Hypocotyl Transcriptome Reveals Auxin Regulation of Growth-Promoting Genes through GA-Dependent and -Independent Pathways

Elisabeth J. Chapman1*, Kathleen Greenham1*, Cristina Castillejo1, Ryan Sartor1, Agnieszka Bialy1, Taiping Sun2, Mark Estelle3*

1 Section of Cell and Developmental Biology, Division of Biological Sciences, University of California San Diego, La Jolla, California, United States of America, 2 Biology Department, Duke University, Durham, North Carolina, United States of America, 3 Howard Hughes Medical Institute, University of California San Diego, La Jolla, California, United States of America

Abstract

Many processes critical to plant growth and development are regulated by the hormone auxin. Auxin responses are initiated through activation of a transcriptional response mediated by the TIR1/AFB family of F-box protein auxin receptors as well as the AUX/IAA and ARF families of transcriptional regulators. However, there is little information on how auxin regulates a specific cellular response. To begin to address this question, we have focused on auxin regulation of cell expansion in the Arabidopsis hypocotyl. We show that auxin-mediated hypocotyl elongation is dependent upon the TIR1/AFB family of auxin receptors and degradation of AUX/IAA repressors. We also use microarray studies of elongating hypocotyls to show that a number of growth-associated processes are activated by auxin including gibberellin biosynthesis, cell wall reorganization and biogenesis, and others. Our studies indicate that GA biosynthesis is required for normal response to auxin in the hypocotyl but that the overall transcriptional auxin output consists of PIF-dependent and -independent genes. We propose that auxin acts independently from and interdependently with PIF and GA pathways to regulate expression of growth-associated genes in cell expansion.

Citation: Chapman EJ, Greenham K, Castillejo C, Sartor R, Bialy A, et al. (2012) Hypocotyl Transcriptome Reveals Auxin Regulation of Growth-Promoting Genes through GA-Dependent and -Independent Pathways. PLoS ONE 7(5): e36210. doi:10.1371/journal.pone.0036210

Editor: Miguel A. Blazquez, Instituto de Biología Molecular y Celular de Plantas, Spain

Received March 23, 2012; Accepted March 28, 2012; Published May 9, 2012

Copyright: © 2012 Chapman et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the National Institute of Health Institute of General Medicine (GM 43644) and the Department of Energy (De-FG02-02ER15312). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: mestelle@ucsd.edu
† These authors contributed equally to this work.
‡ Current address: Forage Genetics International, West Salem, Wisconsin, United States of America

Introduction

The plant hormone auxin has diverse roles in plant growth and development including, but not limited to, embryogenesis, cell division and expansion, root initiation, tropic responses, apical dominance, flowering, and fruit and seed development [1]. A major challenge in the field of auxin biology is to understand how a small molecule can specify such distinct changes in morphogenesis and growth throughout the life cycle of a plant. Current models suggest that auxin levels are highly regulated through changes in auxin biosynthesis, conjugation and storage, degradative processes initiated through activation of a transcriptional response mediated by the TIR1/AFB family of F-box protein auxin receptors [7,8]. The Aux/IAA proteins contain four zinc-binging domains referred to as Aux/IAA response elements (AuxREs) [7,8]. A TGTCTC sequence motif first identified in the auxin-responsive GHB promoter from soybean was shown to recruit multiple members of the Arabidopsis ARF family, with TGTC being absolutely required for ARF-DNA binding [9]. However, the TGTCTC element is not found in all auxin-responsive promoters. In some cases tandem repeats of the TGTC portion of the AuxRE are sufficient for auxin induction [10,11]. ARF proteins are characterized by a B3-like DNA binding domain, a middle region associated with transcriptional repression or activation, and a C-terminal domain (CTD) involved in homo- and hetero-dimerization [2,7,8]. The CTD region is similar to the C-terminal domains III and IV of the Aux/IAA transcriptional regulators [12].

The Aux/IAAs are a 29 member family of small nuclear proteins in Arabidopsis that are involved in repressing auxin-regulated transcription [13]. Aux/IAA proteins contain four conserved domains (I–IV), of which domains I, II and IV contain nuclear localization motifs. Domain III contains a sequence that is related to the β2a DNA binding domain that is required for Aux/IAA homo- and hetero-dimerization. However, there is currently no evidence that Aux/IAA proteins bind DNA directly [14,15]. Rather, Aux/IAAs are recruited to promoters through interactions with ARF proteins that are mediated by domains III and IV of the two proteins. Domain II of Aux/IAAs is highly conserved and
contains a degron motif that is important for degradation by the SCF<sup>TIR1</sup> E3 ubiquitin ligase complex [12,16]. Mutations in this degron result in stabilization of the protein and reduced auxin response, causing various defects in growth and development [6,16,17].

Functional redundancies within the ARF and Aux/IAA gene families make assigning specific roles of each protein a challenge. However, genetic studies have revealed ARF and Aux/IAA combinations that are essential for certain processes. BDL/IAA12 and MP/ARF5 specify apical-basal polarity during embryogenesis [18]. SLR/IAA14 and NPH4/ARF7 are required for lateral root initiation, and MSG2/IAA19 and NPH4/ARF7 are involved in tropic hypocotyl growth [19]. ARF2, ARF8, and ARF19 are involved in root and hypocotyl growth and development, although Aux/IAA partners in these processes are not clear [20,21,22]. Recently, the apical-basal polarity determinant TOPELESS (TPL) was shown to act as a transcriptional co-repressor with IAA12/BDL to repress ARF5/MP transcriptional activity [23]. It has yet to be seen whether all the Aux/IAAs interact with TPL to repress the auxin response in specific developmental pathways.

Auxin exerts changes in gene expression by interacting with the TIR1/AFB family of auxin receptors. These proteins are the F-box protein subunits of SCF (Skp1/Cullin/F-box) complexes that target the Aux/IAAs for proteasome-mediated degradation [24,25,26]. The Arabidopsis genome encodes 5 proteins related to TIR1, Auxin Signaling F-Box (AFB) proteins AFB1, 2, 3, 4 and 5. Previous work has shown that, like TIR1, AFB1–5 function as auxin receptors that interact with Aux/IAA repressors in an auxin-dependent manner [26,27]. Mutant analysis reveals overlapping functions of TIR1/AFB1–3. The most severely affected tir1 afb1 afb2 afb3 quadruple mutants arrest shortly after germination [26]. The AFB4 clade of receptors, including AFB4 and AFB5, display a unique affinity for the synthetic auxin picloram. The afb5-5 single mutant shows almost complete resistance to picloram-induced hypocotyl growth [27].

In order to develop successful models for auxin regulation of growth and development, it will be important to identify the gene targets of the TIR1/AFB pathway(s) and understand their function in cell growth. Several studies of auxin-responsive transcriptomes have identified large numbers of candidate auxin targets. The results of supporting genetic studies ascribe developmental roles to a small number of these [28]. A potential barrier to identification of distinct auxin pathways from such studies lies in the complexity of the tissue sampled for the experiment. Auxin mediates distinct responses in different tissue types, for example inhibiting primary root elongation while stimulating lateral root initiation and outgrowth [29]. Therefore, auxin-responsive transcriptomes in entire plants are too complex to facilitate separation of distinct developmental pathways.

In this study we focus on the role of auxin signaling in cell expansion. We chose the hypocotyl, which grows entirely by cell expansion, as a model tissue for this study [30]. The hypocotyl elongates in plants overexpressing auxin biosynthetic genes [31] and in response to high temperature [32], due to elevated auxin levels. Hypocotyl elongation is tightly regulated and many signaling pathways overlap to regulate uniform, as well as directional, hypocotyl cell expansion. Light is a major repressor of hypocotyl growth and as a consequence, mutations in the phytochrome light receptors result in seedlings with long hypocotyl phenotypes [33]. Light-activated forms of the phytochromes interact with members of the phytochrome-interacting factor (PIF) family of bHLH transcription factors, signaling rapid phyA- and phyB-mediated degradation of PIF3,4 and 5 in the light [34,35,36]. PIFs have also recently been shown to function in GA signaling [37]. The PIFs appear to be the major positive regulators of hypocotyl growth, as they are required for growth responses to time of day, direction of light source, nutrients, high temperature and other stimuli [38,39,40,41]. PIF mRNA and protein levels are controlled by the circadian clock, light, and GA signaling, such that PIF activities and hypocotyl growth are repressed during the day [39,42,37]. Within the PIF family, several PIF and PIF-LIKE (PIL) genes are implicated in germination and early seedling growth [43]. PIF4 and PIF5 seem to be particularly important for hypocotyl growth as expression of these factors is circadian regulated and correlates with hypocotyl growth [39,42]. In addition the pif4pif5 double mutant has a short hypocotyl phenotype [36].

Here we identify auxin signaling components required for auxin-responsive hypocotyl elongation. In addition we characterize the auxin transcriptome specifically in elongating hypocotyl tissue. Our findings indicate that auxin-induced hypocotyl elongation is associated with regulation of a suite of growth-associated genes and involves GA biosynthesis. Importantly, we also show that auxin works in part through pathways independent of GA and PIF activities.

**Results and Discussion**

**Auxin Promotes Elongation of Arabidopsis Hypocotyls**

To explore the function of auxin in plant growth, we have elected to focus on the Arabidopsis hypocotyl. Our first task was to develop a robust assay for auxin response in this system. Seedlings were grown at 22°C for 5 days in various day-night cycles, exposed to auxin, and measured after different treatment times. Initially, we treated seedlings with the synthetic auxin picloram because earlier studies showed that this compound promoted hypocotyl elongation while the natural auxin indole acetic acid (IAA), generally inhibited elongation [27,44,45]. However, under our conditions we found that both picloram and IAA promoted hypocotyl growth in continuous light (LL), long days (LD), or short days (SD) (Fig. 1A). Unless otherwise stated, LD conditions were used for additional experiments designed to characterize auxin-responsive hypocotyl growth (Fig. 1B). Importantly, in our conditions the auxin dose-response curve for hypocotyl growth of wild-type seedlings is bell-shaped (Fig. 2). This differentiates our growth conditions from those in which auxin treatment or constitutive auxin signaling inhibits hypocotyl elongation [22,44,46]. Interestingly, the bell-shaped response curve is similar to auxin dose response in root system growth modeled previously [47], suggesting that an auxin signaling level optimal for eliciting a growth response may be a common feature among auxin-responsive tissues.

**Auxin-mediated Hypocotyl Elongation Requires Transcriptional Auxin Signaling**

To confirm that the auxin-dependent elongation response requires activation of transcriptional auxin signaling pathways, we measured the response in a series of Aux/IAA gain-of-function mutants in which auxin-regulated transcription is repressed [40,49,50,51,52]. In slr-1/iaa14, axr2-1/iaa7 and axr5-1/iaa1, the auxin response was significantly reduced compared to wild-type plants (Fig. 1C). Interestingly, the response of msg2-1/iaa9 seedlings was similar to that of wild type, even though this mutant is deficient in tropic growth in the hypocotyl. This suggests that different auxin signaling pathways have specific roles in hypocotyl growth. This has been shown previously for apical-basal polarity determination [18] and lateral root initiation [21].
We explored the possibility of functional specialization among the TIR1/AFB auxin receptors in hypocotyl elongation by analyzing the phenotypes of various tir1/afb mutants. We observed slight auxin resistance or hypersensitivity in single tir1/afb receptor mutants (Fig. 2A) with the exception of afb5-5 and afb4-2 afb5-5, which are highly resistant to picloram [27]. The basis for auxin hypersensitivity in afb1-3 and afb3-4 mutants is unclear, however, double mutant combinations among afb1-3, afb2-3, and afb3-4 eliminated this hypersensitivity (Fig. 2B) suggesting that increased growth response may be due to enhanced activity of other TIR1/AFB family members that is lost in the higher order mutants. The afb3-5 mutant overexpresses AFB1 and AFB2 due to alterations in small RNA-mediated regulation, and afb2-3 overexpresses AFB1 and AFB3 [53]. Thus, TIR1/AFB single mutants may not display predictable loss-of-function phenotypes. Future analysis of the expression patterns of the receptors in the single and double mutant backgrounds will be necessary to determine whether elevated receptor activity in TIR1/AFB mutants could explain the hypersensitivity we observed.

Double and triple mutants carrying tir1-1 each displayed increased auxin resistance when compared to the tir1-1 mutant (Fig. 2C). The triple mutant tir1-1 afb2-3 afb3-4 displays an incompletely penetrant phenotype in which a significant percentage of individuals fail to develop basal structures such as roots and hypocotyls [26]. In tir1-1 afb2-3 afb3-4 individuals with developed basal structures, hypocotyls were shorter than those of wild-type plants and displayed the highest degree of resistance to IAA-
mediated elongation of all tir1/afb receptor mutants (Fig. 2C). The reliance of the elongation response on the TIR1/AFB auxin receptors and degradation of Aux/IAA proteins confirms that auxin mediated growth requires transcriptional auxin signaling pathways.

Identification of Auxin-responsive Cell Expansion-associated Genes in Elongating Hypocotyls

Based on our finding that auxin-mediated hypocotyl elongation requires the TIR1/AFB pathway, we hypothesized that elongation is preceded by changes in expression of a suite of auxin-responsive genes. To identify such genes, we profiled auxin-responsive transcription in hypocotyls in a series of microarray experiments. We incorporated several parameters into our microarray design to maximize the likelihood of identifying auxin-regulated genes associated with anisotropic cell expansion. To enrich our dataset for cell expansion genes that may not be identified in whole seedling experiments, we sampled hypocotyl tissue dissected from auxin- or control-treated whole seedlings. To minimize time-of-day and circadian effects and avoid mis-identification of auxin-responsive genes, we treated experimental and control seedlings at the same time of day and limited the dissection time to 30 minutes. To maximize the amplitude of the transcriptional auxin response, we treated seedlings two hours after subjective dawn, when hypocotyl growth is minimal in the absence of exogenous auxin [54]. Finally, we used the synthetic auxin picloram and included the afb5-5 mutant in our microarray design, as this mutant is picloram-resistant but does not otherwise display obvious growth defects [27,55]. We theorized that cell expansion-associated genes differentially expressed in wild-type hypocotyls elongating in response to picloram might not be responsive in afb5-5 hypocotyls, which fail to elongate in response to picloram.

For microarray experiment “a”, we sampled hypocotyls from wild-type plants treated for 30 minutes or 2 hours with picloram or a solvent-only control. For experiment “b”, we sampled hypocotyls from wild-type or afb5-5 plants treated for 2 hours with picloram or a solvent-only control (Fig. 3A, Table S1). Following auxin or control treatment of seedlings, hypocotyls were individually dissected and frozen for subsequent RNA isolation.

To identify genes differentially expressed among the treatments, we used a moderated linear model [56] and an FDR cutoff of <0.05 to filter data from each microarray experiment. From this initial analysis we identified 65 genes differentially expressed following the 30-minute auxin treatment (Table S2), and 3544 (experiment “a”) or 804 (experiment “b”) genes differentially expressed following a 2-hour auxin treatment (Fig. 3A). Consistent with the picloram-resistant phenotype of afb5-5, no differential expression was detected in afb5-5 following picloram treatment using the analysis method described. Interestingly, we were also unable to identify genes differentially expressed between wild-type and afb5-5 untreated samples (Fig. 3A). So far, picloram perception and regulation of picloram-responsive transcription is the only known function of the AFB5 auxin receptor. The identification of additional functions for AFB5 will require alternative experimental approaches. Analysis of genes differentially expressed in wild-type hypocotyls following 30 minutes of picloram treatment indicated that MUR genes, AUX/IAA genes, Ghd3 genes and others shown elsewhere to be early auxin-responsive [57] were induced by picloram and were the predominant genes to be regulated at this time-point (Table S2). For additional insight into gene expression associated with auxin response, we focused on data from the 2-hour time-point samples.

Comparison of gene lists from the 2-hour auxin treatment in experiments “a” and “b” identified 267 genes differentially expressed. Differential gene expression between hypocotyl samples of solvent-
shown.

were selected and average expression values for microarray ‘b’ are combined for identification of 1193 picloram-responsive genes. (B) Aver...are not affected by picloram treatment of afb5-5 mutant plants (Fig. 3B), suggesting that these are indeed downstream targets of picloram-stimulated transcriptional auxin signaling. We focused on the set of 1193 downstream targets of picloram- and IAA-stimulated auxin response similar to that observed with IAA, suggesting that the AuxRE-containing promoter elements in the promoter gene set (Fig. S2). From these results we conclude that picloram regulates the same downstream transcriptional targets as IAA, and therefore promotes hypocotyl elongation through the same transcriptional pathways as IAA. For the remaining experiments, we used picloram and IAA interchangeably or in parallel, and we did not observe qualitative differences in responses to these two auxins.

Picoloram and IAA Regulate a Common Set of Target Genes

The synthetic auxin picloram induces a hypocotyl elongation response similar to that observed with IAA, suggesting that the downstream targets of picloram- and IAA-stimulated auxin signaling are common between these two auxin pathways. The failure of afb5-5 to regulate this set of genes or to elogate in response to picloram is consistent with a model in which the genes are targets of auxin signaling and involved in the elongation response. To confirm this, we performed a comprehensive comparison between our auxin-responsive gene set and publicly available microarray data. Our first comparison was done using the MASTA package available from the BAR website (http://bar.utoronto.ca/welcome.htm) that probes differentially expressed genes against a database of 600 contrasts obtained from publicly available microarray datasets. Of the 740 genes upregulated by picloram in our dataset, 219 were identified as auxin-upregulated in IAA treatment arrays; of 453 genes downregulated by picloram in our dataset, 121 genes were identified as auxin-downregulated in IAA arrays (data not shown). These overlaps are statistically significant (p.value <0.001) and confirm that picloram affects known IAA-responsive genes. We also performed independent comparisons with the Nemhauser et al. [61] and Stepanova et al. [62] auxin treatment datasets (see Methods for details of comparison). In both cases, more than 50% of the IAA-induced genes were induced by picloram in our experiments (Fig. S1A). The Stepanova et al. [62] dataset was obtained from experiments using root tissue suggesting that many of the genes involved in hypocotyl growth are common to root tissue. We would expect these genes to be specifically involved in cell elongation during root growth.

Importantly, our analysis identified many genes that are not presented in other auxin transcriptome datasets (Table S4) [61,62]. Of the 740 induced genes, 521 were not described in the Nemhauser et al. [62] and Stepanova et al. [63] datasets or the 7 IAA treatment arrays found in the MASTA database. Eighty-one of these are not represented on ATH1 chips and not well characterized as auxin responsive. We expect that many of the remaining 440 genes are specifically auxin regulated in the elongating hypocotyl and were not identified in other studies because of the relative complexity of the auxin response in seedlings and roots. Similarly, 332 of the 453 repressed genes had not been described in these other datasets, 68 of which are on the Nimblegen chip but not ATH1.

To further validate the effects of picloram on auxin-responsive genes, we confirmed that a set of auxin “marker” genes, proposed to serve as hallmarks of auxin activity [61], were identified as picloram-responsive in our microarray data analysis. Overall, expression of the marker genes was responsive to picloram in wild-type hypocotyls, but not in hypocotyls from afb5-5 mutant plants (Fig. S1B). We further validated the picloram response of several of these genes, GHE3, GHE5, HAT2, IAA5, IAA19 and SAUR15, by quantitative RT-PCR using wild-type and afb5-5 hypocotyls. Expression of each gene was induced in wild-type hypocotyls by picloram treatment, and induction was dependent upon AFB5 (Fig. S1C). This indicates that picloram and IAA regulate an overlapping set of target genes, although the picloram signal is uniquely transduced by AFB5.

Finally, we analyzed our picloram-responsive gene set for association with auxin Gene Ontology terms and overrepresentation of AuxRE-containing promoter elements. GO terms associated with auxin response and hormone signaling are enriched in the annotations of our auxin-responsive gene set (Table S5), and we identified several overrepresented AuxRE-containing promoter elements in the promoter gene set (Fig. S2). From these results we conclude that picloram regulates the same downstream transcriptional targets as IAA, and therefore promotes hypocotyl elongation through the same transcriptional pathways as IAA. For the remaining experiments, we used picloram and IAA interchangeably or in parallel, and we did not observe qualitative differences in responses to these two auxins.

A Profile of the Transcriptional Auxin Response Preceding Hypocotyl Elongation

Further examination of GO terms associated with our auxin-responsive gene set revealed overrepresentation of genes involved in cell wall maintenance, cell expansion, growth and hormone signaling (Fig. 4A, Table S5, Fig. S3). Enriched GO terms associated with the auxin-induced gene set included cell wall metabolism and gibberellin biosynthesis. Terms associated with the auxin-repressed gene set included carbohydrate metabolism and plastoquinone assembly (Fig. 4A). Representation of these GO processes in our auxin-responsive gene set is consistent with a role for auxin in transcriptional regulation of cell expansion-associated genes. Cell expansion in the hypocotyl, as well as in other growing plant tissues, is gated by the circadian clock and shows non-uniform patterns across a 24-hour period [63,54,39]. This is likely due in part to circadian patterns of expression of many genes involved in auxin signaling, biosynthesis and transport, and varying sensitivity to auxin at different times of day [64]. We theorized that genes we found to be auxin-responsive in elongating hypocotyls may
follow circadian expression patterns. To determine whether circadian-regulated genes are overrepresented in our auxin-responsive gene set, we generated a gene subset consisting of the top 400 auxin-induced genes according to statistical significance, and analyzed this subset using the Phaser tool [http://phaser.cgrb.oregonstate.edu/][63]. We observed significant enrichment of genes showing peak expression during phases 0-2 and 22-23 in LD conditions, during which hypocotyl growth is active (Fig. 4B) [63]. We further explored our auxin-induced gene set for additional determinants of expression profile by analyzing the corresponding promoter set for overrepresented regulatory elements. Interestingly, the predicted MYC/MYB binding site ‘CACATG’ was the most highly overrepresented element identified in this analysis (data not shown). The ‘CACATG’ element was previously identified as the Hormone Up at Dawn (HUD) element enriched in promoters of genes responsive to phytohormones and showing peak expression levels during periods of growth [63]. Together, these findings suggest that auxin promotes hypocotyl growth by regulating expression of cell expansion-associated genes whose expression levels are controlled by the circadian clock. This is consistent with auxin gating by the clock to maintain the diurnal pattern of hypocotyl elongation under normal growth conditions [64].

**Auxin-mediated Hypocotyl Elongation Requires GA Signaling**

A number of studies have shown that auxin and GA interact to regulate elongation growth in stems and hypocotyls [65,66,67,68,69]. For example, in pea stem and Arabidopsis seedlings, auxin regulates the expression of a number of GA metabolic genes including members of the GA20OX and GA20X gene families, involved in synthesis of active GAs, as well as GA3OX genes, involved in GA inactivation [66,70]. In addition, Frigerio et al [66] showed that the long hypocotyl phenotype conferred by overexpression of YUCCA1 is suppressed by the GA biosynthesis inhibitor paclobutrazol, indicating that GA synthesis is required for auxin-dependent hypocotyl growth. We found that GA20OX1, GA20OX2, GA20OX8, and GA3OX1 are auxin-regulated in the hypocotyl (Table S2, Table S3). To expand on the role of GA biosynthesis in auxin-mediated hypocotyl elongation, we tested the effect of adding paclobutrazol to auxin treatment assays. Paclobutrazol inhibited the effects of exogenous auxin on stability of the DELLA protein RGA. Treatment of seedlings expressing RGA-GFP with IAA or GA resulted in loss of RGA protein from hypocotyl cells within 2 hours (Fig. 5C). This auxin effect was abolished by co-treatment with paclobutrazol (Fig. 5C). While it is possible that the observed loss of RGA protein in auxin-treated seedlings is due to an effect of auxin on transcription of RGA, we think this is unlikely as we did not identify RGA as an auxin-downregulated gene in our microarray experiments (although we did identify RGA-LIKE1 (AT1G66350) and RGA-LIKE3 (AT5G17490) as auxin-upregulated genes, see Table S3). DELLA protein abundance is also affected by circadian regulation of GA signaling [75]. However, it is unlikely that circadian regulation fully explains the effects we observed on RGA-GFP levels, as DELLA protein levels increase during the day [75] where we observed a decrease. A more likely possibility is that auxin regulation of GA levels results in degradation of RGA-GFP protein in the hypocotyl throughout the course of the experiment, and therefore that the signal in control seedlings was weaker at the 24-hour time point than at time zero. This is also unlikely to be due to circadian patterns, which follow a 24-hour cycle. It is possible that the overall abundance of RGA-GFP in hypocotyls changes with growth dynamics, and that more sensitive imaging methods could be used to visualize the protein in older tissues.

We further explored the requirement for GA biosynthesis and signaling in auxin response by examining the behavior of the ga20ox1 ga20ox2 double mutant in the hypocotyl elongation assay. Plants compromised in endogenous GA levels due to mutations in GA20OX1 and GA20OX2 showed partial auxin resistance (Fig. 5D). These data suggest that auxin and GA act interdependently in hypocotyl cell expansion.

We noted, however, that in our hormone treatment assays, paclobutrazol did not completely abolish the auxin effect (Fig. 5A, 5B). While this inhibitor may not fully suppress GA accumulation in the seedlings, our results suggest that the elongation-promoting effects of auxin may not be limited to regulation of GA metabolism. We further explored the auxin-GA interaction by testing the ability of GA to restore the short hypocotyl phenotypes of several gain-of-function Aux/IAA mutants. We found that GA did not significantly affect the hypocotyl length of the axr2 mutant, and that the hypocotyl phenotypes of these mutants overall were only partially restored by treatment with GA (Fig. S4A). These data indicate that auxin signaling is required for a growth program independent of the regulation of GA metabolism, and that constitutive repression of auxin signaling in the Aux/IAA mutants represses this program. We propose that auxin promotes hypocotyl growth in part through GA and in part through an unknown independent pathway(s). A mechanism by which auxin can induce
hypocotyl growth independently of GA synthesis may be important for rapid growth responses.

It is important to note that while the results of our paclobutrazol experiments are consistent with a model in which auxin stimulates synthesis of GA, which then contributes to the elongation response, this may be an oversimplification. GA levels are under negative feedback regulation in which expression of GA biosynthesis genes is repressed as GA levels increase [76]. Auxin biosynthesis in turn is regulated in part by PIF4, which is indirectly activated by GA. Dynamic regulation between these two hormone pathways is likely to be important for hormone-mediated cell expansion.

Auxin Promotes Cell Expansion Independent of Time of Day in Part through Regulation of PIF-independent Pathways

As previously mentioned, several signaling pathways are important for controlling hypocotyl growth, including light signaling and the circadian clock, as well as hormone signaling [37,39,42]. Many of the growth-associated downstream genes in these pathways are regulated by PIF transcription factors [37,39,42], recently shown to be required for activation of transcription downstream of GA signaling [37,39,42]. PIF4 and PIF5 are two members of the PIF family that are circadian...
regulated and for which expression level is correlated with hypocotyl growth [39,42,77]. A recent study by Nozue et al. [77] suggests that PIF5 is a modulator of auxin signaling and that PIF4 and PIF5 regulate auxin sensitivity to control hypocotyl growth.

There are several possible mechanisms by which transcriptional auxin signaling may promote growth either by feeding into a PIF4/5-mediated pathway or acting independently. First, auxin might promote PIF4/5 activity by inducing PIF4/5 transcription during the day; second, auxin might indirectly promote PIF4/5 activity by stimulating GA synthesis consequently degrading the DELLA repressors of the PIFs; third, auxin might act independently of PIF4/5 and regulate transcription of PIF4/5 targets during the day; last, auxin might act independently of the PIFs and regulate PIF4/5-independent growth genes. We addressed the first
possibility by analyzing our microarray data. We did not detect a transcriptional response to auxin for the PIF4/5 genes, suggesting that auxin either enhances residual PIF activity that may be present during the day, or acts in parallel to promote elongation independently of these proteins during the day.

We asked whether auxin, GA, and PIF4/5 are required for the initial growth response to a pulse of auxin using a time course elongation assay done during the day. A 2-hour auxin treatment led to an increase in hypocotyl length in wild-type seedlings within 2 hours (Fig. 6A). The response of pif4pif5 mutant seedlings was indistinguishable from that of wild type, suggesting that this initial growth response does not require PIF4/5 protein. This result is not surprising given that PIF4 and PIF5 are rapidly degraded by a phby-dependent mechanism and transcriptionally inhibited by the DELLAs during the day, and so are unlikely to be required for daytime growth [37]. In contrast, both the ga20ox2 double mutant and the avr2-1 are completely resistant to auxin in this assay (Fig. S4B). These results suggest that both auxin and GA are required for the initial growth response.

To address whether the transcriptional targets of auxin signaling are also PIF targets, we performed an extensive comparison of our auxin-responsive cell expansion data set with existing growth-related microarray datasets. Nozue et al. [77] describe a series of global expression analyses in the pif4pif5 mutant to classify sets of “growth” and “stationary” phase genes that are PIF4/5-dependent or -independent. Using the resulting gene lists as well as datasets obtained using various light conditions in wild type and a pif1 pif3 pif5 PIF quadruple mutant [pifg] [78], we compared our gene lists to the growth-regulated genes identified in these selected arrays. For a description of the arrays selected and the method of comparison see Methods, Table 1 and Table S6. We compared our auxin-induced and auxin-repressed gene lists to each array dataset and identified 490 auxin-induced genes and 270 auxin-repressed genes also presented in these growth datasets. We converted these results into a matrix in which each row represents an auxin-responsive gene from our list, and each column represents a microarray condition (Table S6). We then used hierarchical clustering to generate maps of each matrix. We divided each map into ‘growth’ and ‘stationary’ sections to reflect the conditions with which regulation of each gene is associated, as described by Nozue et al. [77] (Fig. 6B). We also included a column of genes associated with cell wall reorganization, ‘CW’ [79].

A pattern that emerges from our matrix maps is that many picloram-induced genes are co-regulated by conditions where growth is occurring. We found that 46% of our auxin-induced genes are induced in wild type 2-day-old seedlings grown in the dark when compared to light-grown seedlings (Fig. 6B column 1), and 21% are repressed by a 2-day red light treatment that inhibits hypocotyl elongation (Fig. 6B column 3). Similarly, the overlap between stationary phase genes and auxin-repressed genes is greater (the sum of values in columns 9–15 is 348 for 269 genes) than between stationary phase genes and auxin-induced genes (the sum of values in columns 9–15 is 191 for 490 genes) (Fig. 6B, left and right maps, columns 11,12,14,15). Therefore, our auxin-induced gene list consists at least in part of genes that are associated with growth, such as ARGOS (AT3G59900) and ARGOS-LIKE (AT2G44080) [80,81], LONGIPOLIA1 (AT5G15580) and LGV2 (AT3G02170) [82] and several EXPANSIN and EXPANSIN-LIKE genes (Table S6) [83,84,85]. The matrix maps highlight a significant overlap between PIF-regulated genes and auxin targets in elongating hypocotyls [Fig. 6B columns 3–6]. This is consistent with previous results from Nozue et al. [77] that show that auxin-regulated genes are overrepresented among genes differentially expressed between pif4pif5 double mutant and wild type plants. Not surprisingly, genes in this category include genes associated with GA pathways including gibberellin biosynthesis genes GAI30X1 and GAI20X2, the GA repressor RGL1, PIF3-LIKE2 (AT3G62900) and SOMNUS (AT1G03790), a germination gene downstream of PIF5 (AT2G20180). Of the 81 genes defined by Nozue et al. [77] as upregulated by growth and PIF4- or PIF5-dependent, 38 are also classified in that study as auxin regulated. Of these 38, 35 are in our auxin-induced list. Our auxin-induced list also includes an additional 17 PIF4/5-dependent genes not classified by Nozue et al. as auxin-responsive [77].

These findings raise the question of whether auxin regulates PIF target genes through induction of GA biosynthesis and consequent PIF activation, through a GA-independent PIF process, or through a PIF-independent mechanism. We predicted that a set of PIF4/5-dependent growth-associated genes might be auxin-regulated in the absence of PIF activity, since the hypocotyl growth response to the transient auxin treatment during the day did not require PIF4/5 (Fig. 6A). We tested the response of a subset of growth-associated genes, including MUR23 (AT5G18060), IAA2 (AT3G23030) and ARGOS, to auxin using qRT-PCR. We found that each of these three genes was induced by a 2 hr IAA treatment in pif4pif5 double mutant seedlings (Fig. 6C). This suggests that these genes are directly regulated by auxin. This has been confirmed for IAA2, which is rapidly induced by auxin in the presence of cyclohexamide [86]. The fact that these three genes are PIF-dependent in growth promoting-conditions can be explained by the recent discovery that PIF4/5 directly regulates auxin biosynthesis at elevated temperature [39,42,77,87,88]. Genes in this category may be direct auxin targets whose expression in growth-promoting conditions, such as elevated temperature, is dependent upon PIF regulation of auxin biosynthesis. However, we do not rule out the possibility that such genes may also be directly regulated by the PIF family in some conditions. Thus, our results support a growth model in which a number of important cell expansion-associated genes are common targets of multiple growth-promoting pathways.

Finally, our analysis also revealed overlap between auxin-responsive genes and growth-upregulated genes that are PIF4/5-independent. More than 200 of our auxin-induced genes are in this category. While this group predictably includes auxin transport (e.g. PINOID-BINDING PROTEIN1, AT5G54490; TOUCHI, AT2G41100 [89] and signaling factors (IAA7, AT3G23050; IAA5, AT1G15380), genes in the GA pathway (GAI, AT1G14920; GA20OX2, AT3G53180), ethylene pathway (ETHYLENE RESPONSE 2, AT3G23150; ETHYLENE RESPONSE SENSOR 1, AT2G40940; ERS2, AT1G04310), and brassinosteroid pathway (BR1/E2R1 HOMOLOG 2, AT1G36780; BRASSINAZOLE-RESISTANT 1, AT1G75080) are also present. Additionally, several genes with roles in cell wall metabolism are present, including XYLULOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 16 (AT3G23730), XTH17 (AT1G65310) and XTH6 (AT1G11543), CELLULOSE SYNTHASE-LIKE D3 (AT3G03050), and CELLULOSE SYNTHASE-INTERACTIVE PROTEIN 1 (AT2G22125). Together, these genes are candidate direct auxin targets involved in growth and in cross-talk with other signaling pathways.

AUXIN REGULATES ADDITIONAL CANDIDATE CELL EXPANSION GENES

In our auxin-responsive gene list, 81 genes we identified as auxin-induced and 70 genes we identified as auxin-repressed are interrogated by the NimbleChip but not by the Affymetrix ATH1 chip (ftp://ftp.arabidopsis.org/home/tair/Microarrays/...
These genes are presented in Table S4. Several genes among these have predicted functions in cell expansion, including BREVIS RADIX (AT1G31880), which promotes leaf, root and shoot growth [90], KIDARI (AT1G26945), which promotes shoot elongation downstream of GA [91], and PAR2 (AT3G58850), a transcription factor induced during the shade avoidance response [92]. Due to a lack of available microarray data, we did not further explore the expression profiles or functions of these genes. However, we confirmed auxin-responsiveness of PAR2 as well as of CTR1 (AT5G03730) and BRIL (AT1G55610) (new candidate growth genes that are not represented on the ATH1 chip) in seedlings using qRT-PCR (Fig. S5).

Concluding Remarks

Our hypocotyl sampling approach enabled us to detect auxin-responsive growth-associated genes that have not been detected...
Table 1. Microarray data selected for comparison to auxin-regulated gene sets.

| Reference | GEO ID | Conditions analyzed |
|-----------|--------|---------------------|
| Leivar et al. 2009 | GSE17159 | wild-type vs. pifq (pif1 pif2 pif3 pif4 pif5 mutant) 2 day dark |
| | | wild-type vs. pifq 2 day dark plus 1 hr red light |
| | | wild-type 2 day dark treatment vs. wild-type 2 day red light |
| | | wild-type seed vs. wild-type 2 day dark |
| Nozue et al. 2011 | GSE21684 | UpG PIF4/5 - Genes up in growth phase and PIF4/5 Dependent |
| | | UpG - Genes up in stationary phase and PIF4/5 independent |
| | | upS PIF4/5 - Genes up in stationary phase and PIF4/5 dependent |
| | | upS - Genes up in stationary phase and PIF4/5 independent |
| Ma et al. 2005 | GSE14648 | 6 day old light grown hypocotyls |
| | | 6 day old dark grown hypocotyls |

doi:10.1371/journal.pone.0036210.t001

in many whole seedling arrays. It is possible that the large number of genes in our auxin-responsive lists that were not found in the MASTA analysis or the comparison with the Nemhauser et al. [61] and Stepanova et al. [62] datasets represent genes that are auxin-responsive in a specific spatio-temporal pattern that is masked in experimental designs using diverse tissue homogenates. Results from this study emphasize the value of tissue-specific analyses when addressing a particular developmental question. We have uncovered a large set of auxin-regulated genes that are expressed in elongating hypocotyls, including several GA biosynthesis enzymes (Fig. 7). Our results suggest that auxin regulates GA biosynthesis to release DELLA-dependent growth repression [37]. Genetic analyses confirmed the importance of auxin-GA cross-talk for a complete hypocotyl growth response, a process that has also been reported in pea [93]. However, we also demonstrated that regulation of GA is not the only mechanism for auxin-stimulated hypocotyl growth and an independent pathway is required for optimal response. Interestingly, auxin-GA interplay is also involved in tropic hypocotyl growth, although in these processes GA is required to attenuate growth through repression of auxin signaling [94]. It will be important for a complete understanding of hormone-regulated growth to assign downstream growth genes to specific hormone pathways or identify mechanisms and conditions in which these genes are downstream of multiple signaling pathways, as we have proposed for LLA2, ARGOS, and other genes.

Under normal growth conditions, the circadian clock maintains diurnal hypocotyl growth by gating auxin response primarily through PIF4 and PIF5 [39,41,42]. However, various stress conditions cause plants to stimulate rapid changes in growth during the day in order to survive. For example, rapid flooding causes changes in hormone levels within 1 h of submergence. Studies in Rumex palustris have revealed the importance of ethylene, IAA and GA in stimulating stem elongation following submergence due to rapid flooding [95]. Our hypocotyl transcriptional analysis was performed under conditions that mimick a rapid increase in auxin levels during the day leading to a hypocotyl elongation response. We have identified many cell elongation genes that are known to be growth-associated but have not been previously described as auxin-responsive (Fig. 7). A subset of these genes is described as being PIF4/5-dependent and we would expect this regulation to be active during normal hypocotyl growth conditions during the night when the PIFs are present [39,42]. However, our results suggest that auxin activates these genes in the absence of PIF4/5 suggesting that auxin promotes hypocotyl growth by an independent pathway during the day. It will be interesting to determine how the activities and expression patterns of these genes are important for the extent and timing of hypocotyl growth. Among our auxin-responsive gene list are genes involved in cell wall biogenesis and secretory pathways known to be important for cell elongation. Using the hypocotyl tissue-specific approach and the NimbleChip, we have also uncovered additional hypocotyl growth genes that may also be important for other cell expansion dependent processes such as petiole growth. With this study we present a transcriptional framework for rapid stimulation of hypocotyl elongation during the day, independent of PIF4 and PIF5, and we provide a genomic basis for the model that auxin, GA, and the PIFs have overlapping roles in regulating growth.

Materials and Methods

Plant Material

Arabidopsis thaliana mutants and transgenic lines used in this study were all in the Columbia (Col-0) ecotype. Mutants nug2-1 [48,96], sb-1 [49], and pif4-101/pif5-1 [36] were described previously. ttr1-1, agh1-3, agh2-3, agh3-4, agh4-2, agh5-5 and higher-order combinations among these mutants were described previously [27,97]. RGA::GFP-RGA (CS16360) was obtained from the Arabidopsis Biological Resource Center at The Ohio State University, and the ga20ox1ox2 mutant [98] was a generous gift from Peter Hedden. For hormone treatment assays and RNA isolations, seeds were plated on ½ Murashige-Skoog medium containing 1% sucrose and 1% agar, and stratified 2–4 days in the dark at 4°C.

Hypocotyl Growth Assays and Imaging

Seedlings were grown under long day photoperiods (16 h light/8 h dark) at 23°C unless otherwise indicated, with white light intensity of ~80 μmol/m²/s. For treatment assays and RNA isolations, 5-day-old seedlings were transferred to plates containing the chemical being tested or the solvent control (DMSO was used for picloram; ethanol was used for IAA, GA3, paclobutrazol and NPA) for an additional 48 hours unless otherwise stated. Hypocotyl images were taken using a Nikon SMZ1500 dissecting scope and all measurements were done using ImageJ software. Data shown represent an average of at least 10 seedlings per treatment; error bars represent standard error.
Seedlings used for visualization of RGA-GFP were grown in long day conditions, and RGA-GFP fluorescence at time zero was analyzed four hours after subjective dawn in two-day-old seedlings. Seedlings were then submerged in liquid medium (½× Murashige-Skoog medium containing 1% sucrose) containing the chemical being tested or the solvent control (ethanol) for an additional 2–24 hours, and a subset of seedlings was removed from treatment and imaged at the time points indicated. GFP fluorescence in pRGA:RGA-GFP was visualized using a Nikon SMZ1500 dissecting scope.

Transcriptome Experiments

Microarray “a”.
Stratified seeds were plated on medium overlaid with sterilized nylon mesh (110 micron pore size; www.smallparts.com). Two hours after chamber lights came on, mesh rafts containing 5-day-old seedlings were transferred to medium containing 5 μM picloram or an equivalent volume of DMSO for 30 min or two hours. Hypocotyls were dissected over a 30-minute period and frozen in liquid nitrogen. Tissue samples were collected over several days and pooled into biological replicates containing at least 400 hypocotyls. RNA extractions were done using Trizol reagent (Sigma) followed by additional phenol extraction and ethanol precipitation steps. mRNA was amplified using the MessageAmp II aRNA Amplification kit (Ambion) and the manufacturer’s protocol. Labeled cDNA was prepared from aRNA using the Superscript ds cDNA synthesis kit (Invitrogen), Cy3- and Cy5-labeled random nonomers (TriLink) and Klenow fragment (Promega). Samples representing three biological replicates were hybridized to the 12-plex NimbleGen chip according to manufacturer’s instructions. Experiment ‘b’ was hybridized to the NimbleGen 12-plex chip using single-color labeling. Microarray ‘b’ was carried out at the GeneChip™ Microarray Core facility at the University of California San Diego.

Transcriptome Analysis

All microarray analysis was done using R (R Development Core Team (2011), http://www.R-project.org/) and Bioconductor [99]. Microarray ‘a’ was annotated based on the TAIR8 version and ‘b’ was annotated based on TAIR10. Annotation packages were built with pdlInfoBuilder using raw data files (.xys) along with a Nimblegen microarray design file (.ndf). All microarrays were RMA normalized using oligo in R with this annotation package. Normalized data for array ‘a’ and ‘b’ were analyzed independently using a linear model method [56] performed in the LIMMA package in R. Differentially expressed genes were chosen based on an Empirical Bayes method and an FDR of less than 0.05. To identify a statistically significant list of differentially expressed genes from microarray ‘a’ and ‘b’, Rank Product method was used due to the difficulty comparing datasets derived from independent experiments [58]. As shown in Vert et al. [58], this method [60,99] often outperforms the linear model when comparing microarray experiments derived from different laboratories. There are several advantages to this method, including the use of pre-processed data, eliminating the requirement for normalizing heterogeneous data together that will often retain ‘lab-effects’ [58]. The Rank Product method includes fewer assumptions under the model, accounts for multiple sources of datasets and performs better with noisy data or a low number of replicates. The expression values are transformed into ranks allowing for the integration of datasets from a variety of platforms [59,60]. Genes or splice forms that were not present on both chips were removed from the analysis. Upregulated and downregulated gene lists from RankProd were used for the comparisons described below. Microarray data have been deposited in NCBI’s Gene Expression Omnibus [100] and are accessible through GEO Series accession number GSE37217 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37217).

Array Comparisons

The MASTA package available from the BAR website (www.bar.utoronto.ca) was used to compare RankProd-generated lists with the 7 IAA wild-type treatment arrays included in the MASTA
package. The IAA root treatment data from Stepanova et al. [62] was downloaded from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/). CEL files were RNA normalized using the Affymetrix package and input into RankProd. Differentially expressed genes with an FDR less than 0.05 were selected and compared to the picloram-responsive gene lists. For the Nemhauser et al. [61] comparisons the genes defined by the authors as auxin-responsive were used.

Microarrays selected for the growth gene comparisons are listed in Table 1, Tables S6 and S7. CEL files were not available for all of the arrays selected; picloram-responsive genes identified in this study were compared with genes defined as differentially expressed according to the publication associated with the data in GEO. Matrices were generated with the picloram-induced and – repressed genes in which each row represents an auxin-responsive gene and each column represents a treatment condition from the array being compared. Genes were assigned a value of 1 if defined as differentially expressed in the associated publication, or a value of 0 if absent from the data set. The resulting matrix was used to generate a hierarchical clustered based map in R. Columns were manually arranged based on conditions where growth is occurring (growth phase) or inhibited (stationary phase). The middle column in each map (cw) includes genes that were defined by Jamet et al. [79] as being involved in cell wall biogenesis or secretory pathways likely important for cell wall expansion.

Quantitative RT-PCR
RNA samples collected from hypocotyl and whole seedling tissue were obtained from tissue frozen in liquid N2 using the INVITROGEN PureLink RNA minikit. RNA yield and quality was quantified using the Thermo Scientific NanoDrop 2000. Equal amounts of RNA were pre-treated with DNase using the DNA-free Kit (Ambion) according to manufacturer’s instructions and used to generate cDNA with SuperScript III First-Strand Synthesis [Invitrogen] with 20-mer oligo(dT) primers. Quantitative RT-PCR was done with SyBR green and the primers listed in Table S8. Primer pairs were evaluated for specificity and efficiency using three serial dilutions of cDNA using the CFX96™ Real-Time PCR Detection System (Biorad). Data were normalized to the reference gene PP2AA3 [101] according to the ΔΔCt method [102]. Primers were designed using QuantPrime [103]. Experiments with hypocotyl or seedling tissue were done with two biological replicates and three technical replicates.

Supporting Information
Figure S1 Picloram and IAA share transcriptional targets. (A) Microarray analysis of picloram-regulated genes in hypocotyls and IAA-regulated genes in seedlings or roots identified common target genes. Venn diagrams of auxin-upregulated genes in IAA-treated materials (IAA-up) and hypocotyls of picloram-treated seedlings (Pic-up) are shown. Numbers of genes identified in common are shown in the overlap sections of each diagram. (B) Auxin marker genes are picloram-responsive in hypocotyls from wild-type, but not afb5-5 mutant, seedlings. Hierarchical clustering result of IAA marker gene expression in hypocotyls of picloram-treated or control wild-type (Col-0) or afb5-5 (afb5) seedlings, as determined by analysis of microarray data using ArrayStar, is shown. (C) IAA marker genes are regulated by picloram in hypocotyls of picloram-treated seedlings. Wild-type (Col-0) or afb5-5 mutant seedlings were treated with picloram or a solvent control for 2 hours and used for hypocotyl dissection and RNA isolation. Expression value of each gene shown, relative to a control gene, was determined by qRT-PCR.

Figure S2 Auxin response elements are overrepresented in picloram-responsive promoters. Statistical significance of overrepresentation of each AuxRE-containing sequence element (p-value) is plotted on the x-axis; the number of promoters containing the element is plotted on the y-axis. Overrepresented sequences were identified using ELEMENT [105].

Figure S3 GO terms newly associated with auxin-responsive transcription. Overrepresented GO terms and enrichment scores were identified using GOMiner [106]. Only GO terms not overrepresented in the AtGenExpress datasets [61] are shown.

Figure S4 GA and auxin act independently and interdependently to regulate hypocotyl elongation. (A) Auxin signaling mutants are partially restored by treatment with GA3. Average hypocotyl length of wild-type seedlings or the indicated mutants grown in long days and treated with 50 μM GA3 was determined following 48 hours of treatment. Statistical significance was determined using a Tukey HSD post hoc comparison among the means on the analysis of variance using type III sums of squares (p<0.05). Error bars indicate standard error. (b) The axr2-1 and ga20ox2 double mutant are deficient in transient auxin response. Average hypocotyl length of wild-type and mutant seedlings treated with 5 μM IAA for two hours was measured each hour for 7 hours. Hypocotyl length at each time point is shown as a percentage of length at time 0. Error bars indicate standard error.

Figure S5 PIF4/5-independent genes are regulated by auxin in seedlings. Wild-type seedlings were treated with IAA or a solvent control for 2 hours and used for RNA isolation. Expression value of each gene shown, relative to a control gene, was determined by qRT-PCR.

Table S1 Microarray experimental design.
Table S2 Genes auxin-responsive at 30 minutes.
Table S3 Genes auxin-responsive at 120 minutes.
Table S4 Newly identified auxin-responsive genes.
Table S5 GO terms associated with auxin-responsive gene lists.
Table S6 Genes and microarray datasets presented in Figure 7B, auxin-induced genes.
Table S7 Genes and microarray datasets presented in Figure 7B, auxin-repressed genes.
Table S8 Primer sequences used for quantitative RT-PCR.
Acknowledgments

Colleen Doherty assisted with microarray analysis. Mon-Ray Shao, Amanda Budiman, Tatiana Manchenkov and Britta Baynes assisted with plant propagation and provided technical assistance. Stuart Grande and Zak Gezon assisted with the statistical analysis. Jason Reed, members of the Estelle lab particularly Luz Irina A. Calderon Villalobos, and anonymous reviewers provided helpful comments on early drafts of the manuscript.

Author Contributions

Conceived and designed the experiments: EJC KG CC ME. Performed the experiments: EJC KG CC RS AB. Analyzed the data: EJC KG CC RS AB. Contributed reagents/materials/analysis tools: T-PS. Wrote the paper: EJC KG CC ME.

References

1. Davies P (2004) The Plant Hormones: Their Nature, Occurrence, and Functions. In: Davies P, ed. Plant Hormones: Biosynthesis, Signal Transduction, Action, and Function. Kluwer Academic Publishers. pp. 1–15.
2. Chapman EJ, Estelle M (2009) Mechanism of auxin-regulated gene expression in plants. Annu Rev Genet 43: 263–285.
3. Leye O (2010) The power of auxin in plants. Plant Physiol 154: 501–505.
4. Tesoriero P, Roche-Rechmann C (2010) Recent progress in auxin biology. C R Biol 333: 297–306.
5. Hagen G, Guillefoyle T (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol 49: 373–385.
6. Overvoorde PJ, Okushima Y, Alonso JM, Chan A, Chang C, et al. (2005) Functional genomic analysis of the AUX/INDOLE-3-ACETIC ACID gene family members in Arabidopsis thaliana. The Plant Cell 17: 3292–3300.
7. Ulmasov T, Hagen G, Guillefoyle TJ (1995) Composite structure of auxin response elements. Plant Cell 7: 1611–1623.
8. Nemhauser JL, Mockler TC, Chory J (2004) Interdependence of brassinosteroid and auxin signaling in Arabidopsis. PLoS Biol 2: E258.
9. Müller P, Koller D (2005) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature 435: 1094–1097.
10. Reed JW (2003) Roles and activities of Aux/IAA proteins in Arabidopsis. Trends Plant Sci 8: 420–425.
11. Bertl F, Krogan NT, Scarpella E (2004) Auxin-signaling-turning genes on and turning cells around. Curr Opin Plant Biol 7: 553–563.
12. Abel S, Oeller PW, Theologis A (1994) Early auxin-induced genes encode short-lived nuclear proteins. Proc Natl Acad Sci U S A 91: 326–330.
13. Kim J, Hater K, Theologis A (1997) Protein-protein interactions among the Aux/IAA proteins. Proc Natl Acad Sci U S A 94: 11786–11791.
14. Ramos JA, Zenser N, Leyser O, Callis J (2001) Rapid degradation of auxin- insensitive auxin receptor proteins requires conserved amino acids of domain II and is proteasome dependent. Plant Cell 13: 2349–2360.
15. Scott TK (1972) Auxins and Roots. Annu Rev Plant Physiol 23: 235–258.
16. Gendreau E, Traas J, Desnos T, Grandjean O, Calboche M, et al. (1997) Cellulose basis of hypocotyl growth in Arabidopsis thaliana. Plant Physiol 114: 295–305.
17. Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, et al. (2002) Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450 CYP79B2 and CYP79B3. Genes Dev 16: 295–304.
18. Gray WM, Ostin A, Sandberg G, Romano CP, Estelle M (1998) High temperature promotes auxin-mediated hypocotyl elongation in Arabidopsis. Proc Natl Acad Sci U S A 95: 7179–7202.
19. Scott TK (1972) Auxins and Roots. Annu Rev Plant Physiol 23: 235–258.
20. Kepinski S, Leyser O (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature 435: 441–445.
21. Okushima Y, Fukaki H, Onoda M, Theologis A, Tasaka M (2007) ARF7 and ARF19 regulate lateral root formation via direct activation of LBD/ASL genes. Nature 453: 1094–1097.
22. Tian CE, Muto H, Higuchi K, Matamura T, Tatematsu K, et al. (2004) TERRITORY-OF-REPRESENTATIVE2 encodes Aux/IAA19, an auxin-regulated protein that functions as a negative regulator of auxin signaling in seedlings. Dev Cell 9: 109–119.
23. Harper RM, Stowe-Evans EL, Luesse DR, Muto H, Tatematsu K, et al. (2000) TOPE2 mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science 319: 1384–1386.
24. Kepinski S, Leyser O (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature 435: 441–445.
25. Dharmsiriporn N, Dharmsiriporn S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. Nature 435: 441–445.
26. Dharmsiriporn N, Dharmsiriporn S, Weijers D, Lechner E, Yamada M, et al. (2005) Plant development is regulated by a family of auxin receptor F-box proteins. Dev Cell 9: 109–119.
27. Goda H, Sinha P, Agarwal A, Parniske M, et al. (2010) A novel aux/IAA28 signaling cascade activates GAT3-GAT2-dependent specification of lateral root founder cell identity. Curr Biol 20: 1679–1706.
72. Wilson AK, Pickett FB, Turner JC, Estelle M (1999) A dominant mutation in Arabidopsis confers resistance to auxin, ethylene and abscisic acid. Mol Gen Genet 262: 377–383.

73. Sc-Ammour A, Windels D, Aru-Boutilieres E, Kutter C, Alhaja J, et al. (2011) miR159 and secondary siRNAs regulate expression of the TRB1/AFB2 auxin receptor clade and auxin-related development of Arabidopsis leaves. Plant physiology 157: 683–691.

74. Dowson-Day MJ, Millar AJ (1999) Circadian dysfunction causes aberrant hyponastic elongation patterns in Arabidopsis. Plant J 17: 63–71.

75. Walsh TA, Neal R, Merlo AO, Houna M, Hicks GR, et al. (2006) Mutations in an auxin receptor homolog AFBS and in SGT1b confer resistance to synthetic picoximate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid in Arabidopsis. Plant Physiol 142: 542–552.

76. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article3.

77. Papoušek IA, Paunov M, Teale W, Menges M, Cházkaborotsev S, et al. (2008) Comprehensive transcriptional analysis of auxin responses in Arabidopsis. Mol Plant 1: 321–337.

78. Vert G, Nemhauser JL, Geldner N, Hong F, Chory J (2005) Molecular mechanisms of steroid hormone signaling in plants. Annu Rev Cell Dev Biol 21: 177–203.

79. Hong F, Breitling R, McEntee CW, Wittner BS, Nemhauser JL, et al. (2006) RankProd: a bioconductor package for detecting differentially expressed genes in multiple levels. J Exp Bot 57: 83–92.

80. Nemhauser JL, Hong F, Chory J (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell 126: 467–475.

81. Stepanova AN, Yun J, Likhacheva AV, Alonso JM (2003) Arabidopsis ARR proteins function in auxin signaling and transport. Proc Natl Acad Sci U S A 100: 21893–21898.

82. Michael TP, Breton G, Hazen SP, Priest H, Mockler TC, et al. (2008) A morning-specific phytohormone gene expression program underlying rhythmic plant growth. PLoS Biol 6: e222.

83. Covington MF, Harmer SL (2007) The circadian clock regulates auxin signaling and responses in Arabidopsis. PLoS Biol 5: e212.

84. Desgagne-Penix I, Sposato VM (2008) Expression of gibberellin 20-oxidase1 (Ag20ox1) in Arabidopsis seedlings with altered auxin status is regulated at multiple levels. J Exp Bot 59: 2057–2070.

85. Frigerio M, Alabadi D, Perez-Gomez J, Garcia-Carcel L, Phillips AL, et al. (2006) Transcriptional regulation of gibberellin metabolism genes by auxin signaling in Arabidopsis. Plant Physiol 142: 533–563.

86. O'Neill DP, Davidson SE, Clarke VC, Yamauchi Y, Yamaguchi S, et al. (2010) Regulation of the gibberellin pathway by auxin and DELLA proteins. Planta 231: 1451–1463.

87. Stepanova AN, Yun J, Likhacheva AV, Alonso JM (2007) Multilevel interactions between ethylene and auxin in Arabidopsis roots. Plant Cell 19: 2189–2198.

88. Hong F, Breitling R, McEntee CW, Wittner BS, Nemhauser JL, et al. (2006) Simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett 575: 83–92.

89. Ross JJ, O'Neill DP, Wolbang CM, Symons GM, Reid JB (2001) Auxin-induced degradation of RGA. Proc Natl Acad Sci U S A 98: 14162–14167.

90. Stepanova AN, Yun J, Likhacheva AV, Alonso JM (2007) Multilevel interactions between ethylene and auxin in Arabidopsis roots. Plant Cell 19: 2189–2198.

91. Veech MJ, Blackhall A, Fei J, Shi Q, Fankhauser C, et al. (2008) Interaction of shade avoidance and auxin responses: a role for two novel atypical bHLH proteins. EMBO J 27: 4766–4776.

92. Ross J, O’Neill DP, Smith J, Kerrick DSJ, Elliot RC (2000) Evidence that auxin promotes gibberellin A1 biosynthesis in pea. Plant J 21: 547–552.

93. Gallego-Bartolome J, Kami C, Fankhauser C, Alabadi D, Blazquez MA (2011) A hormonal regulatory module that provides flexibility to tropic responses. Plant J 65: 1019–1025.

94. Gallego-Bartolome J, Kami C, Fankhauser C, Alabadi D, Blazquez MA (2011) A hormonal regulatory module that provides flexibility to tropic responses. Plant J 65: 1019–1025.

95. Voesenek LA, Benschop JJ, Bou J, Cox MC, Groeneveld HW, et al. (2003) Interactions between plant hormones regulate submergence-induced shoot elongation in the flood-tolerant dicot Rumex palustris. Ann Bot 91 Spec No pp 205–211.

96. Watahiki MK, Yamamoto KT (1997) The massuggle mutation of Arabidopsis identified with failure of auxin-induced growth curvature of hypocotyl confers auxin insensitivity to hypocotyl and leaf. Plant Physiol 115: 419–426.

97. Parry G, Calderon-Villalobos L, Priegu M, Peret B, Dharmasiri S, et al. (2009) Complex regulation of the TRB1/AFB family of auxin receptors. Proc Natl Acad Sci U S A 106: 22540–22545.

98. Rieu I, Ruiz-Rivero O, Fernandez-Garcia N, Griffiths J, Powers SJ, et al. (2008) The gibberellin biosynthetic genes Ag1-20ox1 and Ag-20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. Plant J 53: 488–504.

99. Gentlemen RC, Carey VJ, Bates DM, Bolstad B, Deettling M, et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome 5: R80–R86.

100. Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI resource for biological interpretation of genomic and proteomic data. Genome 42: 14–17.

101. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Transcriptomic complements proteomic data. BMC Genomics 6: R10.

102. Li Y, Jones L, McQueen-Mason S (2003) Expansin and cell growth. Curr Opin Plant Biol 6: 603–610.

103. Cosgrove DJ, Li LC, Cho HT, Hoffmann-Benning S, Moore RC, et al. (2002) The growing world of expansins. Plant Cell 14: 1436–1444.

104. Abel S, Nguyen MD, Theologis A (1995) The PI-II/Avs-like family of early auxin-inducible mRNAs in Arabidopsis thaliana. J Mol Biol 251: 531–549.

105. Mockler TC, Michael TP, Priest HD, Shen R, Sullivan CM, et al. (2007) The DIURNAL project: DIURNAL and circadian expression profiling, model-based pattern matching, and pegaerror analysis. Cold Spring Harb Symp Quant Biol 72: 353–363.

106. Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, et al. (2003) GoMiner: a bioconductor package for detecting differentially expressed genes in microarray experiments. Genome 46: 17–24.

107. Gavin A, Hooper D, Bergmann C, Furniture G, Li B, et al. (2003) The growing world of expansins. Plant Cell 15: 1951–1961.

108. Hu Y, Poh HM, Chua NH (2006) The Arabidopsis ARGOSS-LIKE gene regulates cell expansion of organs in growth. Plant 57: 1–9.

109. Lee YK, Kim GT, Kim J, Park J, Kwak SS, et al. (2006) LONGIFOLIA1 and LONGIFOLIA2, two homologous genes, regulate longitudinal cell elongation in Arabidopsis Development. 133: 4305–4314.

110. Lee DK, Ahn JK, Song NK, Choi YD, Lee JS (2003) Expression of an expansin gene is correlated with root elongation in soybean. Plant Physiol 131: 985–997.

111. Li Y, Jones L, McQueen-Mason S (2003) Expansin and cell growth. Curr Opin Plant Biol 6: 603–610.

112. Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, et al. (2003) GoMiner: a bioconductor package for detecting differentially expressed genes in microarray experiments. Genome 46: 17–24.

113. Prufer K, Holland P, Huber T, Senn H, Hofacker I, et al. (2005) The growing world of expansins. Plant Cell 15: 1951–1961.