5′ and 3′ end of the corresponding cDNA. The *PpGH3-2* gene is 2825 bp in length including the 5′ and 3′ untranslated region. Both genes consist of five exons spaced by four introns (Fig. 1). Comparison of the 5′ untranslated region of the *PpGH3-1* gene with the corresponding cDNA revealed an additional intron near the start codon. This additional intron does not exist in the *PpGH3-2* genomic sequence.

Southern blot analysis was performed to estimate the amount of possible *GH3*-like homologues in *Physcomitrella* (data not shown), as these genes build up families in seed plants (Hagen et al., 1991; Hagen and Guilfoyle, 2002). The analyses were performed under moderate hybridisation conditions (at 50°C and washing twice with 2 × SSC, 0.1% SDS) and did not give any indication of the presence of additional family members. This may support the assumption of a very small gene-family of *GH3*-like homologues in *Physcomitrella* consisting only of the isolated three members.

In order to determine the evolutionary position of the two *Physcomitrella GH3*-like proteins, a phylogenetic tree was constructed including the 19 *GH3*-like protein sequences from *Arabidopsis* (Hagen and Guilfoyle, 2002) known to date, as well as sequences from other seed plants like tobacco, soybean, and rice (Fig. 2). As an outgroup, the *GH3*-like sequence from the cyanobacterium *Synechocystis* was used. The tree splits into three major groups, with group one and three containing *GH3*-like homologues from different plant species, whereas the second group only contains *GH3*-like homologues from *Arabidopsis*. Formation of three groups was already suggested for the *Arabidopsis* homologues by Hagen and Guilfoyle (2002). The two *Physcomitrella GH3*-like proteins cluster in the third group together with one protein from *Oryza sativa* (*OsGH3-1*) and two from *A. thaliana* (*AtGH3-10* and *AtGH3-11*). *AtGH3-11* corresponds to the FIN219 protein described by Hsieh et al. (2000). In this third group, the two *Physcomitrella* proteins take a position at the base of the cluster as putative ancient members.

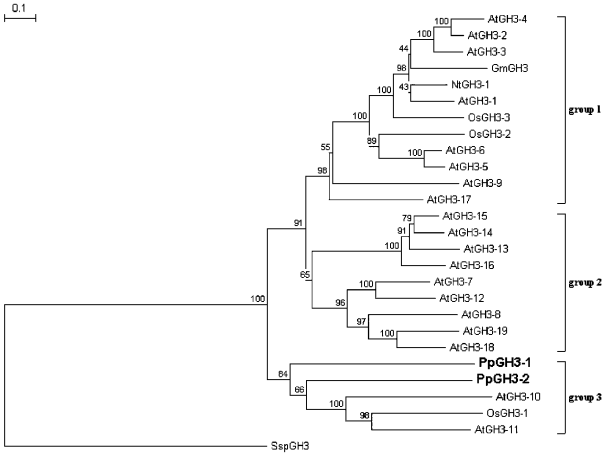

Figure 2. Rooted neighbour-joining tree based on 26 plant *GH3*-like protein sequences. A *Synechocystis GH3*-like protein was used as an outgroup. Bootstrap values out of 500 resampling replicates are shown at the internodes. Plant *GH3*-like proteins form three distinct groups with the two *Physcomitrella* proteins clustering into the third. The following protein sequences (accession numbers are given in parentheses) were used in this study: *AtGH3-1* to *AtGH3-19* (Hagen and Guilfoyle, 2002) from *A. thaliana*; *GmGH3* (S17433) from *Glycine max*; *OsGH3-1* to *OsGH3-3* (BAC15989, BAB92590, BAB63594) from *Oryza sativa* and *Nt-GH3* (AF123503) from *Nicotiana tabacum*.

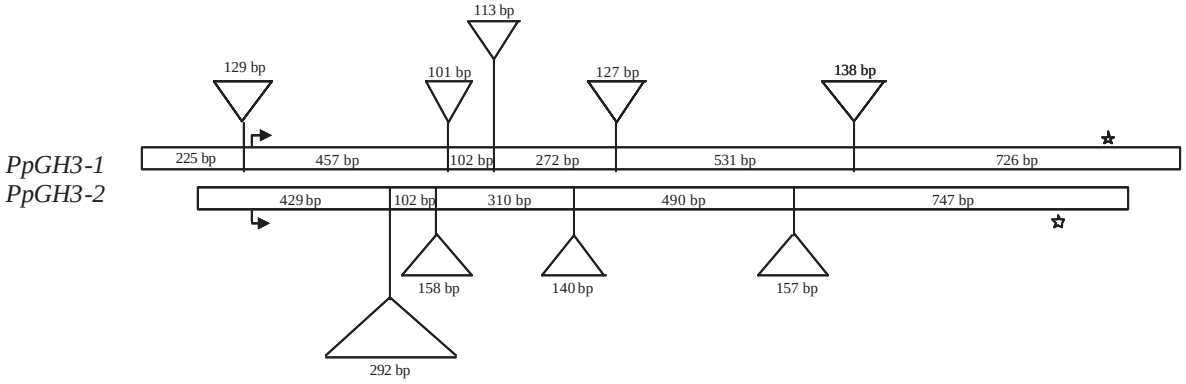

Figure 1. Schematic representation of the *PpGH3-1* and *PpGH3-2* genomic structure. Both genes are built from five exons spaced by four introns. The *PpGH3-1* gene contains an additional intron near the start codon in the 5′ untranslated region. The rectangles represent exons and introns are represented as triangles. Dimensions are drawn to scale. Numbers indicate exon and intron sizes in base pairs. The start codon is presented as an arrow and the stop codon as an asterisk.

Expression analysis

To obtain some insight into a possible tissue-specific expression of the two *GH3*-like transcripts, RT-PCR was performed with cDNA generated from different *Physcomitrella* tissues (Fig. 3A), namely, the filamentous protonema representing the earliest stage in moss development, the leafy moss plant, termed gametophore, gametophores plus sex organs, and gametophores plus sporophytes. Both *GH3*-like homologues were expressed in protonema and gametophores. Nevertheless, these results suggest that both genes are less expressed in protonema tissue than in gametophores. To further characterise the expression of both genes at the beginning of the *Physcomitrella* life cycle, RT-PCR was performed with cDNA produced from regenerating protoplasts (Fig. 3B). The protoplasts grow out to protonema filaments in a similar way as the germinating spore in the first stage of *Physcomitrella* development. Total RNA was taken 1, 4, and 7 days after protoplast isolation. Both *Physcomitrella* genes were expressed in all three stages. Thus, the transcripts were already present in the very beginning of protonema growth.

As most *GH3*-like genes are described as auxin-inducible, the *Physcomitrella* homologues were checked for auxin-inducibility by Northern blot analysis (data not shown). Surprisingly, expression of both genes seemed to be unaffected by exogenous auxin application.

Generation and analysis of knockout plants

To analyse the function of *Physcomitrella GH3*-like homologues, *PpGH3-1* and *PpGH3-2* knockout plants were generated by targeted gene disruption. Two different knockout constructs (A and B) were created for *PpGH3-1* differing in the length of the homologous sequences flanking the selection cassette and one construct was created for *PpGH3-2* knockout (Fig. 4A and 5A). *Physcomitrella* protoplasts were transfected with linearised plasmids containing the *npt*II (neomycin phosphotransferase) CDS flanked by 557 bp (928 bp) and 652 bp (652 bp) stretches of genomic sequence for *PpGH3-1* A (B), and 820 and 829 bp for *PpGH3-2*, respectively.

All 66 *PpGH3-1* transgenic plants were screened by direct PCR. From the analysed plants, 22 were determined as putative knockouts, indicating an

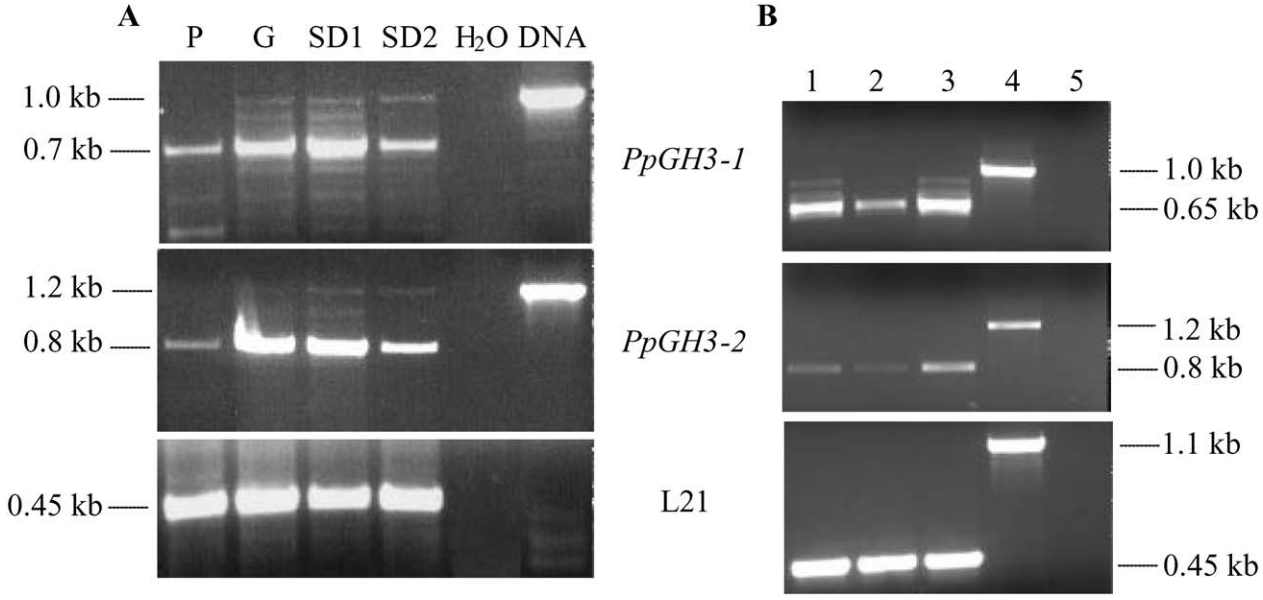

Figure 3. *Physcomitrella GH3*-like homologues are expressed in all stages of gametophytic development. As a loading control, RNA for the ribosomal protein L21 from *Physcomitrella* was used which is constitutively expressed under the described our conditions. To exclude genomic contamination in the RT-PCR, genomic *Physcomitrella* DNA was included as a control and the primers for the amplification spanned an intron, so that a distinction between cDNA and genomic DNA products is guaranteed. (A) Tissue-specific RT-PCR with RNA derived from protonema (P), gametophores (G), gametophores plus gametangia (SD1) and gametophores plus sporophytes (SD2) were performed. *PpGH3-1* and *PpGH3-2* were expressed in protonema and gametophore tissue. H_2O, as a control water was taken instead of PCR template. (B) Expression of *PpGH3-1* and *PpGH3-2* genes was analysed with RT-PCR in regenerating protoplasts. Total RNA was isolated 1 (lane 1), 4 (lane 2) and 7 days (lane 3) after protoplast isolation and transcribed into cDNA. Lane 4, to ascertain amplification of cDNA in lanes 1–3 genomic DNA was used as a template. Lane 5, as a control water was taken instead of cDNA template.

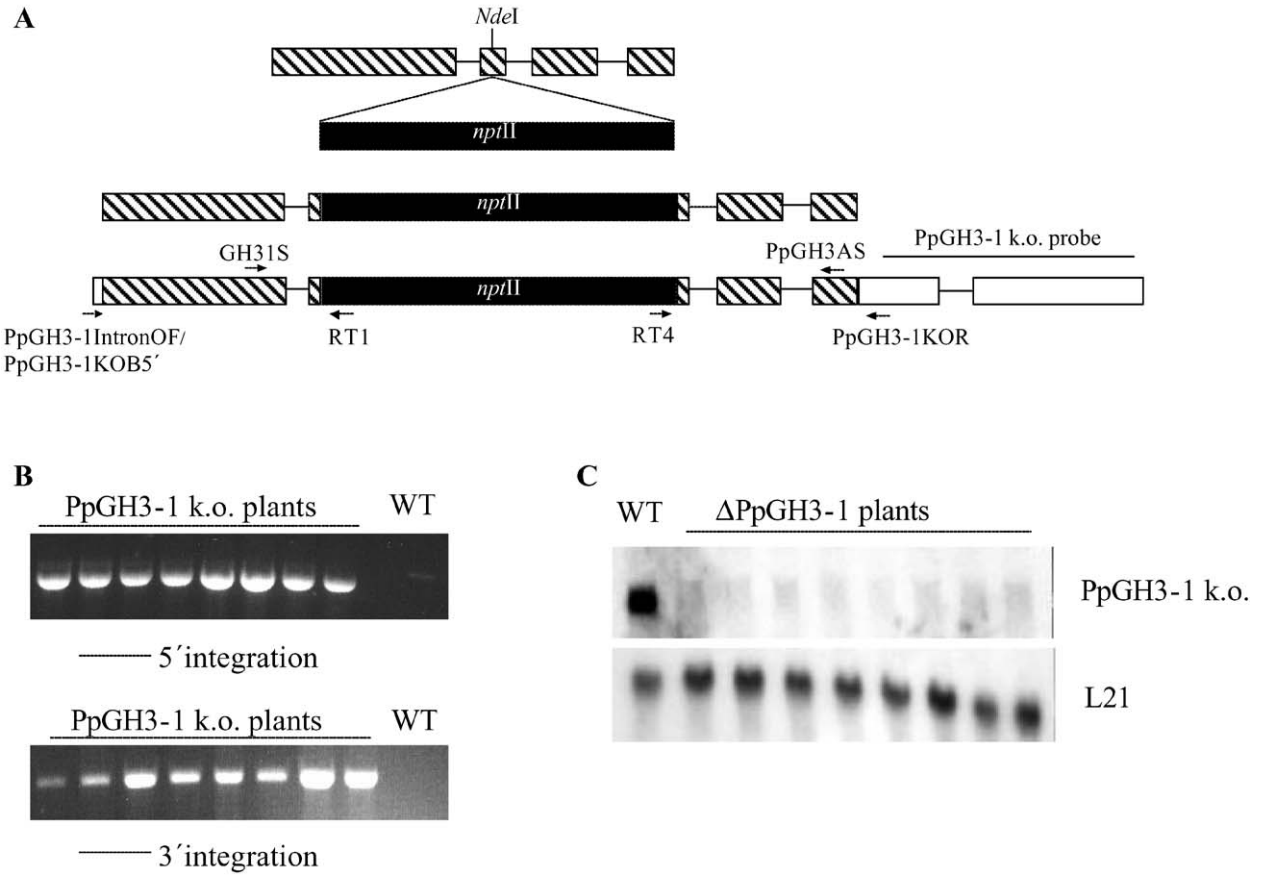

Figure 4. Targeted disruption of the *PpGH3-1* locus in *Physcomitrella*. (A) Schematic representation of the cloning strategy to generate and of PCR primer specifications to analyse *PpGH3-1* knockout plants. A 1209 and 1580 bp genomic fragment was disrupted by insertion of the *npt*II selection cassette (black bar) into the *Nde*I site. Exons included in the knockout constructs are shown in hatched bars, introns are shown as lines. Disruption of the wild type *PpGH3-1* locus was analysed with primers spanning the selection cassette (GH31S/GH3AS) and PCR primers specific for the 5′-integration site (*PpGH3-1*IntronOF, *PpGH3-1*KOB5′/RT1) and 3′-integration site (RT4/PpGH3-1KOR), respectively. Dimensions are drawn to scale. (B) PCR analysis revealed 5′ and 3′ integration of the *PpGH3-1* A and *PpGH3-1* B knockout constructs into the *Physcomitrella* genome. (C) Northern blot analysis with 15 μg of total RNA proved the absence of *PpGH3-1* transcript in four Δ*PpGH3-1* A and four Δ*PpGH3-1* B plants. As a loading control a probe derived from the RNA of ribosomal protein L21 was used. WT, wild type.

efficiency of about 33%. The targeting efficiencies of *PpGH3-1* A and B constructs were in the same range. The efficiency of *PpGH3-1* A was slightly higher with about 35% compared to the second construct with about 31%. Fifty *PpGH3-2* transgenic plants were analysed by PCR and 13 of them were putative knockouts, giving a targeting efficiency of about 26%. The rate of homologous recombination of both genes is in the range of most of the other knockouts reported so far in *Physcomitrella*, which usually have a knockout rate between 20% and 80% (Reski, 1998a). Eight *PpGH3-1* as well as three *PpGH3-2* transgenic plants were further analysed. Flow cytometric analysis confirmed the maintenance of the original haploid state for all of the transgenic plants analysed (data not shown).

To clearly define the transgenic plants as real knockouts, it was necessary to demonstrate the absence of the *PpGH3-1* and *PpGH3-2* transcript. An expression analysis by Northern blot was performed for the selected *PpGH3-1* plants (Fig. 4C) with a probe derived from the 3′ part of the *PpGH3-1* cDNA outside of the knockout construct (Fig. 5A). Wild type yielded a signal of the expected size of about 2.3 kb. In the case of the eight *PpGH3-1* putative knockouts, *PpGH3-1* transcript was no longer detectable. Thus, the *PpGH3-1* expression has been disrupted by the selection cassette. For the three *PpGH3-2* transgenic plants, RT-PCR was performed for expression analysis (Fig. 5C). The existence of full-length cDNA was confirmed by PCR with primer pairs situated in the 5′ region of the *PpGH3-2* gene. PCR was done with primers spanning the region of

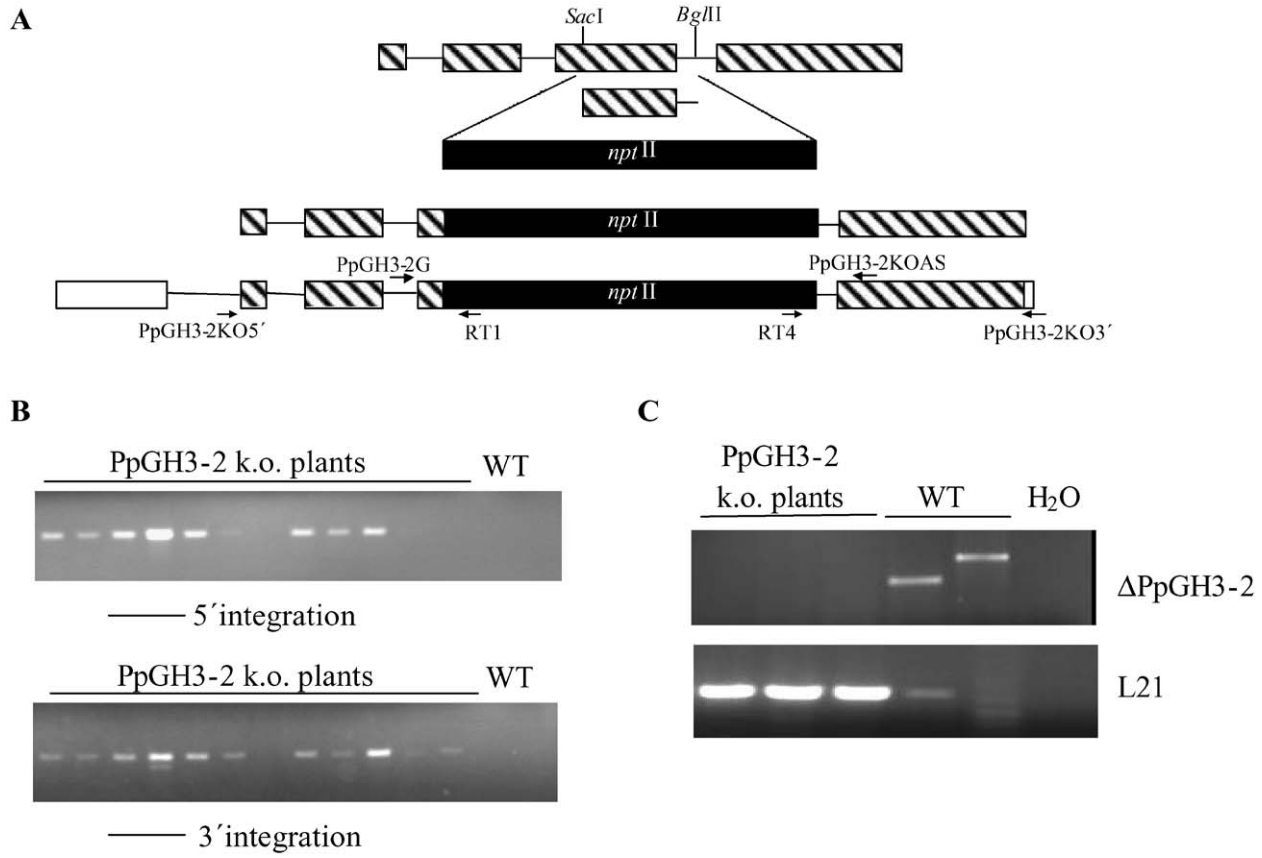

Figure 5. Targeted disruption of the *PpGH3-2* locus in *Physcomitrella*. (A) The knockout construct was generated by replacing a 453 bp fragment by insertion of the *npt*II selection cassette (black bar) into a *Sac*I/*Bgl*II digested 1649 bp genomic *PpGH3-2* fragment. Exons included in the knockout construct are shown by hatched bars, introns by lines. Transgenic plants were screened by PCR with primers spanning the selection cassette (*PpGH3-2*G/*PpGH3-2*KOAS) and primers specific for 5′-integration (*PpGH3-2*KO5′/RT1) and 3′-integration (RT4/*PpGH3-2*KO3′). Dimensions are drawn to scale. (B) Nine putative *PpGH3-2* k.o. plants showed 5′ and 3′ integration of the knockout construct into the *PpGH3-2* gene. **C** RT-PCR demonstrated expression of *PpGH3-2* in the wild type and absence of *PpGH3-2* mRNA in three knockout plants. Amplification of *PpGH3-2* products from WT was shown for cDNA (WT, left) and genomic DNA (WT, right). Internal control is the constitutively expressed RNA for the ribosomal protein L21. WT, wild type.

the *npt*II selection cassette. Wild-type cDNA produced a PCR product of the expected size (500 bp), whereas for the three *PpGH3-2* putative knockout plants no PCR product was visible, confirming the successful disruption of the *PpGH3-2* gene locus. The result of the RT-PCR was further confirmed by Northern blot analysis (data not shown).

Four Δ*PpGH3-1* as well as three Δ*PpGH3-2* plants were analysed for an aberrant phenotype linked to the gene disruption. Development of the knockout plants was investigated on solid medium under standard conditions. A change in the morphological appearance of the knockout plants could not be observed (data not shown). As caulonema development is influenced by auxin (Johri and Desai, 1973), protoplast regeneration in liquid culture under standard conditions with and without the application of exogenous auxin was investigated. The knockout plants did not display any changes in phenotype compared to the wild type with respect to cell morphology or developmental time course (data not shown). As there is a supposed link between the *GH3*-like proteins and phytochrome signaling (Hagen and Guilfoyle, 2002), the development of knockout plants considering protonema and gametophore development under different light conditions was investigated including white, red, and far-red light. In red light the development of protonema and gametophores of wild type and knockout plants was delayed compared to growth under normal white light conditions (Fig. 6). All plants developed gametophores that exhibited an etiolated phenotype (Fig. 7). The observed development of an elongated gametophore in *Physcomitrella* under red light is in agreement with the observations of Imaizumi et al. (2002). Under far-red light conditions this phenotype of wild type and knockout plants was more impressive. Even after 3

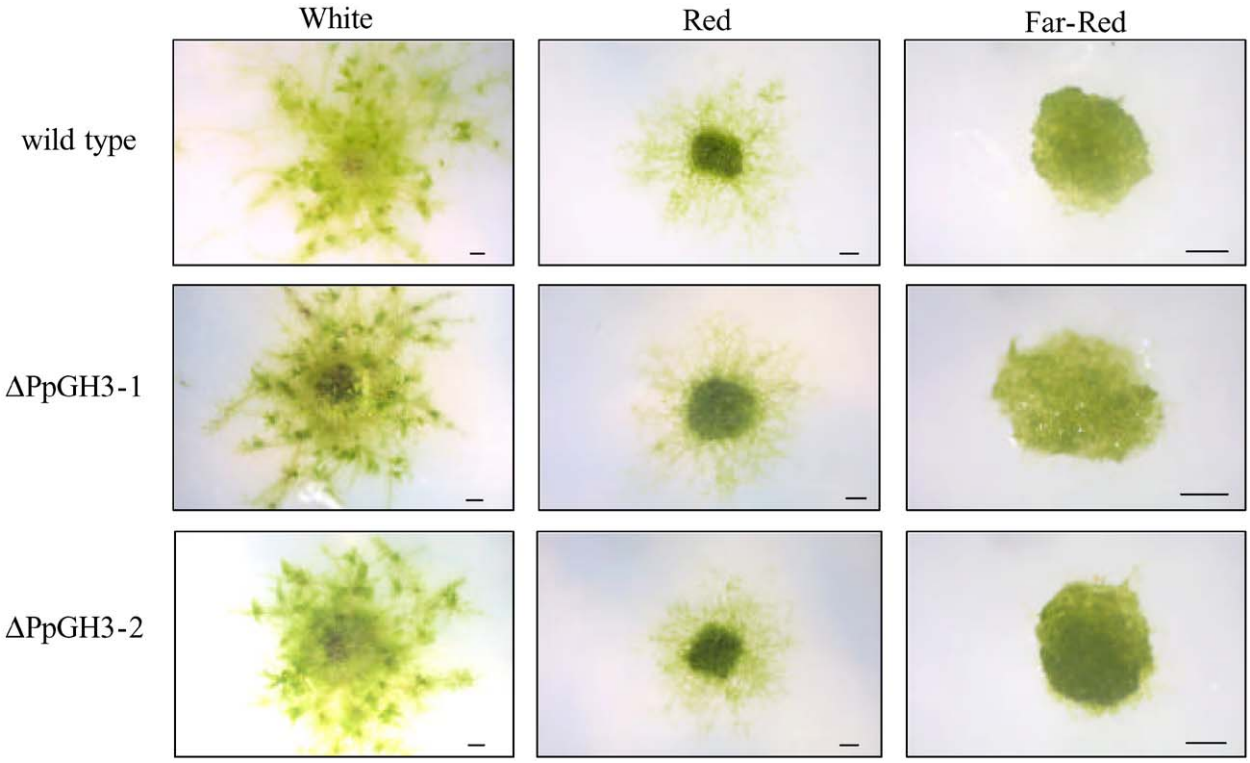

Figure 6. Protonema development under different light conditions. Wild type, Δ*PpGH3-1* and Δ*PpGH3-2* protonema from liquid culture were distributed on Knop agar plates as dots with a similar size and cultivated for up to 3 weeks in different light conditions. Growing wild type and knockout protonema developed gametophores in white light, whereas in red light development and growth was delayed. In far-red light, a further protonema growth and development of gametophores of wild type and knockout plants did not take place. Scale bar 1 mm.

weeks, protonema growth and development of buds and, subsequently, gametophores did not take place (Fig. 6). When protonema was used that had already developed buds and young gametophores a strong elongation of the growing gametophores appeared with short and narrow leaves (Fig. 7). However, also under the diverse light conditions no obviously altered phenotypes of the *PpGH3-1* nor the *PpGH3-2* knockout plants were detectable. Gametophore development was also observed under several fluence points but again no phenotypical differences could be detected between wild type and the two knockouts.

Discussion

Physcomitrella contains a small gene-family encoding GH3-like homologues

The isolated *Physcomitrella GH3*-like homologues *PpGH3-1* and *PpGH3-2* display similarities to those described in seed plants. At first, the proteins do not display any particular features concerning predicted localisation or matches to recognized protein motifs in the databases. Secondly, *PpGH3-1* and *PpGH3-2* contain three weakly conserved motifs that were detected in *JAR1* (*FIN219*, *AtGH3-11*) and mediate adenylation activity (Staswick et al., 2002; Chang et al., 1997). Thirdly, *Physcomitrella* contains more than one *GH3* homologue.

Despite these similarities, the *Physcomitrella* homologues show interesting differences to those previously described from seed plants. Compared to seed plants and the *Physcomitrella PpGH3-2* protein with a predicted molecular weight of about 65–70 kDa (Hagen and Guilfoyle, 2002), *PpGH3-1* is slightly larger with a calculated weight of about 71 kDa. This is mainly due to an additional stretch of amino acids immediately after the translational start of methionine. The *PpGH3-1* gene contains an additional noncoding exon separated by a 5′ intron from the first coding exon. This was never described so far for any other *GH3*-like gene. Introns in the 5′ untranslated region are known from other plants, such as the actin 1 gene from rice (McElroy et al., 1990). McElroy et al. (1990) showed a connection between the 5′ intron and

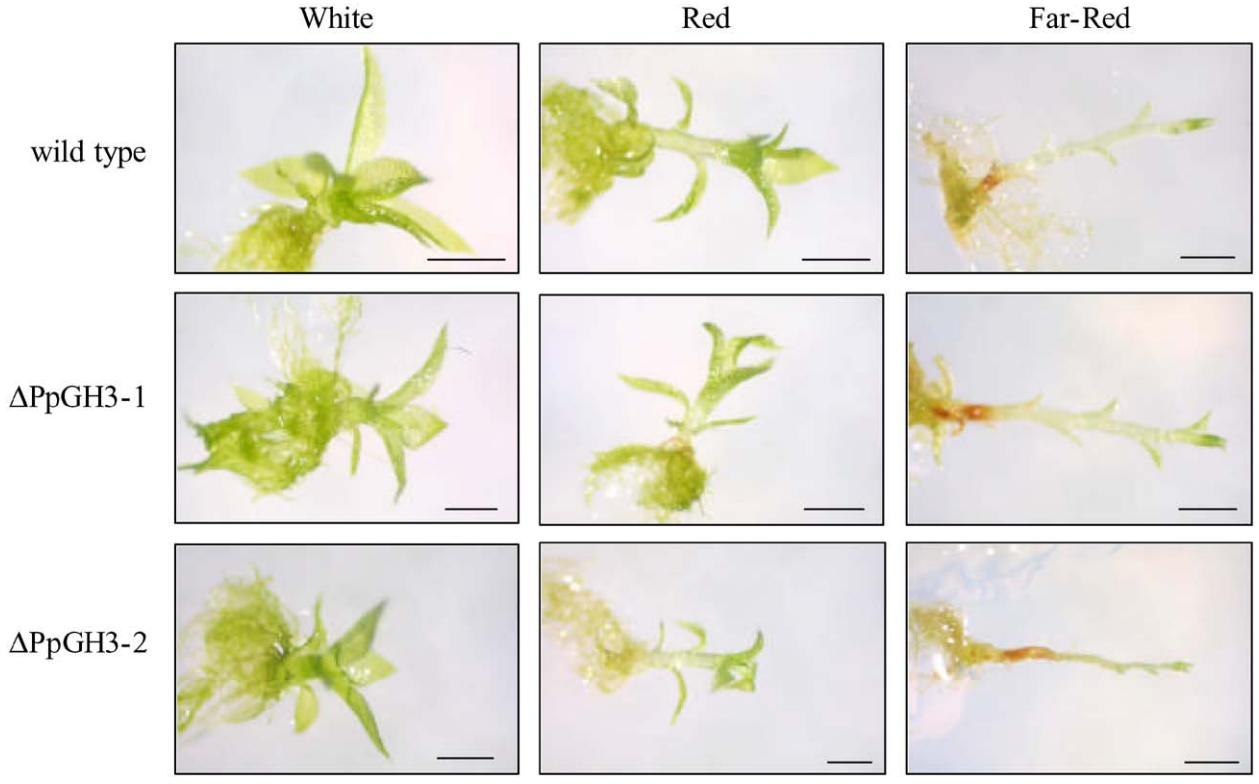

Figure 7. Gametophore development of wild type and knockout plants under white, red and far-red light. Protonema with developing buds was cultivated up to 3 weeks under different light conditions. Under red light, the developing gametophores of wild type and knockout plants were elongated compared to white-light conditions. The elongated phenotype was even stronger for the analysed plants under far-red light and leaves were extremely short and narrow. Scale bar 0.5 mm.

regulation of gene expression. This could also be the function of the *PpGH3-1* 5′ intron but this has yet to be investigated.

GH3-like proteins from seed plants are distributed over three groups in a phylogenetic tree. The two *Physcomitrella GH3* homologues cluster together in the third group, with the *Arabidopsis GH3*-like protein *AtGH3-11* corresponding to *FIN219* (Hsieh et al., 2000). The other two analysed *Arabidopsis GH3*-like proteins are situated in the first group (*AtGH3-6* corresponding to *DFL1*; Nakazawa et al. (2001) and *AtGH3-5* corresponding to *AtGH3a*; Tanaka et al. (2002)). These three *Arabidopsis GH3*-like proteins were connected to light responses at the expression level or after mutant phenotype analysis. Therefore, the membership in one of the three groups could not be assigned to a special protein function.

The *Arabidopsis* gene-family of *GH3*-like homologues is quite large, consisting of 19 members (Hagen and Guilfoyle, 2002). Contrary to this, we assume that *Physcomitrella* possesses only three *GH3* homologues, the two isolated genes *PpGH3-1* and *PpGH3-2* and a third one, which was identified as a partial cDNA sequence within the *Physcomitrella* EST database (data not shown). As our EST database covers most of the *Physcomitrella* transcriptome and the average size of *Physcomitrella* gene-families is significantly reduced compared to *Arabidopsis* (Rensing et al., 2002), the probability of the existence of additional *GH3*-like homologues in *Physcomitrella* might be low. The smaller amount of *GH3*-like homologues in *Physcomitrella* compared to *Arabidopsis* favours further analysis to determine their function in plant development.

PpGH3-1 and PpGH3-2 are expressed in protonema and gametophores

PpGH3-1 and *PpGH3-2* are expressed in the same developmental stages of *Physcomitrella*, namely, the protonema and the leafy gametophore. In addition, they are expressed at the same time points during protonema development, showing an identical spatial and temporal pattern of gene expression. As both transcripts already occured one day after protoplast isolation, the function of both proteins seems to start very early in *Physcomitrella* development.

Most of the analysed *GH3*-like genes from seed plants are described as auxin-inducible (Hagen and Guilfoyle, 2002). Induction could occur after a few minutes and could hold on for a few hours (Roux and Perrot-Rechenmann, 1997; Hsieh et al., 2000). There is one *Arabidopsis* family member (*AtGH3-17*) that is expressed in the absence of auxin treatment and the expression levels of this member could not be further elevated by application of auxin (Hagen and Guilfoyle, 2002). *PpGH3-1* and *PpGH3-2* were similarly expressed under physiological cultivation conditions without auxin application and further enhancement of transcription was not possible by addition of auxin. Thus, their behaviour resembled that of *AtGH3-17*. Contrary to this, Imaizumi et al. (2002) found for *PpGH3L1*, which is identical to *PpGH3-2*, an induction of expression 24 h after application of 10 μM naphthalene acetic acid (NAA). The difference in these expression data might be explained by different culture or experimental conditions. In contrast to cultivation with a light–dark regime of 16:8 h, Imaizumi et al. used permanent light and some medium additives such as ammonium tartrate and glucose, which were not used in our study. Furthermore, they applied auxin to 5-day-old cultures while we started already after 3 days.

Analysis of PpGH3-1 and PpGH3-2 knockout plants

Precise knowledge about the function of *GH3*-like proteins in plant development is still missing. At least for some *GH3*-like proteins, it is assumed that they represent a possible link between auxin and phytochrome/light pathways, which could be concluded from the analysis of *Arabidopsis* mutants (Hagen and Guilfoyle, 2002). To elucidate the possible function of *GH3*-like proteins in the moss *Physcomitrella*, we started a knockout approach with the two isolated homologues, *PpGH3-1* and *PpGH3-2*. There was no obviously altered phenotype visible for single knockouts of both of the genes under our experimental conditions, thus indicating no essential gene function for *PpGH3-1* and *PpGH3-2* in *Physcomitrella* development. A missing phenotype of the *PpGH3-1* and *PpGH3-2* knockout plants leads to the assumption that a functional redundancy between the two genes could exist in *Physcomitrella*. The fact that the two genes group together in the same phylogenetic cluster and that they display the same spatial and temporal expression pattern lends support to this assumption. This is surprising, because they are quite divergent on the nucleotide and aa sequence levels. An *Arabidopsis* mutant with a disturbed *AtGH3a* gene also did not show an aberrant phenotype under the analysed conditions (Tanaka et al., 2002). In contrast to this, *df11-D* and *FIN219* plants carrying a mutation in two different *AtGH3* genes both exhibited altered hypocotyl elongation under different red-light conditions (Hsieh et al., 2000; Nakazawa et al., 2001), revealing overlapping functions of the two genes in phytochrome-mediated signaling. As these *GH3* homologues were suggested to be negative regulators of hypocotyl elongation, we expected a more extreme response of the *Physcomitrella GH3* knockouts to the different light conditions (i.e. a more pronounced elongation). Nevertheless, there were also differences in phenotype appearance in *Arabidopsis* mutants. Over-expression of *DFL1* resulted in a dwarf phenotype (Nakazawa et al., 2001), which was not visible in *FIN219* over-expressing plants (Hsieh et al., 2000). From these results it can be assumed that *GH3*-like proteins have partially redundant functions as well as possibly non-redundant roles in auxin and/or light signaling comparable to another class of auxin-inducible genes, namely the *Aux/IAA* family (Hagen and Guilfoyle, 2002). The missing phenotype of the two *Physcomitrella* knockouts could mean that a possible non-redundant function of *PpGH3-1* and *PpGH3-2* was not detectable under our conditions or that the *Physcomitrella GH3*-like proteins fulfill functions other than the analysed *Arabidopsis* genes.

A functional redundancy between the two *Physcomitrella GH3* homologues could be overcome by creating a double knockout of the two genes. Over-expression of the genes could further help to elucidate a possible function, as the known *dfl1-D* mutant and *Arabidopsis* plants over-expressing the soybean *GH3* show an easily recognizable phenotype exhibiting an exaggerated dwarf phenotype (Nakazawa et al., 2001; Hagen and Guilfoyle, 2002).

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