Constitutive auxin response in *Physcomitrella* reveals complex interactions between Aux/IAA and ARF proteins

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**Abstract** The coordinated action of the auxin-sensitive Aux/IAA transcriptional repressors and ARF transcription factors produces complex gene-regulatory networks in plants. Despite their importance, our knowledge of these two protein families is largely based on analysis of stabilized forms of the Aux/IAAs, and studies of a subgroup of ARFs that function as transcriptional activators. To understand how auxin regulates gene expression we generated a *Physcomitrella patens* line that completely lacks Aux/IAAs. Loss of the repressors causes massive changes in transcription with misregulation of over a third of the annotated genes. Further, we find that the aux/iaa mutant is blind to auxin indicating that auxin regulation of transcription occurs exclusively through Aux/IAA function. We used the aux/iaa mutant as a simplified platform for studies of ARF function and demonstrate that repressing ARFs regulate auxin-induced genes and fine-tune their expression. Further the repressing ARFs coordinate gene induction jointly with activating ARFs and the Aux/IAAs.

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**Introduction**

The plant hormone auxin plays a central role in plant growth and development. Depending on the context, the cellular response to auxin can be very different, including changes in cell division, cell expansion, and differentiation. The hormone acts by regulating the transcription of auxin responsive genes. Strikingly, auxin-regulated gene sets can vary significantly between different cell types consistent with cell specific cellular responses (Bargmann et al., 2013). In the absence of auxin, transcription of auxin-regulated genes is repressed by members of a family of repressors called Auxin/INDOLE-3-ACETIC-ACID (Aux/IAA) proteins. The Aux/IAAs are recruited to promoters through an interaction with AUXIN RESPONSE FACTOR (ARF) transcription factors. Repression is relieved when auxin binds to a co-receptor complex consisting of a TRANSPORT INHIBITOR RESISTANT 1/AUXIN F-BOX (TIR1/AFB) F-box protein and an Aux/IAA protein. The Aux/IAA protein is degraded, permitting ARF-dependent transcription to occur (Calderon Villalobos et al., 2012; Dharmasiri et al., 2005a; 2005b; Kepinski and Leyser, 2005; Tan et al., 2007) reviewed in (Salehin et al., 2015; Wang and Estelle, 2014). The elucidation of the auxin co-receptor mechanism provided the molecular link between auxin perception at the cellular level and subsequent changes in gene expression. However, the mechanisms by which the interactions between TIR1/AFBs, ARFs and Aux/IAAs results in the induction of specific gene sets, as well as the establishment of gene regulatory networks, are not known.
The Aux/IAAs contain, in most cases, three domains: an N terminal repression domain required for the recruitment of the co-repressor TOPLESS (TPL) (Szemenyei et al., 2008) domain II, required for interaction with the TIR1/AFB co-receptors; and a C terminal region (designated domains III and IV) that forms a Phox and Bem1 (PB1) domain (Guilfoyle and Hagen, 2012).

ARF proteins are generally comprised of an N terminal B3-type DNA binding domain, a variable middle region (MR), and a C terminal PB1 domain. The ARFs have been characterized as activating or repressing based on their behavior in transient protoplast assays (Guilfoyle and Hagen, 2007; Tiwari et al., 2003; Ulmasov et al., 1999). It was recently shown that Arabidopsis ARF5, an activating ARF, interacts with SWI/SNF chromatin remodeling ATPases through its MR, and that this interaction mediates changes in chromatin required for gene activation (Wu et al., 2015). On the other hand the repressing activity of the putative repressing ARFs has not been demonstrated in plants. Similar, the mechanism of repression is unknown.

ARF-Aux/IAA interactions occur through their PB1 domains. In addition, the PB1 domain permits homo- and heterodimerization both within and between the Aux/IAA and ARF families. Recent structural studies revealed that ARFs can form higher order ARF and Aux/IAA protein complexes through both their DNA binding domain [ARF-ARF dimerization (Boer et al., 2014)] and the acidic and basic faces of their PB1 domains [ARF-ARF and ARF-Aux/IAA multimerization (Dinesh et al., 2015; Han et al., 2014; Korasick et al., 2014; Nanao et al., 2014) reviewed in (Korasick et al., 2015; Wright and Nemhauser, 2015)]. This combinatorial diversity may result in complex regulation of gene expression. Further, tightly regulated negative feedback loops in which the Aux/IAA genes are regulated by the ARFs, present an additional layer of complexity.

The body plan of the early-diverged moss Physcomitrella patens (P. patens) is relatively simple. The vegetative gametophyte is composed of only a few cell types and developmental stages. Auxin was shown to have a central role in the vegetative growth of P. patens (Prigge and Bezanilla, 2010). In the filamentous protonemal stage, auxin promotes the differentiation of chloroplast-rich filaments called chloronemata into elongated filaments with fewer chloroplasts called caulonemata (Ashton et al., 1979). The development of leafy gametophores is also affected by auxin including stem elongation (Eklund et al., 2010; Fujita et al., 2008), elongation of the gametophore leaves (Bennett et al., 2014; Decker et al., 2006), formation of rhizoids from gametophore epidermal cells (Ashton et al., 1979), and gametophore branching (Coudert et al., 2015). We previously
demonstrated that the molecular mechanism of auxin signaling is conserved between *P. patens* and Arabidopsis (Lavy et al., 2012; Prigge et al., 2010). This finding, together with its morphological simplicity establishes *P. patens* as a powerful model for studies of auxin signaling.

Here we utilized a *P. patens* aux/iaa null mutant to determine the effects of the complete loss of Aux/IAA-based transcriptional repression. Our analysis reveals that the Aux/IAAs are essential for auxin regulation of transcription indicating that at least in moss, auxin does not affect transcription independently of the Aux/IAAs. Complete loss of Aux/IAA repression dramatically alters the transcriptome, including expression of a large number of genes that are not affected by auxin treatment. In addition, our studies revealed new features of repressing ARF function. We demonstrate that auxin induction of gene expression is controlled by complex interactions between the Aux/IAAs and both activating and repressing ARFs.

**Results**

**Loss of the Aux/IAA repressors result in an auxin-constitutive phenotype**

Flowering plants possess large families of Aux/IAA genes (29 in Arabidopsis). Genetic studies of these genes have relied almost entirely on gain-of-function mutations in the degron motif (Prigge et al., 2010; Mockaitis and Estelle, 2008). These dominant mutations prevent interaction between the Aux/IAA and the TIR1/AFB co-receptors resulting in stabilization of the affected Aux/IAA and reduced sensitivity to auxin (Figure 1G). Very few loss-of-function mutants have been described in flowering plants, presumably because of gene redundancy and a plant completely lacking the entire Aux/IAA family is not available. In a recent study, the single Aux/IAA gene in the early diverging Liverwort *Marchantia polymorpha* (*M. polymorpha*) was knocked down using an artificial microRNA (Flores-Sandoval et al., 2015). The resulting lines were auxin hypersensitive. However, because these plants retained some auxin responsiveness, there are unlikely to be nulls. The genome of *P. patens* encodes only three Aux/IAA genes: IAA1A, IAA1B, and IAA2. To study the role of these genes in auxin response and plant development, and to simplify the study of auxin response mechanisms, we generated a mutant lacking all three genes. To generate the triple mutant, we replaced the IAA1B coding region with a β-glucuronidase (GUS) marker gene, followed by deletion of the IAA1A and IAA2 coding regions (P_{IAA1B}:GUS aux/iaaΔ) (Figure 1A and Figure 1—figure supplement 1). Because expression of the IAA1B gene is induced by auxin (Lavy et al., 2012; Prigge et al., 2010), we could use the P_{IAA1B}:GUS gene as an auxin reporter. Following auxin treatment, very low levels of GUS staining was detected in the P_{IAA1B}:GUS line, whereas GUS staining was high in the P_{IAA1B}:GUS aux/iaaΔ line both in the absence and presence of applied auxin, indicating that this line displays a constitutive auxin response (Figure 1B).

The resulting aux/iaa triple knockout mutant (aux/iaaΔ) displayed a phenotype that was similar to that of WT plants grown on high levels of auxin for one month, but more extreme (Figure 1C). When grown on minimal medium (BCD), WT plants grew leafy gametophores (Figure 1D-left panel), while in the presence of auxin, these plants produced gametophores consisting of shoot structures with ectopic brown-pigmented rhizoids and without leaves (Figure 1D-right panel). The aux/iaaΔ mutant produced gametophores with ectopic rhizoids and was completely insensitive to auxin (Figure 1C). To characterize plants at the filamentous protonemal stage, we compared WT, a highly auxin-resistant IAA2 degron mutant (iaa2-P328S), and the aux/iaaΔ mutant. The plants were grown for one month on medium supplemented with ammonium tartrate to allow for slower protonemata differentiation (BCDAT medium) (Figure 1E–H). Under these conditions, WT plants responded to exogenous auxin by developing long caulonemal filaments (Figure 1E,F). Whereas the differentiation of the ia2-P328S auxin-resistant mutant arrested at the primary chloronemal stage (Figure 1G), aux/iaaΔ plants contained disorganized brown-pigmented filaments (Figure 1H). Interestingly, the phenotype of aux/iaaΔ plants was highly variable. Each plant developed heterogeneous filaments with varying levels of chloroplasts and brown pigment (Figure 1H, left and right panel illustrates the range of phenotypes). Since the aux/iaaΔ mutant displayed a phenotype similar to that of auxin treated plants, we also tested its response to reduced endogenous auxin levels using the auxin biosynthesis inhibitor L-Kynurenine (L-Kyn) (He et al., 2011). Differentiation of chloronema to
Figure 1. The aux/iaaΔ mutant displays a severe phenotype. (A) Scheme representing the auxin-signaling pathway in WT plants (left panel) and in the aux/iaaΔ mutant (right panel). (B) GUS expression in P\textsubscript{IAA1B}:GUS and aux/iaaΔ plants carrying the P\textsubscript{IAA1B}:GUS reporter. Arrows denote GUS expression. (C) WT and the aux/iaaΔ mutant grown for one month on BCD medium, stimulating gametophore development without auxin or with different concentrations of 1-naphthalene-acetic acid (NAA). (D) Microscopic enlargement of WT gametophores: left panel-leafy gametophores grown without exogenous auxin. Right panel-ectopic rhizoids emerging from a gametophore grown on 12.5 μM NAA. (E–H) Plants grown for one month on BCDAT medium to promote filamentous growth. (E, F) WT plant grown without auxin or with 12.5 μM NAA, respectively. (G) iaa2-P328S degron-motif mutant. (H) The aux/iaaΔ mutant has a variable phenotype. (I) WT and aux/iaaΔ mutant grown for one month on BCDAT or BCDAT supplemented with 10 μM L-Kyn. Scale bars: 1 mm (B), 0.5 cm (C, H, I), the scale bar in H also corresponds to E–G, 0.5 mm (D).

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Figure 1 continued on next page
caulonemal filaments and gametophore development in WT plants were slower in the presence of L-Kyn, whereas the aux/iaa D mutant was insensitive to the inhibitor (Figure 1I).

Next, we characterized the aux/iaa D mutant at earlier stages of growth following either protoplast recovery or tissue homogenization. Following protoplast recovery, WT plants had a higher growth rate compared to the mutant (Figure 1—figure supplement 2A). After seven days both chloronemal and caulonemal cells were observed in WT plants, whereas clearly differentiated cell types, either chloronemata or caulonemata, were not observed in the aux/iaa D mutant (Figure 1—figure supplement 2B,C). A comparison between WT chloronemal cells and the aux/iaa D mutant cells, seven days following tissue homogenization, revealed that the mutant cells are significantly wider (Figure 2A,B) suggesting that polarized cell growth is impaired. The chlorophyll concentration at that stage was lower in the mutant compared to WT (Figure 2C). Despite these significant morphological and physiological differences, the mutant filamentous growth appeared robust (Figure 2—figure supplement 1).

The Aux/IAAs have a profound role in gene expression

The availability of the aux/iaa D mutant provided a unique opportunity to fully separate auxin perception from auxin response. Taking advantage of this opportunity, we profiled auxin-responsive transcription in WT and aux/iaa D protonemata by RNA sequencing (RNAseq) and compared the transcriptomes.

Our mutant analysis revealed that the differences between WT and the aux/iaa D mutant are more significant in later developmental stages. To minimize these differences, we performed RNAseq on protonemata grown on BCDAT for seven days after tissue homogenization (Figure 2A).

We first analyzed the WT auxin-responsive gene set following five hours of IAA treatment and identified 723 upregulated and 762 repressed genes [1.5 fold or more; p value adjusted (padj) <0.01] (Supplementary file 1A–C). Among the upregulated genes were several well-characterized early auxin response genes including the Aux/IAA genes as well as several genes homologous to SAURs and LBD/ASLs (Paponov et al., 2008). The P. patens genome encodes only two GH3 homologs with a conserved function in IAA-conjugate synthesis (Ludwig-Muller et al., 2009). Neither of these genes displayed differential regulation in the auxin-responsive gene set and one of them was downregulated in the aux/iaa D mutant. A few ARF genes in Arabidopsis have been shown to respond to auxin (Lau et al., 2011; Paponov et al., 2008). Five out of sixteen P. patens ARFs (including both activating and repressing) were upregulated by auxin in the WT protonemata. Nine additional ARFs were upregulated in the aux/iaa D mutant compared to WT, indicating the presence of an ARF-dependent feedback loop that may have evolved in the ancestral land plant lineage. The larger number of auxin regulated ARFs in our datasets may reflect the fact that by comparing the aux/iaa D mutant to WT, we uncover all Aux/IAA-repressed genes, whereas the Arabidopsis transcriptome data describes the effect of exogenous auxin on a complex tissue and under specific conditions.

Strikingly, our results show that the aux/iaa D mutant is completely insensitive to auxin, with no changes in gene expression upon auxin treatment under our experimental conditions and statistical threshold (Figure 2D). A less stringent statistical threshold of padj <0.05 revealed only a few genes displaying a low fold change further validated this finding (Supplementary file 1D). These results imply that any factors that regulate auxin-dependent changes in gene expression must act through the Aux/IAAs, emphasizing the central role of these proteins in auxin signaling.

The aux/iaa D transcriptomic analysis revealed that a third of all annotated genes were differentially regulated in the mutant compared to WT (with 3752 and 4031 up- and downregulated genes, respectively), demonstrating the broad role of the TIR1-Aux/IAA pathway in land plant growth and
Figure 2. Loss of the Aux/IAA genes results in dramatic changes in gene expression at the filamentous developmental stage. (A) Confocal images of WT and aux/iaaΔ mutant protonemata seven days after tissue homogenization (Left panels—chloroplasts are visualized in green. Right panels—cell. Figure 2 continued on next page
structures are visualized with DIC). Scale bar: 200 μm. (B) Average length and width (μm) of WT protonemal cells and aux/iaaΔ mutant cells proximal to branch points. Error bars represent s.e.m. **p<0.001 (t-test), n=30. (C) Total chlorophyll concentration in WT and aux/iaaΔ protonemata seven days after tissue homogenization. Error bars represent s.e.m. *p<0.05 (t-test), n=3. (B) Venn diagram showing the overlap between the four data sets of differentially expressed genes (padj <0.01, fold change ≥1.5). (D) Hierarchical clustering of genes displaying differential expression between auxin treated and untreated WT plant samples with fold change ≥2 compared to their expression levels in the aux/iaaΔ mutant.
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The following figure supplements are available for figure 2:

Figure supplement 1. Protonemal tissue grown under the growth conditions used for the RNAseq experiment.
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Figure supplement 2. qPCR showing the expression levels of auxin responsive genes in WT plant and aux/iaaΔ mutant in mock- or 10 μM IAA-treatment for one hour.
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Figure supplement 3. Graphical representation of enriched cellular components associated with auxin-responsive genes.
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development. Strikingly, approximately 80% of these genes were not differentially regulated by auxin in WT plants whereas most of the regulated genes in WT were presented in the aux/iaaΔ mutant dataset (Figure 2D). Thus, our analysis allowed us to identify genes that were not revealed by auxin treatment. These may include genes that are regulated in a specific developmental, temporal, or environmental context as well as genes that are indirectly affected by the loss of the Aux/IAAs. Additionally, some genes may be revealed only when the Aux/IAAs are completely absent. For example, it is possible that very low levels of Aux/IAAs are sufficient to repress expression of some genes while for others the Aux/IAAs may be protected from auxin dependent-degradation. Finally, some genes may be represented by a small number of transcripts and/or exhibit a low level of differential expression, making them difficult to identify with standard procedures.

As illustrated by a hierarchical clustering of differentially expressed genes, the absence of the Aux/IAAs also had a strong effect on transcript levels (Figure 2E). Numerous up- and downregulated genes identified in WT plants displayed higher or lower expression levels in the aux/iaaΔ mutant respectively, indicating that many genes can be expressed over a broad range. For example, while the highest fold-change of differentially expressed genes following auxin treatment was fifteen, some of the same genes were differentially expressed in the aux/iaaΔ mutant with a fold-change of up to 430 compared to WT. These results indicate that significant levels of the Aux/IAA are present even after auxin treatment, further illustrating the robust nature of the auxin system. Several up- and downregulated genes that were previously described (Lavy et al., 2012), and genes displaying differential expression in this analysis, were selected as representative auxin-responsive gene markers, and used to validate the transcriptomic analysis (Figure 2—figure supplement 2).

The moss auxin-responsive transcriptome may facilitate discovery of ancestral auxin gene targets. We analyzed Gene Ontology (GO) terms associated with auxin responsive genes and found specific enriched categories for up- and downregulated genes. Analysis of upregulated genes revealed over-representation of genes involved in regulation of transcription and biosynthesis, whereas downregulated genes were highly enriched for processes occurring in the chloroplast and associated with photosynthesis, including light responses and carbon fixation (Supplementary file 2A,B and Figure 2—figure supplement 3). The set of downregulated genes differentially expressed in the mutant but not in WT presented a higher level of enrichment of these processes. Thus, our GO analysis suggests that in P. patens photosynthetic tissues, auxin signaling mediates separation of auxin-induced responses from energy production via induction of specific gene sets and the concurrent repression of others.

**The repressing ARFs regulate auxin-induced genes**

The constitutive auxin response displayed by the aux/iaaΔ mutant and the broad effect of loss of Aux/IAA function on gene regulation highlight the central role of ARF transcription factors on gene expression. Based on sequence analyses and transient activity assays (Tiwari et al., 2003; Ulmasov et al., 1999), the ARF proteins were classified as either activators or repressors of transcription. While it is clear that the activating ARFs do activate transcription in plants and their mode
of action is beginning to emerge (Wu et al., 2015), this is not the case for the repressing ARFs. It has not been clearly demonstrated that the repressing ARFs act as transcriptional repressors in plants and a conceptual understanding of their role is lacking. For example, it is not known if repressing ARFs require the Aux/IAAs for their function or if the repressing and activating ARFs regulate the same genes. In addition it is not known if the repressing ARFs have a specific role in targeting downregulated genes.

The aux/iaaΔ mutant allows us to study the function of the repressing ARF in a relatively simple context lacking Aux/IAA repression and the negative feedback loops associated with their activity. Phylogenetic analyses reported that the P. patens ARF protein family consists of three clades (Plavskin and Timmermans, 2012) shared between all land plants and a fourth, non-seed-plant-specific clade characterized by the lack of the DNA binding domain ([Paponov et al., 2009], Figure 3—figure supplement 1). One of the clades, comprising four proteins, groups with Arabidopsis repressing ARFs, whereas another clade, comprising seven ARF proteins, groups with Arabidopsis activating ARFs. The M. polymorpha ARF proteins were recently shown to affect transcription in a transient expression assay in accordance with their phylogenetic classification (Kato et al., 2015) strongly suggesting that ARF function is conserved in land plants. Furthermore, in P. patens, higher levels of putative repressing ARFs resulted in decreased auxin response (Plavskin et al., 2016). We selected ARFb4 as a representative repressing ARF, and overexpressed an ARFb4 c-Myc fusion in the aux/iaaΔ mutant background (ARFb4OEaux/iaaΔ). We found that overexpression of ARFb4 suppressed the constitutive auxin phenotype of the aux/iaaΔ mutant including the formation of green chloronemal-like filaments (Figure 3A). In fact, in a high expressing transgenic line (Figure 3A #1 and Figure 3B—figure supplement 2C), the phenotype was quite similar to that of dominant auxin-resistant aux/iaa mutants [Figure 1G and (Prigge et al., 2010)]. This result demonstrates that repressing ARFs do function as repressors in plants and that this activity does not require the Aux/IAAs. It is worth noting that ARFb4, as well ARFb2 and ARFb3 do not contain any of the known motifs that are thought to recruit TPL and therefore may not affect chromatin remodeling directly. To define the mechanism underlying the suppression of the aux/iaaΔ phenotype we assessed the expression of the Piaa1B::GUS marker and other auxin-responsive genes. The GUS marker (Figure 3B), as well as the additional reporter genes (Figure 3C) had dramatically lower expression levels compared to the aux/iaaΔ mutant indicating that activating ARFs and the repressing ARFs can affect, either directly or indirectly the same target genes. Further, the auxin response genes did not respond to auxin in the ARFb4OEaux/iaaΔ line confirming that overexpression of the repressing ARF converted the constitutive auxin response line to an auxin resistant line. Overexpression of Arabidopsis ARF1 resulted in similar effects, indicating that the role of repressing ARFs is conserved between P. patens and Arabidopsis (Figure 3C and Figure 3—figure supplement 2).

Although ARFb4 overexpression resulted in a clear suppression of the aux/iaaΔ constitutive response, the phenotype was not as dramatic as the aux/iaa degron mutants. Expression of the auxin reporter genes was lower in the iaα2-P328S mutant compared to the ARFb4OEaux/iaaΔ (Figure 3C #5). This result suggests that the repression conferred by the Aux/IAAs is stronger than ARF-based repression.

Our results clearly indicate that auxin cannot stimulate transcriptional response in the ARFb4OEaux/iaaΔ following several hours of treatment. However, it is possible that auxin can promote protonemal development through other mechanisms or by longer treatment. To explore this possibility we grew the ARFb4OEaux/iaaΔ #1 line on NAA. Filament differentiation was not observed after one month of auxin treatment (Figure 3D) indicating that the Aux/IAA function is required for developmental transition in protonema.

We next examined how repressing and activating ARFs interact to regulate the same gene targets. Several attempts to overexpress the activating ARFs P. patens ARFa8 and Arabidopsis ARF7 in the aux/iaaΔ mutant were unsuccessful. We only recovered one ARFa8 transgenic line with weak expression of ARFa8 suggesting that further gene activation, beyond that observed in the aux/iaaΔ mutant, is lethal. As an alternative approach we selected one ARFb4OEaux/iaaΔ line (Figure 3A #1) and introduced an inducible ARFa8-glucocorticoid receptor (GR) fusion gene into this line (ARFa8-GR_ARFb4OEaux/iaaΔ). Two resulting lines were selected for further analysis (ARFa8-GR_ARFb4OEaux/iaaΔ #1 & #2) (Figure 4A–C). Following dexamethasone (DEX) treatment these lines developed gametophores with ectopic rhizoids indicative of auxin hypersensitivity (Figure 4A,B). Expression of the auxin reporter genes supported the phenotypic analysis since expression levels of
Figure 3. Repressing ARFs target auxin-induced genes. (A) WT, aux/iaaΔ and two aux/iaaΔ lines overexpressing ARFb4 (ARFb4OE_aux/iaaΔ) grown for one month on BCDAT. (B) GUS expression in aux/iaaΔ and two ARFb4OE_aux/iaaΔ lines carrying the PIAA1B:GUS reporter. Arrows denote GUS expression. (C) qPCR showing the expression levels of auxin responsive genes in WT, ARFb4OE_aux/iaaΔ (line #1), AtARF1_aux/iaaΔ, aux/iaaΔ, and iaa2-P328S in presence of 10 μM IAA-treated for five hours or mock. Error bars represent s.e.m. a/b/c=P<0.05 (t-test), n=3. a=t-test comparing the lines to WT, b=t-test comparing the lines to aux/iaaΔ. c=t-test comparing the lines to aux/iaaΔ. Figure 3 continued on next page
the upregulated genes in the DEX treated plants were higher compared to both the ARFb4OE_aux/iaaΔ background and untreated ARFa8-GR_ARFb4OE_aux/iaaΔ transgenic lines (Figure 4D). Similarly, the level of a representative downregulated gene was lower (Figure 4D-CBS). This demonstrates that ARFb4 and ARFa8, as representatives of repressing and activating factors respectively, display opposing activity on the same gene targets.

Activating and repressing ARFs may target the same genes by competing for the same promoter elements, by binding to different regions within the same promoters, or through an indirect mechanism. To distinguish between these possibilities we performed an electrophoretic mobility shift assay with DNA sequences from the DR5 reporter and representative auxin responsive gene promoters, IAA1A, IAA1B, and ARFb4. The results indicated that ARFb4 and ARFa8 DNA binding domains can bind to the same DNA sequences (Figure 4E).

The ARFs and the Aux/IAAs display complex interactions

Our results demonstrate that both the repressing ARFs and Aux/IAAs are capable of gene repression, albeit to different extents. To learn more about these two types of repression, we determined the effects of loss of repressing ARFs either in the presence or absence of the Aux/IAAs. The coding regions of ARFb2 and ARFb4 were deleted in WT as well as in the iaa1b iaa2Δ double and aux/iaaΔ triple mutants (Figure 5—figure supplement 1). The loss of ARFb2 and ARFb4 in the aux/iaaΔ line (arf2 arfb4Δ aux/iaaΔ) did not result in any morphological changes or a clear trend with respect to changes in gene expression (Figure 5—figure supplement 2). Conversely and surprisingly, the arfb2 arfb4 knockout in both the WT and the iaa1b iaa2Δ double mutant (arf2 arfb4Δ and arfb2 arfb4Δ iaa1b iaa2Δ, respectively) resulted in lower expression levels of some of the upregulated auxin responsive genes, and higher levels of downregulated genes compared to their backgrounds (Figure 5A). Although the expression of these genes in both untreated and auxin-treated arfb2 arfb4Δ plants was reduced, the fold change was either similar or even higher compared to the WT and iaa1b iaa2Δ backgrounds (Figure 5A, RSL4: 7–3 fold higher and Dox: 2–1.4, respectively). Phenotypic analysis of the arfb2 arfb4Δ revealed developmental defects including shorter filaments and fewer leafy gametophores consistent with reduced auxin response (Figure 5—figure supplement 3). These findings further support our hypothesis that repressing ARFs can affect the same genes as activating ARFs. However, they revealed an unexpected trend in which the loss of repressing ARFs affects transcription in the same direction as their overexpression. To further confirm these contradictory results we also used transient RNAi (Bezanilla et al., 2005) targeting the four repressing ARFs (arf1-4). The RNAi construct was expressed in a transgenic plant expressing the synthetic auxin-responsive reporter DR5:DsRED. In agreement with the analysis of the arfb2 arfb4Δ stable lines the resulting transformants displayed a lower expression level of the DR5:DsRED reporter compared to the control plants when treated with auxin, indicative of reduced auxin sensitivity of the arfb1-4 RNAi knockdown lines (Figure 5B).

These contradictory results may be explained by the indirect effect of negative feedback loops, in which the extensive production of Aux/IAA proteins could result in an overall decrease in auxin response. However, this possibility does not appear to explain the phenotype of arfb2 arfb4Δ lines, as the transcript levels of IAA1A (Figure 5A-IAA1A) are low. Alternatively, it is possible that both activating and repressing ARFs as well as the Aux/IAAs jointly coordinate gene induction. This hypothesis is consistent with our analysis of the arfb2 arfb4Δ lines. In the presence of the Aux/IAAs, loss of ARFB2 and ARFb4 results in reduced auxin response. However, this effect is suppressed by
the deletion of the Aux/IAA genes in the arfb2 arfb4Δ aux/iaaΔ line (Figure 5—figure supplement 2 compared to Figure 5A). Since the activating and repressing ARFs can target the same genes, loss of the repressing ARFs may result in increased levels of activating ARFs on the auxin-responsive promoters. This may have the unexpected effect of recruiting more Aux/IAA repressors to these promoters. To test this idea we overexpressed the activating ARFa8, in WT plants (ARFa8OE).
Figure 5. Both repressing and activating ARFs affect auxin response in the same direction in the presence of the Aux/IAAs. (A) qPCR showing the expression levels of auxin responsive genes in WT, arfb2 arfb4Δ (arf2,4), iaa1b iaa2Δ (iaa1b,2), and arfb2 arfb4 iaa1b iaa2Δ (iaa1b,2 arfb2,4), mock- or 10 μM IAA. 

Figure 5 continued on next page.
Plant biology

Genes and chromosomes

Groups results in a wide range of gene expression levels that contribute to a dynamic and context specific auxin response. ARFs. The repressing ARFs act to buffer gene expression by attenuating the activity of the activating ARFs. The interplay between the three protein groups results in a wide range of gene expression levels that contribute to a dynamic and context specific auxin response. ARFs. The repressing ARFs act to buffer gene expression by attenuating the activity of the activating ARFs. The interplay between the three protein groups results in a wide range of gene expression levels that contribute to a dynamic and context specific auxin response.

resulting lines displayed a phenotype characteristic of auxin-resistant plants and exhibited reduced expression of auxin responsive genes (Figure 5C,D), which supports our hypothesis. Collectively, our results support a model in which repressing ARFs can affect the occupancy of activating ARFs and Aux/IAAs on auxin responsive promoters. These interactions may provide buffering capacity that allows fine-scale regulation of auxin responsive genes (Figure 5E).

Discussion

In this study we exploited the relative simplicity of P. patens to address the complexity of auxin-regulated transcription. The Aux/IAA proteins have a central role in auxin signaling, serving as both auxin co-receptors and transcriptional repressors. However, genetic analysis of these genes has been limited in flowering plants because of genetic redundancy. By deleting all three Aux/IAAs in moss we have determined the effects of the complete absence of Aux/IAA function on plant growth and auxin response. Remarkably, we found that the Aux/IAAs have a broad impact on the expression levels of a great number of genes, the majority of which do not respond to auxin treatment of moss protonemata. Thus our analysis demonstrates that existing transcriptomic studies conducted in flowering plants may significantly underestimate the effects of auxin on the genome and illustrates the central role of the TIR-Aux/IAA pathway on plant growth and development. While our work highlights the remarkably broad impact of the Aux/IAA proteins on gene expression, it also demonstrates an absolute requirement for the Aux/IAAs in auxin-responsive transcription. Some auxin-mediated signaling pathways have been proposed to affect transcription independently of the TIR1/AFB-Aux/IAA signaling cascade. These include a negative role of Mitogen-Activating Protein Kinase (MAPK) activity on auxin-regulated genes (Lee et al., 2009), and the effect of the F-box protein SKP2A on the stability of cell division transcription factors (Jurado et al., 2010). Our findings indicate that at least in moss, any possible effect of these pathways on auxin-regulated transcription requires Aux/IAAs’ function.

In P. patens, auxin-dependent modulation of gene expression triggers developmental transitions and differentiation. Our mutant analyses reveal that the transition from chloronema to caulonema is regulated by a dynamic balance of quantitative auxin responses. When auxin response is low, as in the auxin-resistant mutants, protonema growth is not accompanied by developmental transition to the caulonemal stage. In contrast, a constitutive auxin response, as exhibited by the aux/iaaΔ mutant, results in rapid and abnormal maturation.

It has been proposed that repressing and activating ARFs might compete for binding to the same promoters (Vernoux et al., 2011). Structural studies of Arabidopsis ARF1 and ARF5 revealed that both classes of ARF can potentially bind to similar cis elements and form high order oligomers of

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The following figure supplements are available for figure 5:

Figure supplement 1. qPCR showing the expression levels of auxin responsive genes in WT and aux/iaaΔ mutant background, (iaa1b iaa2Δ, respectively), n=3. (B) Three independent arfb1-4 RNAi and control lines mock- or 10 μM IAA-treated. Each line is presented by two images (DsRED fluorescence (red), chlorophyll auto-fluorescence (blue). Each image is from projection stacks of multiple confocal sections. Scale bar: 100 μm. (C) WT and an ARFa8 overexpression line (ARFa8OE) grown for one month on BCDAT without auxin or with 12.5 μM NAA. Scale bar: 0.5 cm. (D) qPCR showing the expression levels of auxin responsive genes in WT and ARFa8OE mock- or 10 μM IAA-treated for five hours. Error bars represent s.e.m. *p<0.05 (t-test comparing the WT or aux/iaaΔ, respectively), n=3. (E) Model for auxin regulation of transcription. The expression level of an auxin responsive gene is determined by the interplay between the Aux/IAAs, the repressing ARFs, and the activating ARFs. At low auxin levels the Aux/IAAs provide stringent repression. At high auxin levels, the Aux/IAAs are degraded resulting in increased transcription through the action of the activating ARFs. The repressing ARFs act to buffer gene expression by attenuating the activity of the activating ARFs. The interplay between the three protein groups results in a wide range of gene expression levels that contribute to a dynamic and context specific auxin response.
ARFs and Aux/IAAs (Boer et al., 2014; Korasick et al., 2014). Our work provides experimental evidence that auxin responsive genes are targeted by both repressing and activating ARFs, which in turn coordinate their levels of expression. The structural studies as well as our results can support two models of ARF activity. Repressing and activating ARFs can either compete on the same binding sites or cooperatively induce transcription by forming heterodimers. Despite its classification as an activating ARF, the Arabidopsis ARF5 was shown to repress some of its identified gene targets (Zhang et al., 2014; Zhao et al., 2010). Given that ARF transcription factors may display opposing roles in different contexts, ARF activity can result in a very complex gene expression pattern. These mechanisms, by which different ARFs coordinate transcriptional responses, can explain the broad effect of auxin on gene expression and the complexity of the resulting transcriptional networks as each gene can display a wide range of expression levels. Recent studies in liverwort support this hypothesis. The M. polymorpha encodes only one Aux/IAA and one member of each ancient ARF clade yet these transcription factors are sufficient to pattern a complete body plan (Flores-Sandoval et al., 2015; Kato et al., 2015).

Unlike the activating ARFs, our understanding of the repressing ARFs is limited. For some repressing ARFs, the presence of an EAR domain suggests that repression could involve recruitment of the TPL co-repressor. However, many repressing ARFs, including PpARFb2, PpARFb4, and AtARF1 used in this work do not have this motif. We show that repression provided by these repressing ARFs is weaker compared to the repressing effect of the Aux/IAAs. Based on this observation, we suggest that repressing ARFs may fine-tune gene expression. The Aux/IAAs repress gene expression through interactions with co-repressors such as TPL and the subsequent recruitment of chromatin remodeling factors (Kagale and Rozwadowski, 2011). This may provide stable and long term repression when auxin levels are low. In contrast, when auxin levels are high and/or when auxin response needs to be dynamic, the repressing ARFs may provide a less stable repression that fine-tunes auxin response in the absence of the Aux/IAAs. The fact that the repressing ARF genes are induced by auxin and therefore constitute a negative feedback module is consistent with this idea.

In flowering plants, auxin integrates light signals and growth responses (Halliday et al., 2009). While we have some knowledge of how light affects auxin synthesis and distribution, the effects on auxin transcriptional responses are less known. In moss, the interplay between light, auxin, and growth has been implicated in the transition from chloronema to caulonema. In addition to auxin, light intensity and the availability of nutrients affect the transition from one cell type to the other. High light and glucose triggers caulonema formation while low light inhibits caulonemal growth and stimulates chloronemal branching (Thelander et al., 2005). These observations suggest that favorable conditions promote the differentiation of elongated caulonemal cells, whereas low energy conditions promote the formation of chloroplast-rich chloronemal cells thus increasing photosynthetic capacity and energy production. We found that the auxin downregulated gene set is dominated by genes involved in photoperception and carbon fixation. This finding establishes the molecular basis for auxin signaling in response to light stimuli and suggests that auxin may function as a molecular switch between energy production and growth.

Our model cannot explain a direct effect of the ARF transcription factors on downregulated genes. Only a few repressed genes have been identified after a short auxin treatment (Chapman et al., 2012; Paponov et al., 2008). Based on the GO analysis presented here it seems likely that transcription factors that are directly induced by auxin repress many processes downstream of auxin, particularly those associated with photosynthesis. These auxin-induced regulators and their downstream targets are yet to be defined.

**Materials and methods**

**Moss strains and growth conditions**

WT ‘Gransden-2004’ and mutant *P. patens* strains were grown at 25°C under continuous light at an intensity of 40–70 μmol/m2/s on BCD or BCDAT (BCD supplemented with 5 mM Ammonium Tartrate) media. Growth and differentiation of protonema is slower on BCDAT compared to BCD. For each experiment, a medium was selected to allow for analysis of either protonema development or filamentous and gametophyte differentiation, respectively.
Molecular cloning of gene disruption and expression constructs

PCR-amplified DNA fragments were cloned into pENTR-D/TOPO (Life Technologies), and were subsequently cloned either as digested fragments or by LR-Clonase-mediated recombination (Life Technologies) into the final vectors as detailed in the following procedures (Primer pairs used for amplification are listed in Supplementary file 3. backbone vectors are listed in Supplementary file 4). Gene knockout constructs: Approximately one kilobase of genomic sequence upstream (5’ region) and downstream (3’ region) to the genes coding region were amplified and cloned into either pBNRF, pBNRF-GUS, or pBHRF2 as specified below:

| Genes | Primers | Cloning procedure | Backbone |
|-------|---------|-------------------|---------|
| IAA1A | 5’ region | PML61, 62 | BamHI ligation | pBHRF2 |
| 3’ region | PML59, 60 | SpeI ligation |
| IAA1B | 5’ region | PML52, 53 | BamHI ligation | pBNRF-GUS |
| 3’ region | PML59, 60 | SpeI ligation |
| IAA2 | 5’ region | PML63, 64 | BamHI ligation | pBNRF |
| 3’ region | PML65, 66 | SpeI ligation |
| ARFb4 | 5’ region | PML599, 600 | BamHI ligation | pBNRF-GUS/ pBHRF |
| 3’ region | PML601, 602 | SpeI ligation |
| ARFb2 | 5’ region | PML677, 678 | BamHI ligation | pBHRF2 |
| 3’ region | PML679, 680 | SalI ligation |

Gene replacement construct: To create a mutant degron motif of IAA2 (iaa2-P328S) the PpIAA2 genomic region was amplified from the ppiaa2-183 mutant (Prigge et al., 2010) using the PpIAA2 genomic region-F’ and R’ primer pair. The fragment was subcloned as an AvrII and BamHI fragment.

Overexpression and inducible expression constructs: Constructs for protein-expression were generated by LR-Clonase-mediated recombination between pENTR-D/TOPO plasmids and destination vectors. To create PpARFb4, PpARFa8, and AtARF1 c-myc fusions, the genes’ coding regions were amplified using the primer pairs PML455 & 457, PML461 & 463, and PML776 & 777 respectively and recombined into pMP1377. To create ARFa8 and glucocorticoid receptor fusion (ARFa8-GR), the rat glucocorticoid receptor DNA fragment was inserted into theAscI site of ARFa8-pENTR-D/TOPO plasmid to create an ARFa8-GR fusion. The resulting recombinant fusion was recombined into pTHUBiGate.

To create a construct carrying the auxin responsive marker (DR5:DsRED2) eight repeats containing the DR5rev element (ggGAGACAttt) were inserted ahead of a minimal CaMV 35S promoter (-50 to +26). This promoter fragment was fused to the DsRED2 coding region by PCR then inserted together as a BamHI fragment into pMP1432 (replacing the Hsp:Gateway cassette).

Moss transformation and screening of transgenic lines

Protoplast isolation and PEG-mediated transformation of P. patens was performed as described in (Nishiyama et al., 2000). Three to five days after regeneration, transformants were selected on BCDAT medium containing 20 mg/l of either G418 or hygromycin, or 150 mg/l Gentamycin. Plants transformed with iaa2-P328S fragment were identified based on their phenotype on media containing 20 μM NAA.

Transgenic knockout lines were screened by PCR for the presence of both left and right transgene-endogenous sequence junctions to verify the insertion of the transgene to the targeted locus and for the absence of the corresponding coding region. cDNA was detected by RT-PCR to confirm the absence of a transcribed product. Genomic DNA extracted from over- and inducible expression transgenic lines was detected by PCR to confirm the presence of the transgene, and recombinant protein expression was detected by immunoblot as described in Supplemental Experimental Procedures. iaa2-P328S lines were genotyped by derived Cleaved Amplified Polymorphic sequences (dCAPS) (Table S7).

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All knockout mutants expressed either nptII or Hygromycin selectable marker genes. To create higher order mutants, the selectable marker cassettes were first excised using the site-specific recombination Cre/lox system (Schaefer and Zryd, 2001) to allow recycling of the selectable marker genes. The resulting lines are listed in the following table.

### Strains used in this study

| Description                              | Name in the text                  | Source                        |
|------------------------------------------|-----------------------------------|-------------------------------|
| $P_{IAA1B}$::GUS/iaa1bΔ                 | $P_{IAA1B}$::GUS                  | This work                     |
| $P_{IAA1B}$::GUS/iaa1bΔ iaa2Δ           | iaa1b iaa2Δ                       | This work                     |
| $P_{IAA1B}$::GUS/iaa1bΔ iaa2Δ iaa1aΔ    | aux/iaaΔ                          | This work                     |
| $P_{ARFb4}$::GUS/arfβ4Δ                 |                                  | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ4Δ          | Arfb2 arfβ4Δ                      | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ2Δ          |                                  | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ2Δ iaa2Δ    | arfβ2 arfβ4Δ iaa1b iaa2Δ          | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ2Δ iaa1aΔ   | arfβ2Δ aux/iaaΔ                   | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ2Δ iaa2Δ    | iaa2-Δ                            | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ2Δ iaa1aΔ   |                                  | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ2Δ iaa2Δ iaa1aΔ |                                  | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ2Δ iaa1aΔ   |                                  | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ2Δ iaa1aΔ   |                                  | This work                     |
| $P_{ARFb4}$::GUS/arfβ2Δ arfβ2Δ iaa1aΔ   |                                  | This work                     |

### RNA-sequencing

Protonemal tissue comprised mainly of chloronemata from WT and aux/iaa knockout mutant plants was homogenized in a Waring blender and plated in triplicate on BCDAT plates with cellophane overlays for seven days. The cellophane overlays covered with protonemal tissue were transferred into liquid BCD medium containing either 10 μM IAA or the equivalent amount of ethanol solvent and incubated at 25°C under continuous light for 5 hr. Total RNA was isolated using the RNAeasy plant mini kit (Qiagen) and treated with DNA-free™ DNase removal kit (Life technologies). The three biological replicates were sequenced on Illumina HiSeq2500 by New York Genome Center, resulting in 30 million 50 bp paired-end reads per sample. Total reads were mapped to the P. patens genome (version 1.2.1) using RNAseq aligner STAR (Dobin et al., 2013). Genes were quantified with featureCounts (Liao et al., 2014), using v6 annotation the GTF file corresponding to the annotation v6 (obtained from http://phytozome.jgi.doe.gov/). Differential Expression was determined using the DESeq2 (Love et al., 2014). The sequences reads have been deposited to the sequence Read Archive (SRA) database (BioProject accession PRJNA317343).

### RNA-sequencing, additional analysis tools

GO enrichment analysis was carried out using AgriGO (Du et al., 2010) with GO annotation obtained from https://www.cosmoss.org. Hierarchical clustering was performed with DNASTAR.

### RNA interference

RNA interference was carried out as described in Bezanilla et al. (2005). Overlapping PCR fragments from PpARFB4 (PML506, 507) and PpARFB3 (PML508, 509) were fused by PCR. Overlapping PCR fragments from PpARFB1 (PML510, 511) and PpARFB2 (PML512, 513) were fused by PCR. The resulting two fragments were fused together by PCR and cloned into pENTR-D/TOPO. The hairpin
expression plasmids were generated by LR-Clonase-mediated recombination between the resulting pENTR plasmid and pUGGi. A pUGi vector lacking the gateway cassettes was used as control. The hairpin expression plasmids were transformed into DR5:DsRED strain which expresses a nuclear-localized GFP-GUS fusion protein. Following protoplast regeneration, the transformants, carried on cellophane overlays, were transferred to BCD media with Hygromycin and grown for eight days and then transferred into liquid BCD medium containing either 10 μM IAA or ethanol and incubated for 32 hr. Transformants lacking GFP fluorescence were selected and photographed. Fluorescence signals were detected for GFP (excitation 488 nm, emission 493–535 nm), DsRED (561 nm excitation, 566–623 nm emission), and chlorophyll auto-fluorescence (excitation 633 nm, emission 703–735 nm), using laser scanning confocal microscope (Zeiss MLS 710).

qRT-PCR
Protonemal tissue was grown in triplicate or quadruplicates on BCDAT plates with cellophane overlays for seven days. For IAA or DEX treatment, plant tissue was transferred into liquid BCD medium containing either 10 μM IAA or 10 μM DEX or the equivalent amount of ethanol. Following incubation, the tissue was collected and total RNA was isolated using RNeasy plant mini kit (Qiagen). 500 μg RNA was reverse transcribed using the Superscript III First Strand cDNA Synthesis System (Life Technologies). 20 μl RT reaction was diluted with water to a final volume of 200 μL. PCR samples contained 4 μl diluted cDNA were detected using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The following primer pairs were used to amplify the target genes: ARFa8 (PML399, 400), ARFa6 (PML409, 419), RSL4; Pp1s164_82V6.1 (PML614, 615), GCN5; Pp1s20_204V6.1 (PML618, 619), SAUR; Pp1s4_222V6.1 (PML624, 625), AUX1; Pp1s56_28V6.1 (PML626, 627), CBS domain; Pp1s11_325V6.1 (PML810, 811), Dox; Pp1s15_259V6.1 (PML812, 813), Epimerase; Pp1s189_34V6.3 (PML814, 815), bHLH TF; Pp1s231_17V6.1 (PML818, 819), Ubiquitin Ligase; Pp1s37_28V6 (PML822, 823), IAA1A (IAA1A-F’, IAA1A-R’), IAA1B (IAA1B-F’, IAA1B-R’), IAA2 (IAA2-F’, IAA2-R’), EF1α; Pp1s84_186V6.1 (EF1α-F’, EF1α-R’). The sequences are listed in Supplementary file 3. Normalized expression (ΔΔC(t) method) was calculated using the Bio-Rad CFX manager software using PpEF1α as a reference gene and plotted as relative values ± SEM. Each analysis included three biological and four technical replicates. qPT-PCR analysis comparing gene expression levels between arfb2 arfb4Δ aux/iaaΔ and aux/iaaΔ lines included four biological replicates.

Electrophoresis mobility shift assay (EMSA)
cDNA fragments encoding ARFb4 and ARFa8 DNA binding domains were amplified using primer pairs PML 455 & 851 and PML 461 & 852 respectively, and cloned into pDEST15 (Invitrogen). Recombinant GST fusions were expressed in Escherichia coli strain BL21-AI (Invitrogen) and purified by GST-agarose affinity. The eluted proteins were dialyzed in 100 mM Tris pH 7.5, 100mM KCl, 5 mM MgCl₂. Electrophoresis mobility shift assay was carried out using LightShift Chemiluminescent EMSA Kit (Pierce, 20148). DNA oligonucleotides were biotinylated using Biotin 3’ End DNA Labeling Kit (Thermo, 89818), annealed and used as DNA probes. For each binding reaction 150 fmol of DNA probe was incubated with x protein at room temperature for 20 min in a final volume of 20 μl containing binding buffer (20 mM Tris pH 7.5, 100 mM KCl, 2 mM DTT, 5% glycerol, 0.1 NP40, 10 MgCl₂), 0.5 μg Pol (d+IC), and in the presence or the absence of excess molar ratio of specific or mutated unlabeled DNA competitor. The resulting protein-DNA complexes were electrophoresed on 5% native polyacrylamide gels, and then transferred to a Hybond N+ nylon membrane (GE Healthcare). Biotin labeled DNA detection was carried out according to the LightShift Chemiluminescent EMSA Kit manufacturer’s instructions.

| Gene promoter region | Specific DNA probe | Mutated oligonucleotide |
|----------------------|-------------------|-------------------------|
| DR5                  | PML 839, 840      | PML 841, 842            |
| ARFB4                | PML 1018, 1009    | PML 1110, 1111          |
| IAA1A                | PML 1082, 1083    | PML 1084, 1085          |
| IAA1B                | PML 1068, 1069    | PML 1094, 1095          |
Plant growth assay
Following protoplast recovery, cellophane overlays were transferred to BCD plates. Same plants were imaged every day for five days using a Nikon SMZ1500 dissecting scope for five days and their length was measured using ImageJ.

Cell measurement
Following tissue homogenization, plants were grown for seven days on BCDAT plates with cellophane overlays and imaged by confocal microscope (Zeiss MLS 710). Cells proximal to filament branches were selected. The cell length and width of thirty cells from different confocal photos were measured using ImageJ.

Chlorophyll measurement
Plant tissue grown for seven days on BCDAT plates with cellophane overlays was extracted in 80% Acetone for 24 hr. Light absorbance of chlorophyll a and b were measured at wavelengths 663 and 646 nm using spectrophotometer and total chlorophyll concentration was calculated.

GUS staining
Tissue was stained in GUS staining solution (50 mM NaH2PO4 (pH7.0), 0.5 mM X-Gluc, 0.5 mM K3Fe(CN) 6, 0.5 mM K4Fe(CN)6, 0.05% Triton X-100) at 37°C for 5 hr following the auxin treatment of PIAA10/GUS background lines. 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 65 mM Tris pH6.8, 2% SDS, and 10% glycerol. The resulting protein extract was centrifuged for 10 min 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 monoclonal anti-c-Myc 9E10 (Roche) or anti-GR P-20 (Santa Cruz) antibodies were used for protein detection. Proteins were visualized using ECL Plus Western Blot Detection System (GE healthcare).

Phylogenetic analysis
Full-length ARF protein sequences were extracted from the Physcomitrella patens, Selaginella moellendorfii, and Arabidopsis thaliana protein databases downloaded from the Joint Genome Institute plant genome website (http://www.phytozome.org, accessed 14 Jan, 2014). The sequences were aligned using T-Coffee (Notredame et al., 2000), and poorly aligned regions were removed from the alignment. The tree was inferred using MrBayes [v3.2.2.x64; (Ronquist et al., 2012)] with the following parameters: aamodelpr=mixed, nst = 6, rates = invgamma, nruns = 4, nchains = 4, and ngen = 2000000. The consensus tree was visualized and exported using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Gene identifiers are as follows:

AtARF1, At1g59750; AtARF2, At5g62000; AtARF3_ETT, At2g33860; AtARF4, At5g60450; AtARF5_MP, At1g19850; AtARF6, At1g30330; AtARF7_NPH4, At5g20730; AtARF8, At5g37020; AtARF9, At4g23980; AtARF10, At2g28350; AtARF11, At2g46530; AtARF12, At1g34310; AtARF13, At1g34170; AtARF14, At1g35540; AtARF15, At1g35520; AtARF16, At4g30080; AtARF17, At1g77850; AtARF18, At3g61830; AtARF19, At1g19220; AtARF20, At1g35240; AtARF21, At1g34410; AtARF22, At1g34390; AtARF23, At1g43950; PpARFa1, Pp3c1_14480V3.1/Pp1s68_1V5_1; PpARFa2, Pp3c1_14440V3.1/—; PpARFa3, Pp3c2_25890V3.1/Pp1s119_25V5_1; PpARFa4, Pp3c13_4720V3.1/Pp1s133_57V5_1; PpARFa5, Pp3c26_11550V3.1/Pp1s6_230V5_3; PpARFa6, Pp3c17_19900V3/Pp1s65_225V5 (edited); PpARFa7, Pp3c14_16990V3.15/Pp1s148_142V5_1; PpARFa8, Pp3c1_40270V3/Pp1s63_120V5_1 (edited); PpARFb1, Pp3c27_60V3.1/Pp1s280_7V5_1; PpARFb2, Pp3c16_6100V3.1/Pp1s341_4V5_1; PpARFb3, Pp3c5_9420V3.1/Pp1s64_136V5_1; PpARFb4, Pp3c6_21370V3.1/Pp1s316_22V6_1; PpARFc1B, Pp3c4_13010V3.1/—; PpARFc2, Pp3c6_26890V3.1/Pp1s279_8V5_1; PpARFd1, Pp3c9_21330V3.1/Pp1s316_22V6_1;
SmARFa1, Selmo117217 (edited); SmARFa2, Selmo424114 (edited); SmARFa3, Selmo181406 (edited); SmARFb1, Selmo437944 (edited); SmARFb2, Selmo81992 (edited); SmARFc1, Selmo61688 (edited); SmARFc2, Selmo51695 (edited); SmARFd1, Selmo1d28604 (edited); and SmARFd2, Selmo1d115320 (edited).

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Author contributions
ML, Designed the experiments, Performed the experiments, Analysis and interpretation of data, Wrote the manuscript, Conception and design, Drafting or revising the article; MJP, Designed the experiments, Performed the experiments, Analysis and interpretation of data, Conception and design, Drafting or revising the article; ST, Performed the experiments, Conception and design, Analysis and interpretation of data, Drafting or revising the article; SS, Performed the experiments, Drafting or revising the article; AK, Performed the experiments, Analysis and interpretation of data, Drafting or revising the article; KK, Performed the experiments, Conception and design, Drafting or revising the article, Contributed unpublished essential data or reagents; ME, Designed the experiments, Analysis and interpretation of data, Wrote the manuscript, Conception and design, Drafting or revising the article

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Additional files

Supplementary files
- Supplementary file 1. (A) Differentially expressed genes between mock- and IAA treated WT protonemata, (fold change ≥1.5; padj <0.01). (B) Differentially expressed genes between mock treated WT protonemata and the aux/iaaΔ mutant (fold change ≥1.5; padj <0.01). (C) Differentially expressed genes between IAA treated WT protonemata, and the aux/iaaΔ mutant (fold change ≥1.5; padj <0.01). (D) Differentially expressed genes between mock- and IAA treated aux/iaaΔ mutant (padj <0.05).
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- Supplementary file 2. (A) GO terms of biological processes (P), molecular function (F), and cellular component (C) associated with auxin-regulated genes. (B) GO terms of biological processes (P), molecular function (F), and cellular component (C) associated with genes regulated between WT and the aux/iaaΔ mutant.
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• Supplementary file 3. Primer sequences used in this study. DOI: 10.7554/eLife.13325.020
• Supplementary file 4. Backbone vectors used in this study. DOI: 10.7554/eLife.13325.021

Major datasets
The following dataset was generated:

| Author(s)         | Year | Dataset title                  | Dataset URL                                                      | Database, license, and accessibility information |
|-------------------|------|--------------------------------|-----------------------------------------------------------------|--------------------------------------------------|
| Meirav Lavy, Mark Estelle | 2016 | Physcomitrella patens Raw Sequence Reads | http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA317343         | Publicly available at NCBI BioSample (accession no: PRJNA317343) |

References
Ashton NW, Grimsley NH, Cove DJ. 1979. Analysis of gametophytic development in the moss, physcomitrella patens, using auxin and cytokinin resistant mutants. Planta 144:427–435. doi: 10.1007/BF00380118
Bargmann BO, Vanneste S, Krouk G, Navy T, Efroni I, Shani E, Choe G, Friml J, Bergmann DC, Estelle M, Birnbaum KD. 2013. A map of cell type-specific auxin responses. Molecular Systems Biology 9. doi: 10.1038/msb.2013.40
Bennett TA, Liu MM, Aoyama T, Bierfreund NM, Braun M, Coudert Y, Dennis RJ, O’Connor D, Wang XY, White CD, Decker EL, Reski R, Harrison CJ. 2014. Plasma membrane-targeted PIN proteins drive shoot development in a moss. Current Biology 24:2776–2785. doi: 10.1016/j.cub.2014.09.054
Bezanilla M, Perroud PF, Pan A, Klueh P, Quatrano RS. 2005. An mni system in physcomitrella patens with an internal marker for silencing allows for rapid identification of loss of function phenotypes. Plant Biology 7:251–257. doi: 10.1055/s-2005-837597
Bierfreund NM, Reski R, Decker EL. 2003. Use of an inducible reporter gene system for the analysis of auxin distribution in the moss physcomitrella patens. Plant Cell Reports 21:1143–1152. doi: 10.1007/s00299-003-0646-1
Boer DR, Freire-Rios A, van den Berg WA, Saakii T, Manfield IW, Kepinski S, López-Vidriero I, Franco-Zorrilla JM, de Vries SC, Solano R, Weijers D, Coll M. 2014. Structural basis for DNA binding specificity by the auxin-dependent ARF transcription factors. Cell 156:577–589. doi: 10.1016/j.cell.2013.12.027
Calderón Villalobos LJ, Lee S, De Oliveira C, Iveta A, Brandt W, Armitage L, Sheard LB, Tan X, Parry G, Mao H, Zheng N, Napier R, Kepinski S, Estelle M. 2012. A combinatorial tir1/afb-aux/iaa co-receptor system for differential sensing of auxin. Nature Chemical Biology 8:477–485. doi: 10.1038/nchembio.926
Chapman EJ, Greenham K, Castillejo C, Sartor R, Bialy A, Sun TP, Estelle M. 2012. Hypocotyl transcriptome reveals auxin regulation of growth-promoting genes through ga-dependent and -independent pathways. PloS One 7:e36210. doi: 10.1371/journal.pone.0036210
Coudert Y, Palubicki W, Ljung K, Novak O, Leyser O, Harrison CJ. 2015. Three ancient hormonal cues coordinate shoot branching in a moss. eLife 4:e06808. doi: 10.7554/eLife.06808
Decker EL, Frank W, Sarnighausen E, Reski R. 2006. Moss systems biology en route: Phytohormones in physcomitrella development. Plant Biology 8:397–405. doi: 10.1055/s-2006-923952
Dharmasiri N, Dharmasiri S, Estelle M. 2005a. The f-box protein TIR1 is an auxin receptor. Nature 435:441–445. doi: 10.1038/nature03543
Dharmasiri N, Dharmasiri S, Werejers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jürgens G, Estelle M. 2005b. Plant development is regulated by a family of auxin receptor F box proteins. Developmental Cell 9:109–119. doi: 10.1016/j.devcel.2005.05.014
Dinesh DC, Kovernam M, Gopalswamy M, Hellmuth A, Calderón Villalobos LJ, Lilie H, Balbach J, Abel S. 2015. Solution structure of the psia4d oligomerization domain reveals interaction modes for transcription factors in early auxin response. Proceedings of the National Academy of Sciences of the United States of America 112:6230–6235. doi: 10.1073/pnas.1424077112
Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: Ultrafast universal rna-seq aligner. Bioinformatics 29:15–21. doi: 10.1093/bioinformatics/bts635
Du Z, Zhou X, Ling Y, Zhang Z, Su Z. 2010. agrIGo: A GO analysis toolkit for the agricultural community. Nucleic Acids Research 38:W64–W70. doi: 10.1093/nar/gkq310
Eklund DM, Thelander M, Landberg K, Stådal V, Nilsson A, Johansson M, Valsecchi I, Pederson ER, Kowalczyk M, Ljung K, Ronne H, Sundberg E. 2010. Homologues of the arabidopsis thaliana SHI/STY/LRP1 genes control auxin biosynthesis and affect growth and development in the moss physcomitrella patens. Development 137:1275–1284. doi: 10.1242/dev.093954
Flores-Sandoval E, Eklund DM, Bowman JL. 2015. A simple auxin transcriptional response system regulates multiple morphogenetic processes in the liverwort marchantia polymorpha. PLoS Genetics 11:e1005207. doi: 10.1371/journal.pgen.1005207
Fujita T, Sakaguchi H, Hiwatashi Y, Wagstaff SJ, Ito M, Deguchi H, Sato T, Hasebe M. 2008. Convergent evolution of shoots in land plants: Lack of auxin transport in moss shoots. *Evolution & Development* **10**:176–186. doi: 10.1111/j.1525-142X.2008.00225.x

Guilfoyle TJ, Hagen G. 2007. Auxin response factors. *Current Opinion in Plant Biology* **10**:453–460. doi: 10.1016/j.cbpb.2007.08.014

Guilfoyle TJ, Hagen G. 2012. Getting a grasp on domain III/IV responsible for auxin response factor-IAA19 protein interactions. *Plant Science* **190**:82–88. doi: 10.1016/j.plantsci.2012.04.003

Halliday KJ, Martinez-Garcia JF, Josse EM. 2009. Integration of light and auxin signaling. *Cold Spring Harbor Perspectives in Biology* **1**:a001586. doi: 10.1101/cshperspect.a001586

Han M, Park Y, Kim I, Kim EH, Yu TK, Rhee S, Suh JY. 2014. Structural basis for the auxin-induced transcriptional regulation by aux/iaa17. *Proceedings of the National Academy of Sciences of the United States of America* **111**:18613–18618. doi: 10.1073/pnas.1419525112

He W, Brumos J, Li H, Ji Y, Ke M, Gong X, Zeng Q, Li W, Zhang X, An F, Wen X, Li P, Chu J, Sun X, Yan C, Yan N, Xie DY, Raikhel N, Yang Z, Stepanova AN, et al. 2011. A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in arabidopsis. *The Plant Cell* **23**:3944–3960. doi: 10.1105/tpc.111.098902

Jurado S, Abraham Z, Manzano C, Lopez-Torrejon G, PACIOS LF, Del Pozo JC. 2010. The arabidopsis cell cycle f-box protein SKP2a binds to auxin. *The Plant Cell* **22**:3891–3904. doi: 10.1105/tpc.107.097872

Kagale S, Rozwadowski K. 2011. EAR motif-mediated transcriptional repression in plants: An underlying mechanism for epigenetic regulation of gene expression. *Epigenetics* **6**:141–146. doi: 10.4161/epi.6.2.13627

Kato H, Ishizaki K, Kouno M, Shirakawa M, Bowman JL, Nishihama R, Kohchi T. 2015. Auxin-mediated transcriptional system with a minimal set of components is critical for morphogenesis through the life cycle in marchantia polymorpha. *PLoS Genetics* **11**:e1005084. doi: 10.1371/journal.pgen.1005084

Kepinski S, Leyser O. 2005. The arabidopsis f-box protein TIR1 is an auxin receptor. *Nature* **435**:446–451. doi: 10.1038/nature03542

Koralski DA, Jez JM, Strader LC. 2015. Refining the nuclear auxin response pathway through structural biology. *Current Opinion in Plant Biology* **27**:22–28. doi: 10.1016/j.cbpb.2015.05.007

Koralski DA, Westfall CS, Lee SG, Nanao MH, Dumas R, Hagen G, Guilfoyle TJ, Jez JM, Strader LC. 2014. Molecular basis for AUXIN RESPONSE FACTOR protein interaction and the control of auxin response repression. *Proceedings of the National Academy of Sciences of the United States of America* **111**:5427–5432. doi: 10.1073/pnas.140007411

Lau S, De Smet I, Kolb M, Meinhardt H, Jürgens G. 2011. Auxin triggers a genetic switch. *Nature Cell Biology* **13**:611–615. doi: 10.1038/ncb2212

Lavy M, Prigge MJ, Tigyi K, Estelle M. 2012. The cyclophilin DIAGEOTROPICA has a conserved role in auxin signaling. *Development* **139**:1115–1214. doi: 10.1242/dev.074631

Lee JS, Wang S, Sritubtim S, Chen JG, Ellis BE. 2009. Arabidopsis mitogen-activated protein kinase MPK12 interacts with the MAPK phosphatase IBR5 and regulates auxin signaling. *The Plant Journal* **57**:975–985. doi: 10.1111/j.1365-313X.2008.03741.x

Liao Y, Smyth GK, Shi W. 2014. featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**:923–930. doi: 10.1093/bioinformatics/btt656

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for rna-seq data with sesq2. *Genome Biology* **15**: doi: 10.1186/s13059-014-0550-8

Ludwig-Müller J, Jülke S, Bierfreund NM, Decker EL, Reski R. 2009. Moss (physcomitrella patens) GH3 proteins act in auxin homeostasis. *The New Phytologist* **181**:323–338. doi: 10.1111/j.1469-8137.2008.02677.x

Mockaitis K, Estelle M. 2008. Auxin receptors and plant development: A new signaling paradigm. *Annual Review of Cell and Developmental Biology* **24**:55–80. doi: 10.1146/annurev.cellbio.23.090506.123214

Nanao MH, Vinos-Poyo T, Brunoud G, Thévenon E, Mazzoleni M, Mast D, Lainé S, Wang S, Hagen G, Li H, Guilfoyle TJ, Parcy F, Vernoux T, Dumas R. 2014. Structural basis for oligomerization of auxin transcriptional regulators. *Nature Communications* **5**:doi: 10.1038/ncomms4617

Nishiyama T, Hiwatashi Y, Sakakibara I, Kato M, Hasebe M. 2000. Tagged mutagenesis and gene-trap in the moss, physcomitrella patens by shuttle mutagenesis. *DNA Research* **7**:9–17. doi: 10.1093/dnares/7.1.9

Notredame C, Higgins DG, Heringa J. 2000. T-coffee: A novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology* **302**:205–217. doi: 10.1006/jmbi.2000.4042

Paponov IA, Paponov M, Teale W, Menges M, Chakrabortee S, Murray JA, Palme K. 2008. Comprehensive transcriptome analysis of auxin responses in arabidopsis. *Molecular Plant* **1**:321–337. doi: 10.1093/mp/ssm021

Paponov IA, Teale W, Lang D, Paponov M, Reski R, Rensing SA, Palme K. 2009. The evolution of nuclear auxin signalling. * BMC Evolutionary Biology* **9**: doi: 10.1186/1471-2148-9-126

Plavskin Y, Nagashima A, Perroud PF, Hasebe M, Quatrano RS, Atwal GS, Timmermans MC. 2016. Ancient transacting siRNAs confer robustness and sensitivity onto the auxin response. *Developmental Cell* **36**:276–289. doi: 10.1016/j.devcel.2016.01.010

Plavskin Y, Timmermans MC. 2012. Small rna-regulated networks and the evolution of novel structures in plants. *Cold Spring Harbor Symposia on Quantitative Biology* **77**:221–233. doi: 10.1101/sqb.2013.77.014878

Prigge MJ, Bezania M. 2010. Evolutionary crossovers in developmental biology: Physcomitrella patens. *Development* **137**:3535–3543. doi: 10.1242/dev.049027

Prigge MJ, Lavy M, Ashton NW, Estelle M. 2010. Physcomitrella patens auxin-resistant mutants affect conserved elements of an auxin-signaling pathway. *Current Biology* **20**:1907–1912. doi: 10.1016/j.cub.2010.08.050
Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. Mrbayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61:539–542. doi: 10.1093/sysbio/sys029

Salehin M, Bagchi R, Estelle M. 2015. SCFTIR1/AFB-based auxin perception: Mechanism and role in plant growth and development. The Plant Cell 27:9–19. doi: 10.1105/tpc.114.133744

Schaefer DG, Zryd JP. 2001. The moss physcomitrella patens, now and then. Plant Physiology 127:1430–1438. doi: 10.1104/pp.010786

Szemenyei H, Hannon M, Long JA. 2008. TOPLESS mediates auxin-dependent transcriptional repression during arabidopsis embryogenesis. Science 319:1384–1386. doi: 10.1126/science.1151461

Tan X, Calderon-Villalobos Li, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N. 2007. Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446:640–645. doi: 10.1038/nature05731

Thelander M, Nilsson A, Olsson T, Johansson M, Girod PA, Schaefer DG, Zryd JP, Ronne H. 2007. The moss genes ppski1 and ppski2 encode nuclear srk1 interacting proteins with homologues in vascular plants. Plant Molecular Biology 64:559–573. doi: 10.1007/s11103-007-9176-5

Thelander M, Olsson T, Ronne H. 2005. Effect of the energy supply on filamentous growth and development in physcomitrella patens. Journal of Experimental Botany 56:653–662. doi: 10.1093/jxb/eri040

Tiwari SB, Hagen G, Guilfoyle T. 2003. The roles of auxin response factor domains in auxin-responsive transcription. The Plant Cell 15:533–543. doi: 10.1105/tpc.008417

Ulmasov T, Hagen G, Guilfoyle TJ. 1999. Activation and repression of transcription by auxin-response factors. Proceedings of the National Academy of Sciences of the United States of America 96:5844–5849. doi: 10.1073/pnas.96.10.5844

Vernoux T, Brunoud G, Farcot E, Morin V, Van den Daele H, Legrand J, Oliva M, Das P, Larrieu A, Wells D, Guédon Y, Armitage L, Picard F, Guyomarc’h S, Cellier C, Parry G, Koumprogoulou R, Doonan JH, Estelle M, Godin C, et al. 2011. The auxin signalling network translates dynamic input into robust patterning at the shoot apex. Molecular Systems Biology 7. doi: 10.1038/msb.2011.39

Vidali L, Augustine RC, Kleinman KP, Bezanilla M. 2007. Profilin is essential for tip growth in the moss physcomitrella patens. The Plant Cell 19:3705–3722. doi: 10.1105/tpc.107.053413

Wang R, Estelle M. 2014. Diversity and specificity: Auxin perception and signaling through the TIR1/AFB pathway. Current Opinion in Plant Biology 21:51–58. doi: 10.1016/j.pbi.2014.06.006

Wright RC, Nemhauser JL. 2015. New tangles in the auxin signaling web. F1000prime Reports 7. doi: 10.12703/P7.19

Wu M-F, Yamaguchi N, Xiao J, Bargmann B, Estelle M, Sang Y, Wagner D. 2015. Auxin-regulated chromatin switch directs acquisition of flower primordium founder fate. eLife 4. doi: 10.7554/eLife.09269

Zhang JY, He SB, Li L, Yang HQ. 2014. Auxin inhibits stomatal development through MONOPTEROS repression of a mobile peptide gene STOMAGEN in mesophyll. Proceedings of the National Academy of Sciences of the United States of America 111:E3015–3023. doi: 10.1073/pnas.1400542111

Zhao Z, Andersen SU, Ljung K, Dolezal K, Miotk A, Schultheiss SJ, Lohmann JU. 2010. Hormonal control of the shoot stem-cell niche. Nature 465:1089–1092. doi: 10.1038/nature09126