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The cyclophilin DIAGEOTROPICA has a conserved role in auxin signaling

Meirav Lavy[1], Michael J. Prigge[1], Kristof Tigyi[1] and Mark Estelle[1,2,*]

SUMMARY

Auxin has a fundamental role throughout the life cycle of land plants. Previous studies showed that the tomato cyclophilin DIAGEOTROPICA (DGT) promotes auxin response, but its specific role in auxin signaling remains unknown. We sequenced candidate genes in auxin-insensitive mutants of *Physcomitrella patens* and identified mutations in highly conserved regions of the moss ortholog of tomato *DGT*. As *P. patens* and tomato diverged from a common ancestor more than 500 million years ago, this result suggests a conserved and central role for DGT in auxin signaling in land plants. In this study we characterize the *P. patens dgt* (*Ppdgt*) mutants and show that their response to auxin is altered, affecting the chloronema-to-caulonema transition and the development of rhizoids. To gain an understanding of PpDGT function we tested its interactions with the TIR1/AFB-dependent auxin signaling pathway. We did not observe a clear effect of the *Ppdgt* mutation on the degradation of Aux/IAA proteins. However, the induction of several auxin-regulated genes was reduced. Genetic analysis revealed that *dgt* can suppress the phenotype conferred by overexpression of an AFB auxin receptor. Our results indicate that the DGT protein affects auxin-induced transcription and has a conserved function in auxin regulation in land plants.

KEY WORDS: Auxin, *Physcomitrella patens*, DGT

INTRODUCTION

The plant hormone auxin has a central role in all aspects of plant growth and development. Auxin acts by regulating transcription through the action of three protein families: the TIR1/AFB auxin receptors (Dharmasiri et al., 2005b; Mockaitis and Estelle, 2008; Greenham et al., 2011), the Aux/IAA transcriptional repressors (Overvoorde et al., 2005) and the ARF transcription factors (Guilfoyle and Hagen, 2007). The TIR1/AFB receptors are F-box proteins and subunits of ubiquitin ligase $SCF^{TIR1/AFB}$ complexes (Santner and Estelle, 2010). Upon auxin binding, the TIR1/AFB protein interacts with the highly conserved degron domain of the Aux/IAA proteins, resulting in their degradation (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Tan et al., 2007; Calderon-Villalobos et al., 2010). The degradation of Aux/IAA proteins releases the repression of ARFs, leading to ARF-dependent transcription.

Our understanding of auxin signaling is based primarily on work in *Arabidopsis*. Less is known about auxin signaling in non-seed plants. The completion of the genome sequence for the moss *Physcomitrella patens* (Rensing et al., 2008), together with efficient targeted gene insertion (Schaefer, 2002; Prigge and Bezanilla, 2010), have established *P. patens* as a useful experimental tool for studying the ancestral role of auxin.

Mosses are one of three lineages referred to as bryophytes, which are early diverging non-vascular land plants (Kenrick and Crane, 1997). The moss life cycle is dominated by a haploid gametophyte. Following germination of a haploid spore, protonemal filaments are produced and proliferate by apical division. Initially, protonemata consist of chloroplast-rich filaments called chloronemata. Later, chloronemal filaments can differentiate to form a distinct type of filament, called caulonemata, which are more elongated and contain fewer chloroplasts. Subapical caulonemal cells, in turn, can form branches that either develop into a secondary chloronemal filament, a caulonemal filament or into a leafy gametophore (Ashton et al., 1979).

Auxin plays a central role throughout the life cycle of *P. patens*. In the filamentous protonemata stage, exogenous auxin promotes the transition of a chloronemal filament into a caulonemal filament (Ashton et al., 1979). In the adult plant, auxin treatment stimulates stem elongation (Fujita et al., 2008; Eklund et al., 2010), elongation of gametophore phyllids (leaves) (Decker et al., 2006) and the formation of rhizoids from epidermal cells of the gametophore (Ashton et al., 1979).

In a screen carried out three decades ago, Ashton and colleagues (Ashton et al., 1979) isolated *P. patens* mutants with an altered response to the synthetic auxin 1-naphthalene-acetic acid (NAA). These NAA-resistant (*nar*) mutants were isolated based on their reduced sensitivity to a high concentration of auxin or by their morphological abnormalities. Although the affected genes remained unknown, the mutants were utilized to characterize the auxin response in moss.

The *P. patens* genome encodes four auxin receptors, three Aux/IAA and fifteen ARF proteins (Rensing et al., 2008; Paponov et al., 2009; Parry et al., 2009; Prigge et al., 2010). Recently, we demonstrated that the mechanism of auxin action is conserved in *P. patens*. DNA sequencing revealed eight independent *nar* mutants harboring gain-of-function mutations in the conserved degron motif of the three *PpAux/IAA* genes, similar to comparable mutations in *Arabidopsis*. The moss mutants display an auxin-insensitive phenotype that includes a significant reduction in the number of caulonemal cells and the subsequent absence or reduction in the development of leafy gametophores. Comparable phenotypes were also identified as a result of silencing of the four *PpAFB* receptor genes. Additionally, PpAFB and PpAux/IAA proteins interact in

[1]Section of Cell and Developmental Biology and [2]Howard Hughes Medical Institute, University of California San Diego, La Jolla, CA 92093, USA.

*Author for correspondence (mestelle@ucsd.edu)

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the presence of auxin, indicating that the mechanism of auxin perception is conserved in non-seed plants (Prigge et al., 2010). This work demonstrates the significant contribution of moss genetic analyses to the study of auxin signaling. In the current work we describe the discovery and analysis of *P. patens diageotropica* (*dgt*) auxin-resistant mutants.

Tomato *dgt* mutants exhibit a pleiotropic phenotype that includes a slow gravitropic response, lack of lateral roots, reduced apical dominance, shortened internodes, altered vascular development and reduced fertility and fruit growth (Zobel, 1973; Mito and Bennett, 1995; Rice and Lomax, 2000; Balbi and Lomax, 2003; Ivanchenko et al., 2006). Application of auxin to tomato *dgt* mutants does not stimulate shoot elongation and the effect on root growth inhibition is reduced compared with the wild type (WT). Ethylene production is also reduced in auxin-treated *dgt* tomato plants (Bradford and Yang, 1980; Kelly and Bradford, 1986). However, the rate of polar indole-3-acetic acid (IAA) transport in *dgt* hypocotyls and roots is similar to that of the WT parent (Daniel et al., 1989; Muday et al., 1995) and levels of IAA in *dgt* shoot apices were shown to be normal (Fujino et al., 1988). These results generally support the hypothesis that tomato *dgt* is altered in auxin perception or response. The effect of the mutant *dgt* on the expression of auxin-induced genes is variable. Whereas the induction of several *Aux/IAA* genes was shown to be normal, the induction of others was significantly reduced (Nebenfuhr et al., 2000; Balbi and Lomax, 2003). The subset of *Aux/IAA* genes showing reduced expression differs depending on the developmental stage and tissue (Balbi and Lomax, 2003). Similarly, the expression of genes encoding 1-aminocyclopropane-1-carboxylic acid synthases (ACSs), which are key regulatory enzymes of ethylene biosynthesis, is differentially affected in the tomato *dgt* mutants (Balbi and Lomax, 2003; Coenen et al., 2003; Vidoz et al., 2010). The induction of a *SMALL AUXIN UP-REGULATED RNA* (*SAUR*) gene also displays reduced induction compared with WT (Mito and Bennett, 1995; Coenen et al., 2003). The tomato *DGT* gene was mapped and determined to encode a type A cyclophilin (CyPA), designated CYP1 (Oh et al., 2002; Oh et al., 2006). Each of the three known *dgt* mutant alleles was shown to contain a single point mutation that generates either an amino acid substitution or an early stop codon (Oh et al., 2006).

Cyclophilins are represented in every organism and belong to a highly conserved large family of peptidyl-prolyl cis-trans isomerase (PPIase) proteins that catalyze the cis-trans isomerization of peptide bonds preceding a proline residue (Galat, 2003). The cellular roles of the cyclophilins have been difficult to determine. In mammals, they have numerous functions, including protein folding, chaperone activities, cell signaling, protein trafficking, ligand-receptor recognition, regulation of transcription and splicing (Wang and Heitman, 2005). The archetypal CyPA has attracted interest due to its involvement in diverse pathological conditions (Nagy et al., 2011; Lee and Kim, 2010). Although extensively studied, its targets and specific roles remain obscure. Cyclophilins are abundantly expressed in *Arabidopsis* (Chou and Gasser, 1997; Romano et al., 2004), but the functions of only a few are known.

Here we demonstrate that several *P. patens nar* mutants harbor mutations in the moss ortholog of the tomato *DGT* (*CYP1*) gene and describe their reduced sensitivity to auxin. We also characterize the *P. patens DGT* gene and study its interactions with components of the TIR1/AFB-dependent auxin signaling cascade. We show that DGT has a conserved role in auxin signaling, which affects the induction of auxin-regulated genes downstream of the auxin receptors.

MATERIALS AND METHODS

Moss strains and growth conditions

WT 'Gransden-2004' *P. patens* and other strains listed below were grown at 25°C under continuous light at an intensity of 40-70 μmol/m^2/second on BCD and BCDAT media supplemented with 1.8 μM para-aminobenzoic acid (PAB) to allow growth of the auxotrophic strains *pabA3* and *pabB4*. Strains NAR 23 (*dgt-23*), NAR 26 (*dgt-26*), NAR 171 (*dgt-171 pabA3*), NAR 176 (*dgt-176 pabA3*), NAR 180 (*dgt-180 pabA3*) and *pabB4* were described previously (Ashton and Cove, 1977; Ashton et al., 1979). In this work, the *pabB4* line was used as an auxotrophic control strain because *pabA3* was not available.

Sequencing

Mutations in the *PpDGT* gene were identified by sequencing PCR products from genomic DNA isolated from *nar* mutant lines using PpDGT-F/PpDGT-R primers (supplementary material Table S1).

Molecular cloning

The constructs used for the moss transformations described below are listed in Table 1. Primers are listed in supplementary material Table S1.

To create *pML36*, *GUS:T$_{nos}$* was amplified using *PML9/PML10* primers and cloned into the *Xho*I site of *pBNRF* to create *pBNRF-GUS*. Approximately 1 kb of genomic sequence upstream (5′ flanking region) and downstream (3′ flanking region) to the *DGT* coding region were amplified using the primer pairs *PML132/ PML125* and *PML128/PML129*, respectively, and each fragment was cloned into *pENTR-D/TOPO* (Invitrogen, Carlsbad, CA, USA). The 5′ and 3′ flanking regions were recombined into *Bam*HI and *Spe*I sites in *pBNRF-GUS*, respectively.

To create *pML31*, a fragment consisting of 509 bp upstream sequence together with the *DGT* coding region (*P$_{DGT509}$:DGT*) was amplified using the *PML289/PML301A* primer pair. *pML36* was digested with *Bam*HI and the *DGT* 5′ flanking region was replaced with the *P$_{DGT509}$:DGT* sequence.

To create *pML38*, *PML31* was subject to site-directed mutagenesis using *PML149/PML150*.

To create *pML32*, the *YFP-HA:T$_{OCS}$* fragment was amplified from *pEarleyGate102* (Earley et al., 2006) using *PML378/PML379* primers followed by site-directed mutagenesis to mutate an *Xho*I site using *PML380/PML381* primers. *pML31* was digested with *Xho*I and the *GUS* gene was replaced with *YFP-HA:T$_{OCS}$*.

To create *pML37*, a *P$_{35S}$:HygR:T$_{CaMV}$* hygromycin resistance cassette was amplified from *pMP1159* (http://web.mac.com/mjprigge/Prigge/Moss.html) using *PML371/PML372* primers. The *pBNRF-GUS* construct harboring a *DGT* 3′ flanking region inserted into the *Spe*I site was digested with *Kpn*I to replace the *nptII* gene with a *HygR* gene.

To create *pML53*, *pML36* was digested with *Xho*I to replace a fragment consisting of the *DGT* 5′ flanking region and the *GUS* gene with the *DGT* 5′ flanking region only. The resulting construct was digested with *Kpn*I to replace the *nptII* gene with a *HygR* gene.

To create *pML59*, *PpIAA1B* cDNA was cloned upstream of *GUS:Tnos* before amplifying the *PpIAA1B-GUS:Tnos* fragment using *PML230/PML10* primers. The resulting product was cloned into *pENTR-D/TOPO* and recombined into *pMP1180*.

To create *pML49*, *PpAFB2* cDNA was amplified using *PML203/PML204* primers and cloned upstream of a *c-Myc* tag before amplifying the *PpAFB2-c-Myc* fragment using *PML203/PML208* primers. The resulting product was cloned into *pENTR-D/TOPO* and recombined into *pMP1155*.

Moss transformation and screening of transgenic lines

Protoplast isolation and PEG-mediated transformation of *P. patens* was performed as described (Nishiyama et al., 2000). Five days after regeneration, transformants were selected on BCDAT medium containing 20 mg/l G418 or hygromycin.

Transgenic lines were screened by PCR for the presence of both left and right transgene-endogenous sequence junctions to verify the insertion of the transgene at the targeted locus. Genomic DNA extracted from *dgtΔ* lines was analyzed by PCR to confirm the integration of the transgene into the *DGT* locus and the absence of the *DGT* coding region (confirmation of the transgene integration is shown in supplementary material Fig. S3O). cDNA (extracted as described below) was detected by RT-PCR to confirm the absence of a transcribed product.

Table 1. Vectors used in this study

Name	Description	Backbone	Resistance gene	Source
pBNRF			*nptII*	(Schaefer et al., 2010)
P_{GmGH3}:GUS			*nptII*	(Bierfreund et al., 2003)
pMP1180	*pUK-Pp108B-nptII-GmP_{HSP}17.3B:Gateway:e9*		*nptII*	http://web.mac.com/mjprigge/Prigge/Moss.html
pMP1155	*pUK-Pp108B-nptII-P_{UBi}:Gateway:T_{OCS}*		*nptII*	http://web.mac.com/mjprigge/Prigge/Moss.html
pBNRF-GUS			*nptII*	This study
pML36	*P_{DGT}:GUS/PpdgtΔ*	pBNRF	*nptII*	This study
pML31	*P_{DGT}:PpDGT-GUS*	pBNRF	*nptII*	This study
pML38	*P_{DGT}:Ppdgt-176-GUS*	pBNRF	*nptII*	This study
pML32	*P_{DGT}:PpDGT-YFP-HA*	pBNRF	*nptII*	This study
pML37	*P_{DGT}:GUS/PpdgtΔ*	pBNRF	*HygR*	This study
pML53	*PpdgtΔ*	pBNRF	*HygR*	This study
pML59	*P_{HSP}:PpIAA1B-GUS*	pMP1180	*nptII*	This study
pML49	*P_{UBi}:PpAFB2-c-Myc*	pMP1155	*nptII*	This study

Quantitative RT-PCR

Protonemal tissue composed mainly of chloronemata was homogenized in a Waring blender and plated in triplicate on BCDAT plates with cellophane overlays for 7 days. For hormone treatment, the cellophane overlays covered with protonemal tissue were transferred into liquid BCD medium containing 10 μM IAA or 10 μM ABA or the equivalent amount of ethanol or methanol solvent, respectively. For heat shock treatment, the cellophane covered with protonemal tissue was transferred into liquid BCD medium and incubated at 37°C (or 25°C control). The tissue was collected after 5 hours and total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). RNA (500 μg) was reverse transcribed using the Superscript III First-Strand cDNA Synthesis System (Invitrogen). Then 20 μl of the reverse transcription reaction was diluted with water to a final volume of 200 μl. PCR reactions were performed with 4 μl diluted cDNA and the primers listed in supplementary material Table S1 and detected using the CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Normalized expression [ΔΔC(t) method] was calculated using Bio-Rad CFX Manager software with *PpACTIN* as a reference gene and plotted as values relative to mock-treated WT ± s.e.m.

PpIAA1B-GUS degradation assay

To express the PpIAA1B-GUS fusion protein, plants were grown on BCDAT plates with cellophane overlays, transferred to liquid BCDAT medium and incubated at 37°C for 1 hour and 30 minutes, followed by a recovery of 30 minutes at 25°C. Tissue was transferred to liquid BCDAT media containing 50 μM IAA or the equivalent amount of ethanol solvent for 5 hours and then stained for GUS.

GUS staining

Tissue was stained in GUS staining solution [50 mM NaH_2PO_4 pH 7.0, 0.5 mM X-Gluc, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.05% Triton X-100] at 37°C for 45 minutes or overnight following auxin treatment of *P_{GH3}:GUS* lines or the degradation assay, respectively. Plants were cleared in 70% (v/v) ethanol and imaged using a Nikon SMZ1500 dissecting scope.

Protein immunoblot analysis

Proteins from protonemal tissue were extracted in 50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% NP40 and complete protease inhibitor (Roche, Indianapolis, IN, USA). The resulting protein extract was centrifuged for 10 minutes at 10,000 ***g*** and the supernatant was collected. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were stained with Ponceau S to standardize the input and an HRP-conjugated anti-c-Myc antibody was used for protein detection (Roche). Proteins were visualized using the ECL Plus Western Blot Detection System (GE Healthcare, Piscataway, NJ, USA).

RESULTS

Moss *dgt* mutants have reduced auxin sensitivity

To discover which genes are mutated in the *P. patens nar* mutants, we took a candidate gene approach and sequenced the moss orthologs of known auxin-related genes. A moss gene encoding the closest homolog to tomato DGT (CYP1) and to human CyPA was identified and designated *PpDGT* (GenBank accession number XM_001778952; supplementary material Fig. S1). Sequencing of *PpDGT* in the auxin-resistant moss mutants revealed five different *dgt* mutant alleles. The mutated alleles contain single-nucleotide point mutations that generate either an amino acid change (dgt-$26^{S\text{-}L}$, dgt-$171^{G\text{-}E}$, dgt-$176^{G\text{-}D}$ and dgt-$180^{C\text{-}Y}$) or a stop codon (dgt-$23^{Q\text{-}stop}$) affecting highly conserved regions (supplementary material Fig. S1).

The *dgt/nar* mutants and WT strains (Grandsen-2004 and *pabB4* serving as controls for the non-auxotrophic and auxotrophic mutants, respectively) were grown on minimal medium supplemented with ammonium tartrate (BCDAT) or on a medium without ammonium tartrate (BCD) to stimulate the growth of protonemata or gametophores, respectively. When WT plants were grown on auxin-containing BCDAT medium for 1 month, caulonemal filaments developed from chloronemal cells (Fig. 1A, WT and *pabB4*). By contrast, the *dgt/nar* mutants developed fewer and much shorter caulonemal filaments than WT. WT plants grown on BCD medium produced more leafy gametophores (Fig. 1B, WT, *pabB4*; supplementary material Fig. S2). When 0.5 μM NAA was added to the medium, only a few fully developed gametophores grew and the plants produced mainly rhizoid-like cells that emerged from small leafless gametophore-like structures. With a higher concentration of exogenous auxin, no gametophores developed and the plants comprised rhizoid-like cells only. Under the same conditions, *dgt/nar* mutant plants grown on BCD developed fewer and smaller gametophores. The effect of exogenous auxin on the *dgt/nar* mutants was less pronounced compared with WT, with fewer rhizoids developing in response to high auxin concentrations. The *dgt-176* mutant showed the strongest phenotype, with no gametophores developing on BCD medium (Fig. 1B).

It has been shown that auxin affects the size of the leaves in moss (Decker et al., 2006). To further characterize the differences between WT and *dgt/nar* mutants in their response to auxin, we quantified the length of the uppermost two or three leaves from plants grown on BCD medium for 1 month (Fig. 1E). Similar to the differences in size between WT and mutant gametophores, the individual leaves of WT gametophores were significantly longer than those of *dgt/nar* mutants. Whereas growth on auxin resulted in a decrease in WT leaf length, mutant leaves were either the same length or longer when grown on auxin as compared with untreated leaves.

To confirm that the phenotype of the *dgt/nar* mutants results from the mutated *dgt*, we replaced the *DGT* coding region with the *GUS* gene by homologous recombination (*P_{DGT}:GUS/dgtΔ*).

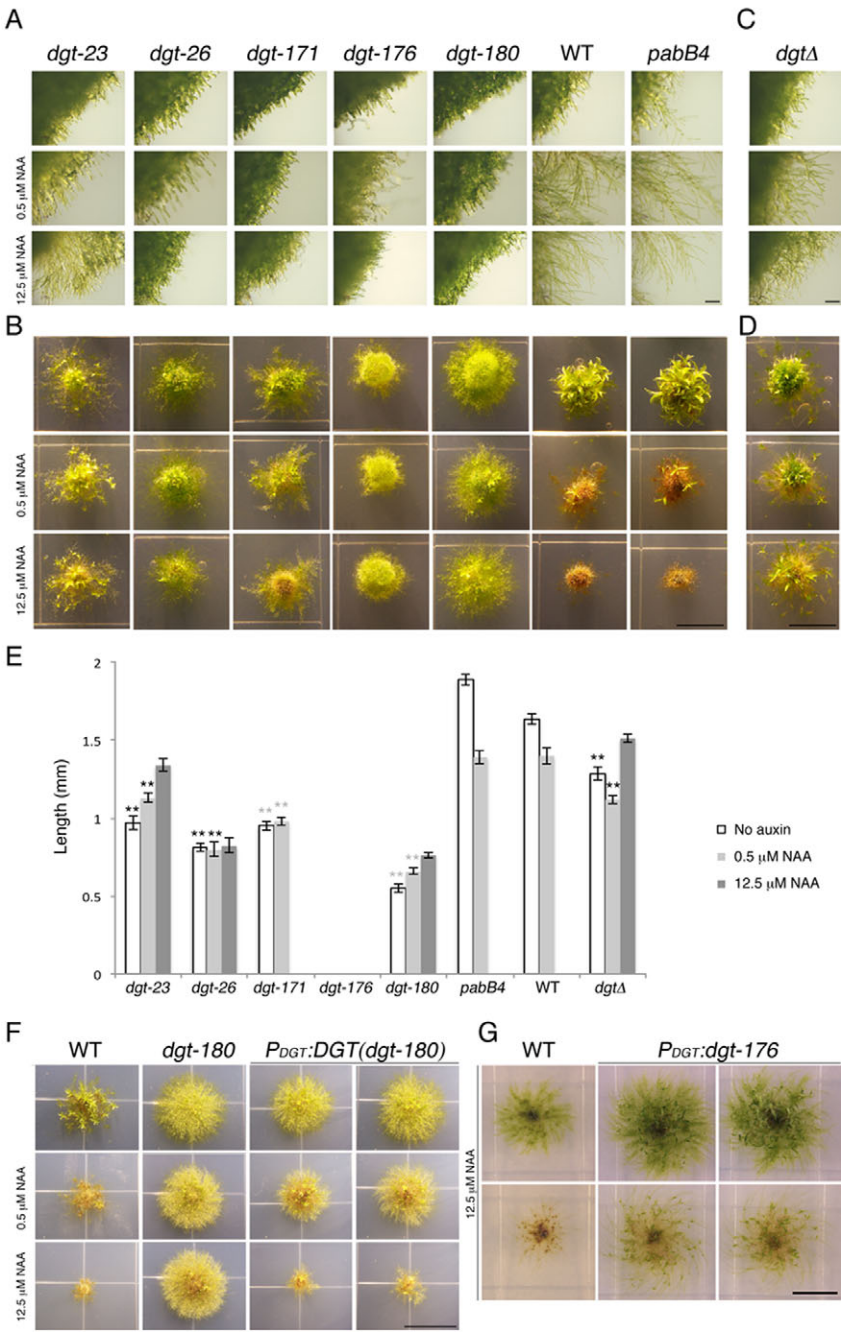

Fig. 1. Phenotypic analysis of *P. patens dgt* mutants. (**A**) The peripheral part of 1-month-old *dgt* mutants (*dgt-23*, *dgt-26*, *dgt-171*, *dgt-176* and *dgt-180*) and non-mutant plants (WT and *pabB4*) grown on BCDAT without auxin or with 0.5 μM or 12.5 μM NAA. (**B**) One-month-old *dgt* mutants, WT and *pabB4* grown on BCD without auxin or with 0.5 μM or 12.5 μM NAA. (**C**,**D**) *dgt* knockout mutants (*dgtΔ*) grown as described in A,B. (**E**) The average length (mm) of 20 leaves taken from *dgt* mutant and WT plants grown on BCD without auxin or with 0.5 μM NAA or 12.5 μM NAA. Error bars represent s.e.m. $^{**}P<0.001$ (*t*-test); black asterisks denote significantly different from WT, gray asterisks denote significantly different from *pabB4*. (**F**) DGT complementation assay comparing 1-month-old WT, *dgt* mutant (*dgt-180*) and two independent lines created by replacing the mutated *dgt* with the native gene in the *dgt-180* mutant [P_{DGT}*:DGT (dgt-180)*]. The plants are grown on BCD without auxin or with 0.5 μM or 12.5 μM NAA. (**G**) One-month-old WT and two independent lines of P_{DGT}*:dgt-176* mutants grown on BCDAT (top row) or BCD (bottom row) with 12.5 μM NAA. Scale bars: 500 μm in A,C; 50 mm in B,D,G; 100 mm in F.

Two and five independent lines were obtained using two different constructs carrying either the neomycin resistance (*nptII*) or hygromycin resistance (*HygR*) gene, respectively. The insertion of the transgene into the targeted locus and the absence of *DGT* were confirmed for all lines. All the lines presented similar phenotypes. The two *nptII*-encoding lines were compared with the *dgt/nar* mutants (Fig. 1C,D; supplementary material Fig. S2). RT-PCR analyses confirmed the absence of a full-length *DGT* transcript in the transgenic line, indicating that the *dgtΔ* strain is a null allele (supplementary material Fig. S3). Loss of the *DGT* gene resulted in reduced sensitivity to auxin. The *dgtΔ* plants developed fewer caulonemal filaments than WT when grown on auxin (Fig. 1C). When grown on medium lacking ammonium tartrate (BCD), the size of gametophores was reduced and the length of their leaves was also reduced compared with WT. Application of auxin also resulted in shorter rhizoids (Fig. 1D,E; supplementary material Fig. S2). However, the *dgtΔ* mutant presented a less severe phenotype than the five *dgt/nar* mutants.

We next tested whether the *dgt/nar* mutations can be complemented with the native *DGT* gene. The mutant locus in *dgt-180* was replaced with a *DGT-GUS* fusion gene and four independent lines were obtained. These lines [P_{DGT}*:DGT-GUS* (*dgt-180*)] were compared with *dgt-180* and WT, and the replacement of *dgt* with the native gene was found to partially restore the native phenotype when grown on auxin, but not when grown without exogenous auxin [two representative P_{DGT}*:DGT-GUS* (*dgt-180*) lines are presented in Fig. 1F]. These data indicate that the auxin-insensitive phenotype of *dgt-180* resulted from the mutation in *dgt*.

The severe phenotypes of the *dgt/nar* mutants might suggest a dominant effect of the mutant alleles. An alternative explanation is that the more severe phenotypes are related to additional N-methyl-N′-nitro-N-nitrosoguanidine (EMS)-induced mutations. This explanation is supported by the failure of the *DGT* gene to complement the phenotype of the *dgt-180* mutant when grown without exogenous auxin. To address this, we knocked out the mutant *dgt* gene in the *dgt-26* and *dgt-180* lines and compared the resulting transgenic lines with the corresponding mutant background lines. In addition, we ectopically overexpressed the *dgt-26* and *dgt-180* genes in WT plants and compared these lines with DGT ectopic overexpression lines. No differences were observed in either case between the test and control lines (data not shown). These results suggest that the severe phenotypes of the *dgt/nar* mutants do not result from a dominant effect.

To compare the phenotypes of the *dgtΔ* null mutant and a *dgt* point mutant in the same background, we replaced the *DGT* coding region with a *dgt-176-GUS* fusion. We found that the *dgt-176-GUS* and *dgtΔ* lines were very similar (two representative P_{DGT}*:dgt-176* lines are shown in Fig. 1G). This indicates that the loss of native DGT protein has the same effect as a mutation that produces a non-functional protein and further associates *DGT* with the phenotype observed in the *nar* mutants.

Because the *dgt/nar* lines appear to have defects unrelated to *DGT*, to further characterize the *dgt* phenotype we focused on the *dgtΔ* null lines. One of each of the *nptII* and *HygR* lines was grown on BCDAT or BCD media with different concentrations of NAA (supplementary material Fig. S4A,B). Both *dgtΔ* mutant lines showed reduced sensitivity to applied auxin. When grown on BCDAT medium, shorter protonemal filaments and fewer gametophores developed compared with WT. In the presence of auxin, WT plants developed fewer gametophores and longer filaments, whereas the *dgtΔ* mutants presented a delayed response (supplementary material Fig. S4A). On BCD medium, WT plants produced gametophores and also caulonemal filaments. The addition of auxin resulted in a decrease in the growth of caulonemata and reduced the number and size of gametophores and leaves (supplementary material Fig. S4B,D,E). Conversely, the growth of rhizoids was increased (supplementary material Fig. S4B). The *dgtΔ* mutants presented a delayed auxin response. In the absence of exogenous auxin, the mutants developed smaller gametophores than WT but more filaments. In the presence of exogenous auxin, longer filaments developed. In the mutant, the proportion of gametophores to rhizoids was not decreased (in contrast to observations in WT) and gametophores could still be detected in the presence of 12.5 μM NAA.

To exclude the possibility that replacement of the *DGT* coding region with a *GUS* insertion affects the *dgt* phenotype, *dgt* knockout lines that do not harbor *GUS* were also generated (these lines were created in a P_{GH3}*:GUS* background for an additional purpose described below). Four independent lines were obtained and the absence of *DGT* transcripts was confirmed (supplementary material Fig. S3B-E). Three independent lines were analyzed further and used to quantify the number of gametophores, the length of leaves, and the proportion of chloronema to caulonema (supplementary material Fig. S4D-G). The phenotype of these lines was indistinguishable from that of the *dgtΔ/GUS* lines.

It is possible that the *dgt* phenotype is related to a defect in auxin uptake or efflux. The *P. patens* genome encodes orthologs of both the AUX1 uptake carrier and the PIN efflux proteins (Rensing et al., 2008). Because NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) are substrates for the efflux and influx carriers, respectively, differential sensitivity to these auxins is indicative of a defect in auxin transport (Delbarre et al., 1996). We found that 2,4-D had a similar effect to NAA on the growth of *dgtΔ*, suggesting that the mutant is not deficient in an aspect of auxin transport (supplementary material Fig. S4C). However, a better understanding of auxin transport in *P. patens* will be needed to address this hypothesis.

Characterization of moss DGT

To gain a better understanding of the role of DGT in moss development we studied its expression pattern using P_{DGT}:GUS (the *dgtΔ/GUS* lines described above). In protonemata, GUS activity was detected in all filaments, with the strongest activity in the apical cells of rapidly growing caulonemata and in side branches differentiating into secondary chloronemata (Fig. 2A,B). In gametophores, GUS activity was detected in rhizoids and in axillary hairs emerging from the adaxial side of the leaves (Fig. 2C,D). To compare the transcription pattern with the expression of the DGT protein, we created transgenic lines harboring a *DGT-GUS* fusion. The DGT-GUS expression pattern was identical to the GUS expression in the *dgtΔ* lines (supplementary material Fig. S5) and was unchanged following auxin treatment (data not shown).

To analyze the subcellular localization of DGT, we generated lines in which a *DGT-YFP* fusion is expressed from the endogenous *DGT* locus (P_{DGT}*:DGT-YFP*). Similar to other CyPA-like proteins (Wang and Heitman, 2005), YFP fluorescence was observed in both the cytoplasm and nucleus (supplementary material Fig. S6). As cyclophilins can function as chaperones and therefore may facilitate the subcellular translocation of their substrate proteins, we examined whether the subcellular localization of DGT is affected by auxin. We treated the P_{DGT}*:DGT-YFP* fusion lines with auxin but did not observe any changes (data not shown).

DGT affects auxin-induced transcription

Tomato *dgt* mutants exhibit reduced auxin induction of several auxin-regulated genes (Mito and Bennett, 1995; Balbi and Lomax, 2003; Coenen et al., 2003; Vidoz et al., 2010). To explore the effect of *P. patens* DGT on auxin-dependent transcription, we utilized an auxin reporter. A transgenic moss plant expressing a *GUS* transcriptional fusion driven by a *Glycine max GH3* promoter (P_{GH3}*:GUS*) was previously used as an auxin reporter and was shown to respond to exogenous auxin within 24 hours (Bierfreund et al., 2003). We knocked out the *DGT* coding region within the P_{GH3}*:GUS* line, as described above, and examined the distribution of GUS protein in the resulting lines (P_{GH3}*:GUS dgtΔ*). Following a 5-hour treatment with IAA, GUS expression in all three representative P_{GH3}*:GUS dgtΔ* lines was lower in both protonemata (Fig. 3A) and gametophores (Fig. 3B) compared with the control line (Fig. 3, P_{GH3}*:GUS*). Moreover, knocking out *DGT* in the P_{GH3}*:GUS* line enabled us to compare the effect of DGT on the distribution of auxin response activity. No differences in the expression pattern were observed, again indicating that DGT does not affect auxin transport.

To further study the effect of DGT on auxin-dependent transcription we examined the expression of several auxin-regulated genes in *dgt* and WT. Seven-day-old plants composed mainly of protonema were treated with auxin for 5 hours and transcript levels of the target genes were determined by quantitative RT-PCR (qPCR). The selected genes are known to be regulated by auxin in *P. patens* [the three *PpAux/IAA* genes (Prigge et al., 2010) and *RHD6-like* (Jang and Dolan, 2011)] or are orthologs of known *Arabidopsis* auxin-regulated genes (*GCN5*, *SAUR*, *ARF19* and *AUX1*). With the exception of *IAA2* and *ARF19-likeA*, the expression levels of all the sampled genes

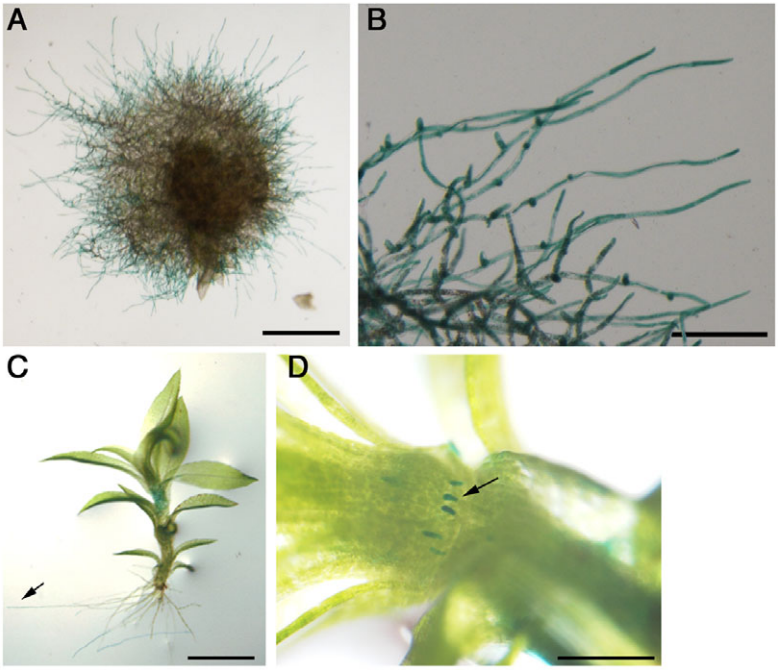

Fig. 2. *DGT* is expressed in protonemata and rhizoids. Gus expression in P_{DGT}*:GUS* transcriptional fusion lines. (**A**,**B**) Two-week-old protonemata grown on BCDAT. (**C**,**D**) Single gametophore taken from a plant grown on BCD for 1 month. The arrows in C and D denote a rhizoid and an axillary hair, respectively. Scale bars: 1 mm in A,C; 200 μm in B; 250 μm in D.

were generally reduced in *dgtΔ* compared with WT. Based on a Student's *t*-test, the basal levels of *IAA1A*, *IAA1B*, *RHD6*, *GCN5*, *SAUR* and *AUX1* and also the auxin-induced levels of *IAA1A*, *IAA1B*, *GCN5*, *SAUR* and *AUX1* were significantly different between *dgtΔ* and WT.

These results indicate a clear deficiency in auxin-regulated transcription in *dgtΔ*. However, a general effect of DGT on gene expression could not be excluded. To test whether DGT has a specific effect on auxin regulation, we tested the response of *dgtΔ* to the plant hormone abscisic acid (ABA) and to a heat shock treatment. We selected several genes that display either an ABA (Cuming et al., 2007; Khandelwal et al., 2010) or a heat shock response and tested their expression following 5 hours of treatment (Fig. 4B,C). No differences were observed between *dgtΔ* and WT with respect to either basal or induced transcript levels, suggesting a specific role for DGT in auxin-regulated transcription.

The role of DGT in Aux/IAA degradation

Reasoning that cyclophilins may function in protein folding, we examined whether the effect of DGT on transcription is due to a role in the interaction between the AFB and Aux/IAA proteins and the subsequent degradation of Aux/IAAs. We created transgenic lines expressing *PpIAA1B-GUS* fusions driven by a soybean heat shock promoter (P_{HSP}*:IAA1B-GUS*). We obtained two independent transgenic lines and first tested their ability to degrade the heat shock-induced protein following auxin treatment. We show that auxin promotes the degradation of IAA1B-GUS (Fig. 5A,D). To test the effect of DGT on IAA1B-GUS, we introduced P_{HSP}*:IAA1B-GUS* into *dgt-26* and *dgt-180* and isolated one and two independent lines, respectively (one of each is shown in Fig. 5B,C). In addition, *dgt* was knocked out in one of the P_{HSP}*:IAA1B-GUS* transgenic lines (Fig. 5D), resulting in three independent lines (two representative lines are shown in Fig. 5E,F; the null mutation is confirmed in supplementary

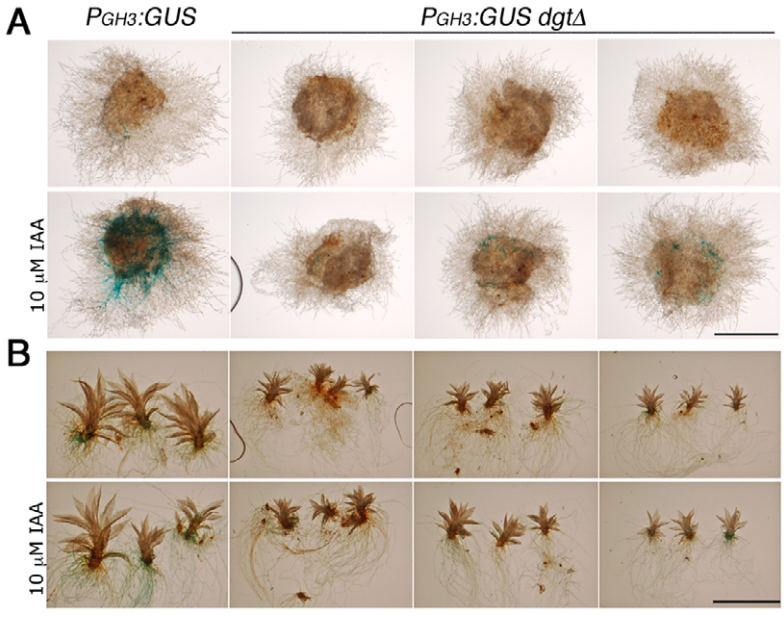

Fig. 3. *dgtΔ* alters the expression of the auxin-responsive *GH3:GUS* marker. GUS expression in the P_{GH3}*:GUS* reporter line and in three independent *dgtΔ* lines in the background of the P_{GH3}*:GUS* line (P_{GH3}*:GUS dgtΔ*). (**A**) Plants were grown for 2 weeks on BCDAT and then either mock-treated or with 10 μM IAA for 5 hours. (**B**) Three individual gametophores of the P_{GH3}*:GUS* reporter line or the three independent lines of P_{GH3}*:GUS dgtΔ*. Plants were grown on BCD for 1 month and then individual gametophores were either mock-treated or with 10 μM IAA for 5 hours. Scale bars: 1 mm in A; 3 mm in B.

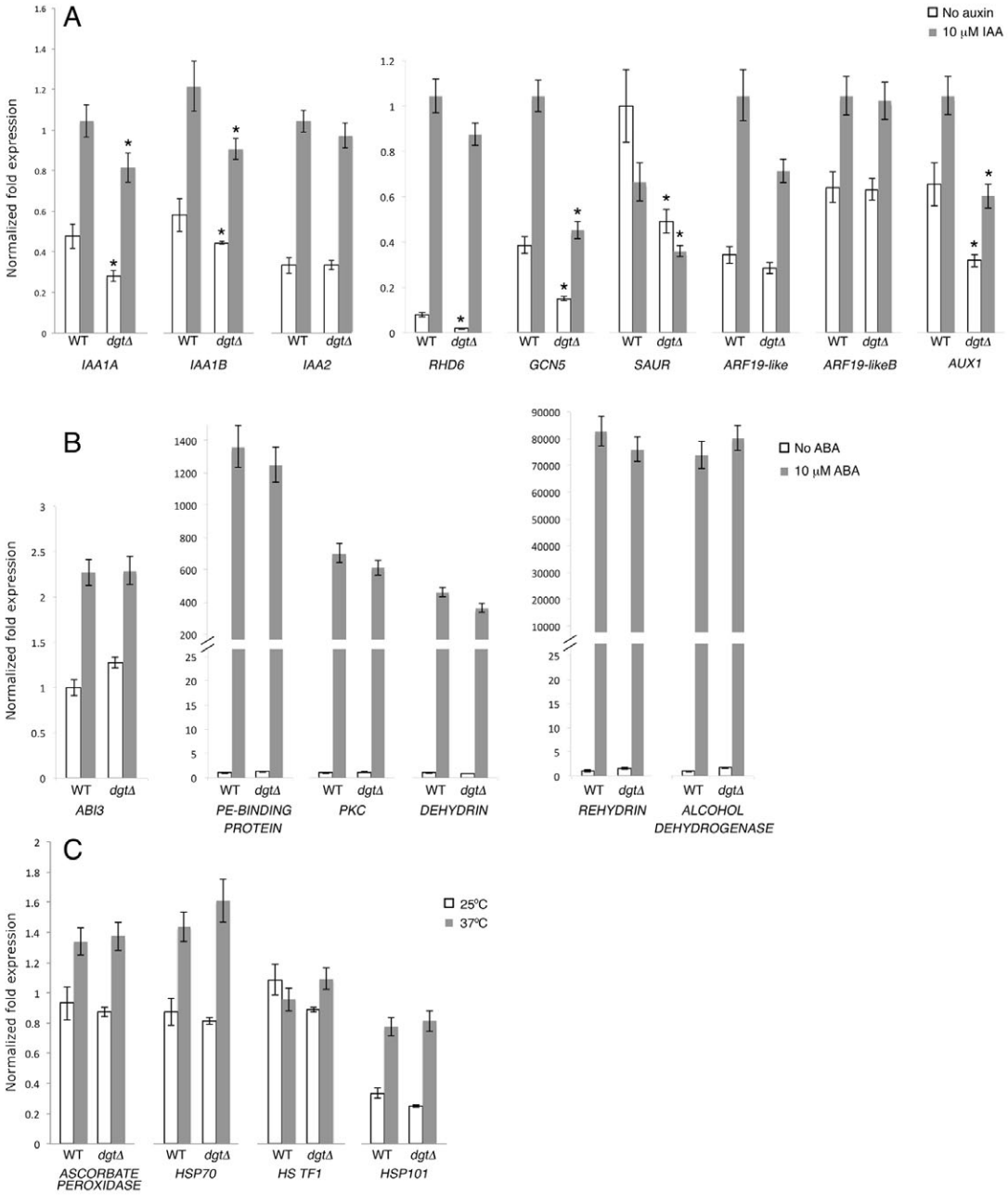

Fig. 4. *DGT* affects auxin-induced transcription. (**A**) qPCR showing the expression levels of auxin-induced genes in protonemal tissue of WT and *dgtΔ* plants that were either mock-treated or with 10 μM IAA for 5 hours. (**B**) qPCR showing expression levels of ABA-induced genes in protonemal tissue of WT and *dgtΔ* plants that were either mock-treated or with 10 μM ABA for 5 hours. (**C**) qPCR showing expression levels of heat shock-induced genes in protonemal tissue of WT and *dgtΔ* incubated for 5 hours at 25°C or 37°C. Each bar in A-C represents three biological replicates with four technical replicates. The expression of target genes was normalized to *PpACTIN*. Error bars represent s.e.m. **P*<0.05 (*t*-test), *n*=3.

material Fig. S3F-H). In all these lines IAA promoted the degradation of IAA1B-GUS in a similar way, suggesting that DGT does not affect the ubiquitin-dependent degradation of Aux/IAA proteins. However, it is possible that a modest change in the IAA1B-GUS level might not be detectable in our assay.

Deletion of *DGT* suppresses the effect of AFB overexpression

To further explore the role of DGT in auxin signaling, we studied the genetic interaction between *DGT* and *PpAFB2*. We first generated transgenic lines overexpressing *PpAFB2* and characterized the resulting phenotype. We targeted an *AFB2-c-Myc* fusion gene driven by a maize ubiquitin promoter (P_{Ubi}*:AFB2-Myc*). Eight lines were selected and the levels of the tagged protein were determined. Two lines with different levels of AFB2-Myc were used for further analysis (Fig. 6A). When these lines were grown on BCDAT medium they developed more and longer caulonemal filaments, which in turn produced more secondary chloronemal filaments, than WT. Furthermore, their phenotype correlated with the levels of AFB2-Myc protein (Fig. 6C): the higher expressing line (P_{Ubi}*:AFB2-Myc #2*) developed more caulonemal filaments and more secondary chloronema than the lower expressing line (P_{Ubi}*:AFB2-Myc #1*).

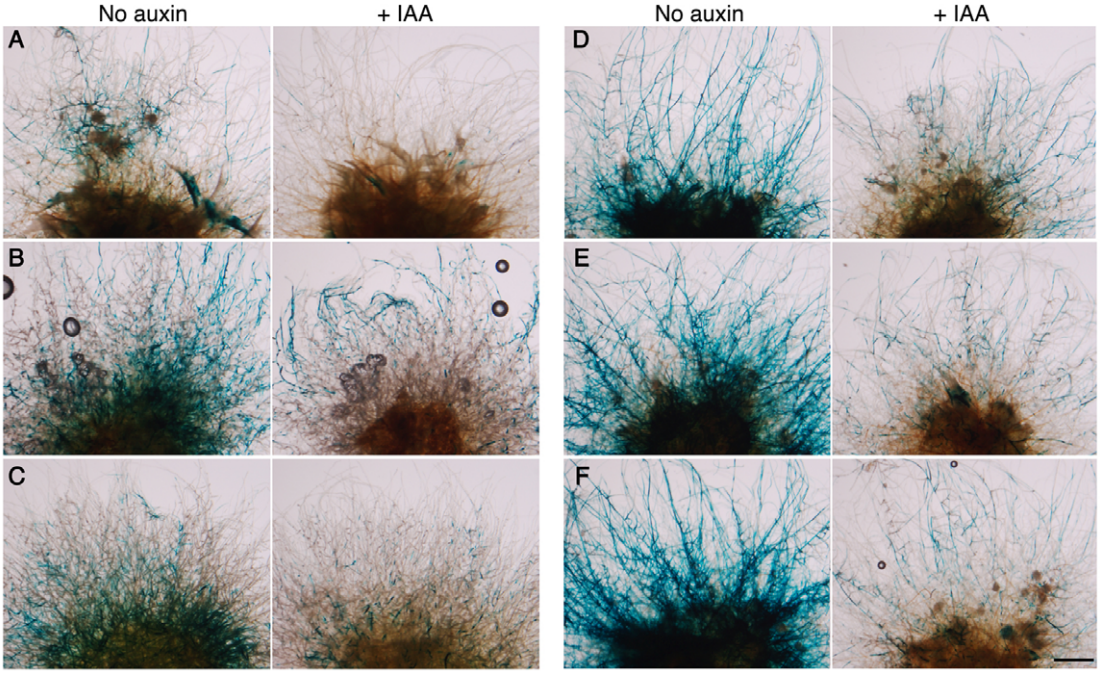

Fig. 5. *dgt* does not affect the degradation of IAA1B protein. GUS expression in P_{HS}*:IAA1B-GUS* lines grown on BCD for 2 weeks and either mock-treated (no auxin) or with 10 μM IAA (+IAA) for 5 hours. (**A**,**D**) Two independent transgenic lines transformed with P_{HSP}*:IAA1B-GUS*. (**B**) *dgt-26* mutant plant transformed with P_{HSP}*:IAA1B-GUS*. (**C**) *dgt-180* mutant transformed with P_{HSP}*:IAA1B-GUS*. (**E**,**F**) Two independent *dgt* knockout lines in the background of the P_{HSP}*:IAA1B-GUS* transgenic line shown in D. Scale bar: 500 μm.

The P_{Ubi}*:AFB2-Myc #2* line was characterized further. The auxin hypersensitive phenotype was also displayed during the development of the gametophore; when no exogenous auxin was added, the leafy gametophores appeared smaller than those of the WT and their leaves were longer and narrower, similar to WT plants grown on auxin-containing media (Fig. 6B,F). Whereas gametophores were still observed on WT plants grown on 0.5 μM NAA, the AFB2 overexpression line developed only rhizoid-like cells, consistent with a hypersensitivity to auxin.

To study the genetic interaction between *DGT* and the *AFB2* auxin receptor we knocked out *DGT* in the P_{Ubi}*:AFB2-Myc #2* line. Two lines were obtained (confirmation of the null mutation is shown in supplementary material Fig. S3I,J) and the AFB2 protein level was analyzed and found to be similar to that of the background line (Fig. 6D). The P_{Ubi}*:AFB2-Myc* overexpression line and the two P_{Ubi}*:AFB2-Myc dgtΔ* lines were grown together with WT on BCD media with or without varying concentrations of auxin (Fig. 6B). In general, deletion of *DGT* from the *AFB2* overexpression lines dramatically reduced auxin sensitivity. Whereas the number of gametophores and the size of the leaves were dramatically affected by auxin in both WT and the *AFB2* overexpression lines, only a mild effect was observed in the P_{Ubi}*:AFB2-Myc dgtΔ* lines. Similar to *dgtΔ*, even on 12.5 μM NAA the P_{Ubi}*:AFB2-Myc dgtΔ* lines produced some gametophores whereas the WT and *AFB2* overexpression background line grew only rhizoid-like cells (Fig. 6E,F). These results suggest that *dgt* can suppress *AFB2* and might be involved in transcriptional regulation.

DISCUSSION

DGT has a conserved function in auxin action

The significant effect of auxin on the development and growth of the moss *P. patens* has been known for many years. The sequencing of the *P. patens* genome revealed that the core components of the auxin signaling machinery and other auxin-related genes are conserved in this early land plant. Recently, a few studies of *P. patens* genes have demonstrated a link between conserved auxin-related genes and auxin-dependent phenotypes. In a recent study (Prigge et al., 2010), we sequenced a set of severe auxin-insensitive mutants and found them to be affected in the three PpAux/IAA proteins. We also found that knockdown of the four *TIR1/AFB* auxin receptor genes resulted in defects in the chloronema-to-caulonema transition. The Arabidopsis *SHORT INTERNODS/STYLISH* (*SHI/STY*) family genes serve as positive regulators of auxin biosynthesis genes including *YUCCA4*. Knockout and overexpression of the *P. patens* homologs of *SHI/STY* resulted in lower and higher levels of IAA, respectively (Eklund et al., 2010). Menand and colleagues (Menand et al., 2007) present a double-knockout mutant of *PpRSL1* and *PpRSL2* (homologs of *Arabidopsis ROOT HAIR DEFECTIVE 6*, which encodes a basic helix-loop-helix transcription factor), which fails to develop caulonemal filaments and rhizoids. A recent study reports that the *RSL* genes are positively regulated by auxin (Jang and Dolan, 2011). In the current study, we show another intriguing example of a conserved role of auxin action that is shared between early land plants and angiosperms. We have sequenced a number of moss mutants displaying an auxin-insensitive phenotype and found five independent alleles of a moss ortholog of the tomato *DGT* gene.

The tomato *dgt* mutated lesion was shown to affect several complex developmental processes in tomato, including root growth, fruit development, ethylene production and the development of adventitious roots (Zobel, 1973; Kelly and Bradford, 1986; Balbi and Lomax, 2003; Ivanchenko et al., 2006; Vidoz et al., 2010). By contrast, the developmental stages and body plan of moss are simple. This suggests that DGT has a basic and central role in the auxin response that is expressed as different, lineage-specific outputs.

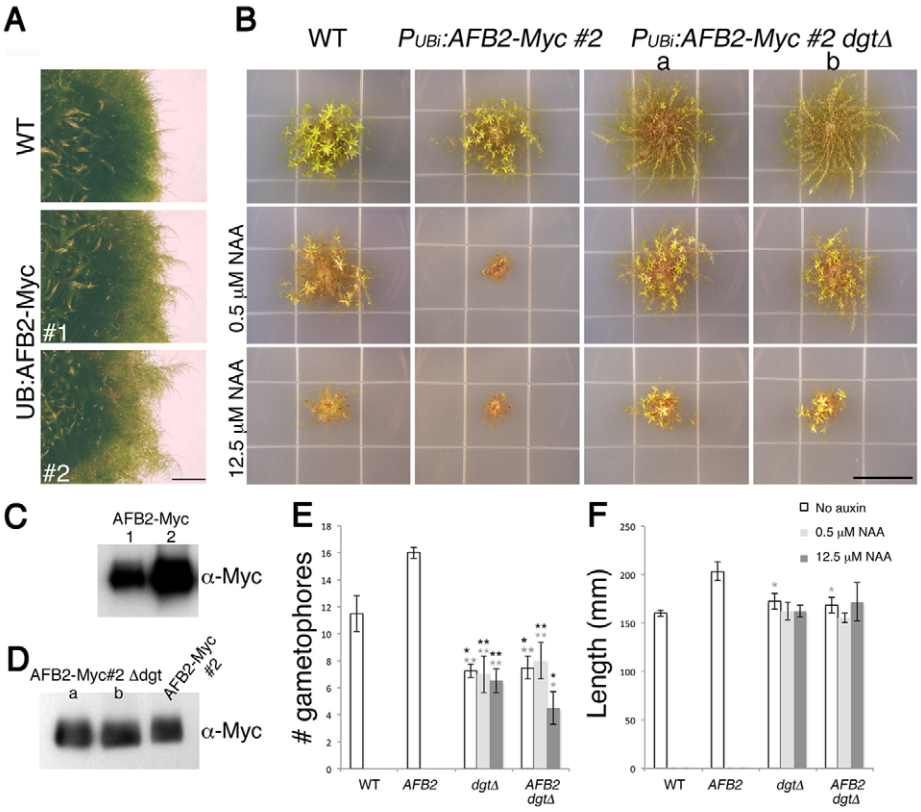

Fig. 6. *DGT* suppresses the phenotype of the *AFB2* auxin receptor. (**A**) WT and two independent P_{Ubi}*:AFB2-Myc* transgenic lines (#1, #2) grown on BCD plates with cellophane overlays for 1 month. (**B**) One-month-old WT, *AFB2-Myc* overexpression line #2 (P_{Ubi}*:AFB2-Myc #2*) and two independent *dgt* knockout lines in the background of the *AFB2-Myc* overexpression line #2 (P_{Ubi}*:AFB2-Myc #2 dgtΔ* a and b), grown on BCD without auxin or with 0.5 μM or 12.5 μM NAA. (**C**,**D**) Immunoblot of total protein extract from plants overexpressing AFB2, detected with a c-Myc antibody. The two *AFB2* overexpression lines #1 and #2 (*AFB-Myc* 1 and 2) are shown in C. The two *dgt* knockout lines in the *AFB2* overexpression #2 line (*AFB2-Myc #2 dgtΔ* a and b) together with the *AFB2* overexpression background line (*AFB2-Myc #2*) are shown in D. (**E**,**F**) The number of gametophores (E) and the average length (mm) of 20 leaves (F) of *AFB2* overexpression #2 line, *dgtΔ* and *AFB2-Myc #2 dgtΔ* lines (*AFB2*, *dgtΔ* and *AFB2 dgtΔ*) grown in four spots each on BCD without auxin or with 0.5 μM or 12.5 μM NAA. Error bars represent s.e.m. **P*<0.05, ***P*<0.001 (*t*-test); black asterisks denote significantly different from WT, gray asterisks denote significantly different from *AFB2-Myc #2*. Some inconsistencies between B, E and F relate to different growth methods. Scale bars: 1 mm in A; 100 mm in B.

Indeed, the *P. patens dgt* mutants display defects in every auxin-dependent process, including the chloronema-to-caulonema transition and the development of rhizoids.

Prior to this study, *dgt* mutants were known only in tomato. The moss *dgt* mutants highlight the central role of this gene in the auxin signaling cascade. The *Arabidopsis* genome encodes four close homologs of DGT, and analysis of single-mutant lines has not revealed noticeable phenotypes. Therefore, it is possible that the clear phenotype of tomato and *P. patens dgt* mutants is due to decreased numbers of *DGT* genes in these two plant species. Higher-order mutant analysis in *Arabidopsis*, a plant for which the auxin signaling cascade is better characterized, might contribute to our understanding of the conserved role of DGT in plants.

How does DGT regulate the auxin response?

The role of DGT in auxin signaling is unknown. Phenotypic studies in tomato together with direct measurements of auxin concentration and transport suggested a role for DGT in auxin signaling rather than in auxin biosynthesis or transport (Fujino et al., 1988; Daniel et al., 1989; Muday et al., 1995). The tomato *dgt* mutants were established as a useful tool to study auxin signaling in tomato and in angiosperms in general. Several studies took advantage of the pleiotropic phenotype of the *dgt* mutants to characterize the auxin signaling pathway as well as the crosstalk between auxin and other hormones. These studies advanced our understanding of the effect of auxin on plant development and growth. However, they did not reveal a specific biological role for DGT. The position of DGT within an auxin signaling cascade also remains unclear. One mechanistic aspect that has been investigated is the effect of DGT on auxin-regulated transcription (Nebenfuhr et al., 2000; Balbi and Lomax, 2003). These studies demonstrate that DGT affects only a subset of auxin-regulated genes, suggesting that it is a component of a specific auxin signal transduction pathway. In this study, we provide evidence that *P. patens dgt* has a broad effect on the induction of auxin-regulated genes. Further studies focusing on the role of DGT in gene expression will elucidate the direct targets of DGT.

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Competing interests statement
The authors declare no competing financial interests.

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