A Kinetic Analysis of the Auxin Transcriptome Reveals Cell Wall Remodeling Proteins That Modulate Lateral Root Development in Arabidopsis

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To identify gene products that participate in auxin-dependent lateral root formation, a high temporal resolution, genome-wide transcript abundance analysis was performed with auxin-treated Arabidopsis thaliana roots. Data analysis identified 1246 transcripts that were consistently regulated by indole-3-acetic acid (IAA), partitioning into 60 clusters with distinct response kinetics. We identified rapidly induced clusters containing auxin-response functional annotations and clusters exhibiting delayed induction linked to cell division temporally correlated with lateral root induction. Several clusters were enriched with genes encoding proteins involved in cell wall modification, opening the possibility for understanding mechanistic details of cell structural changes that result in root formation following auxin treatment. Mutants with insertions in 72 genes annotated with a cell wall remodeling function were examined for alterations in IAA-regulated root growth and development. This reverse-genetic screen yielded eight mutants with root phenotypes. Detailed characterization of seedlings with mutations in CELLULASE3/GLYCOSYLHYDROLASE9B3 and LEUCINE RICH EXTENSIN2, genes not normally linked to auxin response, revealed defects in the early and late stages of lateral root development, respectively. The genes identified here using kinetic insight into expression changes lay the foundation for mechanistic understanding of auxin-mediated cell wall remodeling as an essential feature of lateral root development.

INTRODUCTION

The plant hormone auxin regulates primary root elongation and gravitropism, as well as the initiation, emergence, and elongation of lateral roots (Woodward and Bartel, 2005; Overvoorde et al., 2010). Activation of the signaling pathways that mediate these responses causes profound and rapid increases in gene expression in well characterized gene families, including AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA), GRETCHEN HAGEN3 (GH3), and SMALL AUXIN UP-REGULATED (SAUR) families (Chapman and Estelle, 2009). With the advent of genome-wide transcriptional profiling, additional rapidly regulated auxin-dependent transcripts have been identified (Paponov et al., 2008). Genetic approaches have provided insight into the function of proteins that mediate auxin signaling and transcriptional responses. Auxin binds to the TRANSPORT INHIBITOR RESPONSE1 (TIR1) receptor or another auxin F-box (AFB) protein, which are E3-ubiquitin ligases that add ubiquitin tags to AUX/IAA transcriptional repressor proteins (Chapman and Estelle, 2009). This ubiquitination targets AUX/IAA proteins for proteolytic destruction (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Destruction of AUX/IAA repressors releases auxin response factors, which act as transcription factors that mediate auxin-dependent transcription (Abel et al., 1995; Ulmasov et al., 1999; Shin et al., 2007). Mutations in genes encoding auxin response factors and TIR1/AFBs alter auxin responses, as do mutations that stabilize AUX/IAA proteins, preventing their proteolytic destruction (Dharmasiri et al., 2005b; Lee et al., 2009). The core auxin transcriptional response machinery drives the synthesis of proteins that modulate auxin dependent growth processes.

Few well-characterized downstream targets of auxin transcriptional machinery that mediate the events leading to auxin-dependent changes in growth and development have been identified. One set of targets of auxin-induced gene expression are auxin transport proteins that play essential roles in development (Zazimalova et al., 2010). Mutations in genes encoding auxin transport proteins including PIN-FORMED auxin efflux carriers, AUXIN INSENSITIVE/LIKE AUX1 influx carriers, and ATP BINDING CASSETTE TYPE B efflux carriers lead to profound developmental and growth defects (Zazimalova et al., 2010). The transcription of these genes is positively regulated by auxin, contributing to auxin-dependent increases in auxin transport (Noh et al., 2001; Vieten et al., 2005; Laskowski et al., 2008; Lewis et al., 2011a; Peer et al., 2011). Auxin treatment increases flavonol accumulation via transcription of genes encoding flavonoid biosynthetic enzymes, which in turn modulates auxin transport.
dependent processes, such as gravitropism (Lewis et al., 2011b). Molecular targets of auxin that drive cell division leading to lateral root initiation have been identified, including the transcription factors E2Fa (Berkman et al., 2011), PLETHORA1 (Bilou et al., 2005), and GATA23 (De Rybel et al., 2010). In addition, transcriptional changes linked to auxin’s effect on transcripts encoding cell wall enzymes have been reported (Laskowski et al., 2006; Swarup et al., 2008).

Other downstream targets of auxin-regulated transcription include gene products that function to modulate the properties of the cell wall (Catala et al., 1997; Esmon et al., 2006; Laskowski et al., 2006; Swarup et al., 2008). The cell wall is a dynamic structure that is constantly remodeled to adjust its biomechanical properties, facilitating development and the response to endogenous and exogenous stimuli, including auxin (Cosgrove, 2005; Sanchez-Rodriguez et al., 2010). The cell wall is a reinforced semicrystalline structure composed of three major types of polysaccharides (Carpita and Gibeaut, 1993). Cellulose is the major structural component of the cell wall, while hemicelluloses anchor the cellulose microfibrils to each other and to additional components of the cell wall matrix. The pectin polysaccharide forms a rigid gel in which the microfibrils and hemicelluloses are embedded (Reiter, 2002). According to the acid growth hypothesis, auxin causes cell wall loosening by stimulating the pumping of protons into the cell wall, allowing the cell wall carbohydrate polymers to move relative to each other (Perrot-Rechenmann, 2010). Additionally, auxin may modulate the cell wall structure by transcription of genes encoding cell wall remodeling proteins. Two expansions increase in abundance on the lower side of hypocotyls responding to gravity, where auxin levels are elevated (Esmon et al., 2006). Local auxin accumulation at the point of lateral root emergence enhances accumulation of the GLYCO-SIDE HYDROLASE17 transcript, which is predicted to encode an enzyme that cleaves (1-3)-β-D-glucans (hemicellulose) during lateral root emergence (Swarup et al., 2008).

Our goal was to identify mediators of auxin-dependent growth and developmental changes in Arabidopsis thaliana by examining genome-wide transcript changes in response to auxin over a detailed and physiologically relevant time course. To go beyond previously published studies that focused on rapid and primary auxin response genes, we examined global transcript abundance changes for 24 h following indole-3-acetic acid (IAA) stimulation. Presumably, changes in transcript abundance of transcription factors that regulate downstream target genes would precede expression changes in genes whose proteins regulate or mediate the biochemical reactions that cause the auxin response. This is especially true for lateral root development, which exhibits a 4- to 8-h lag after IAA treatment before lateral root initiation and emergence begin. Therefore, the time courses of growth and developmental changes in response to treatment with auxin were examined in parallel to better place the transcriptional changes in a physiological context. We found that clusters of transcripts differentially regulated later in the time course were enriched with genes linked to cell division, lateral root development, and cell wall–remodeling enzymes.

We conducted an unbiased reverse genetic screen to determine if these downstream target genes linked to cell wall synthesis or remodeling were necessary for auxin-dependent root growth and development. We identify eight insertion lines with root growth or developmental phenotypes and provide a detailed characterization of mutants in two genes, CELULLASE3/GLY-COSYLHYDROLASEB3 (CEL3/GH8B3) and LEUCINE RICH REPEAT EXTENSIN (LRX2), which have altered initiation and emergence of lateral roots, respectively. This kinetic information is used to create and test informed hypotheses about the function of cell wall remodeling–enzymes that mediate auxin-dependent growth and developmental responses. These experiments led to the identification of mediators of auxin-dependent processes.

RESULTS

Time Course of Growth, Development, and Gene Expression Responses to IAA

To define the timeline for physiological responses and gene expression changes in response to IAA treatment, root elongation, lateral root initiation, and an auxin-responsive green fluorescent protein (GFP) reporter were examined over a 24-h time course. We used a dose of 1 μM IAA for these experiments to ensure there were robust transcriptional and developmental responses. This dose is lower than the fivefold to 10-fold higher doses used in several microarray experiments (Okushima et al., 2005b; Overvoorde et al., 2005; Vanneste et al., 2005). A high-resolution morphometric analysis showed a rapid and nearly complete inhibition of elongation growth that became significantly different from untreated control 30 min after treatment with 1 μM IAA (P < 0.05) (see Supplemental Figure 1A online). The formation of lateral root primordia in the presence and absence of IAA was also determined by microscopy observation of roots expressing the auxin-responsive DR5 promoter fused to GFP (DR5:GFP), which is expressed in the earliest stages of lateral root initiation. The IAA-dependent increase in lateral root initiation is evident by 1 h but becomes statistically significant by 8 h (P < 0.05) (see Supplemental Figure 1B online). The distribution of the developmental stages of lateral root primordia for 24 h after IAA treatment was examined using previously defined developmental stages (Malamy and Benfey, 1997). The number of early stage primordia (stages 1 to 3) remained roughly constant over the first 12 h of auxin treatment, while the number of stage 4 and later stage primordia steadily accumulated throughout the time course (Figure 1A). Root tip swelling and excess root hair production were also observed within 4 h after exogenous IAA treatment. This analysis indicates that IAA-dependent increases in initiation of lateral roots and root hairs become evident as early as 4 to 8 h after IAA treatment.

We also examined the fluorescence of the DR5:GFP auxin-responsive reporter along the entire root. A confocal microscope was used in tile scanning mode to capture an image of the whole root and to provide high-resolution images of the root tip and individual lateral root primordia (Figure 1B). Fluorescence intensity changes were quantified in two regions: the elongation/meristematic zone and the zone of lateral root formation (Figure 1C). In the meristematic and elongation zones at the root apex, DR5:GFP fluorescence was significantly increased relative to control at each time point beginning at 1 h after treatment (P < 0.05) and
Figure 1. IAA Increases DR5:GFP Fluorescence and Lateral Root Development.

(A) The abundance of each stage of lateral root development was quantified and reported at each time after IAA treatment, as well as in 0- and 24-h control samples.

(B) Confocal laser scanning microscopy images of DR5:GFP fluorescence after IAA treatment are compared with 0- and 24-h controls. Representative images out of seven roots imaged at each time point are shown. Tile scan micrographs of the entire root (middle) as well as high-magnification images of the most mature lateral root primordia (top) and root tip are shown (bottom). Roots were counterstained with propidium iodide (gray) to show cell outlines. The arrowheads mark the position of lateral root primordia. Bar for lateral root and root tip images = 100 µm. Bar for whole root images = 1 mm.

(C) The intensity of the GFP signal was determined in the primary root tip and mature zone (5 mm from root apex). For (A) and (B), the average and SE from more than six roots per time point are shown.
reached a maximum 10-fold increase over control at 12 h. By contrast, DR5:GFP fluorescence in the lateral root forming zone, located ~5 mm from the root apex, was significantly elevated 0.5 h after auxin treatment (P < 0.05) and was 13-fold elevated at both 12 and 24 h after treatment. The quantified fluorescence of roots 24 h after mock treatment was not significantly different from time 0 (P > 0.6), suggesting that the baseline auxin signaling activity is stable over the time course.

**Auxin Treatment Induces a Burst of Transcriptional Activity**

The physiological and auxin-responsive reporter expression results were used to set the duration and time resolution of the transcriptome analysis. To identify gene expression events temporally linked to rapid responses, like growth inhibition, and slowly induced responses, such as increases in lateral root initiation and emergence, we chose eight time points spanning 24 h, concentrated within the first 4 h after treatment and becoming more distantly separated as the experiment progressed. Seedlings were transferred to media with and without 1 μM IAA and, at the indicated times, root samples were used for RNA isolation and microarray analysis. Each of three replicates was analyzed individually using a stringent filtering approach that considers the statistical significance of the signal and defines a minimal change relative to controls. A distinctive feature of this analysis that is specific to detailed time profiles is the requirement of consistent magnitude and pattern of change across the replicates and over the complete time course (Olex et al., 2010). This differential expression (DE) filtering analysis (Olex and Fetrow, 2011) (outlined in Supplemental Figure 2 online) identified significant and reproducible transcript abundance changes in other time-course microarray experiments (Olex et al., 2010).

Application of the DE filtering process identified 1246 consistently and significantly IAA-responsive transcripts (see Supplemental Data Set 1 online). A global view of the auxin-induced transcriptional changes is shown in the distribution of the SLR (signal log ratio of treatment over control using log base 2) for these 1246 transcripts at each time point (Figure 2A). At the 0-h time point, the vast majority of transcripts exhibit an SLR close to 0, which is consistent with the expected similarity in the 0-h control and IAA samples. At the early time points of 0.5 and 1 h, the peak is still centered on an SLR of 0, but the peak height decreases while the distribution width increases. During the 2-, 4-, and 8-h time points, a bimodal distribution is observed, indicating that some transcripts decrease and some increase relative to control. By 12 and 24 h, most transcripts return to control accumulation levels (distribution again centered at zero). This pattern indicates that the global response to auxin consists of a burst of transcriptional activity, predominately consisting of upregulation (the positive mode is larger than the negative mode; Figure 2A), peaking between 2 and 8 h after auxin treatment, followed by a gradual return to global expression levels similar to that observed at 1 h after auxin treatment.

**Cluster Analysis Reveals Groups of Transcripts with Similar Kinetic Responses**

To identify genes with similar time response profiles, the filtered list of 1246 transcripts was clustered using the latest version of SC2ATmd (Olex and Fetrow, 2011). Consensus clustering revealed 60 clusters with two or more members and 209 singleton genes. The average profile for each cluster was calculated, and the average SLR at each time point for all genes in the cluster is shown in the heat map in Figure 2B. Using depth-first-search to identify consensus clusters from an adjacency matrix allowed us to visualize the clustering results as networks (Figure 3), where each node represents a gene and each edge indicates that the two connected genes were found in the same cluster in at least 90% of the clustering iterations. The edges in these networks represent cluster membership only and do not indicate physical protein interactions or any other molecular interaction. The edge length is based on the number of node connections, where few connections result in longer edges (as determined by Cytoscape’s layout algorithm) (Shannon et al., 2003). This view exhibits several advantages over a traditional heat map view. Cluster networks provide a means to differentiate core cluster genes (those connected by many edges) from fringe genes (those connected by only a few). DE clusters exhibiting a highly connected core (e.g., clusters 1 and 6) tend to have more consistency in gene expression levels over time than those without such a core (e.g., clusters 3 and 5). Additionally, viewing the cluster networks in series reveals the dynamics of each cluster relative to the other clusters (see Supplemental Figure 3 online for the full time course).

Analysis of the DE cluster networks and the summary heat map (Figures 2B and 3, respectively) revealed a variety of responses to auxin treatment. The majority of the filtered transcripts (68%) were included in the largest 10 consensus DE clusters (clusters 1 to 10). The average temporal profile after IAA treatment for each cluster reveals a range of dynamic gene expression events (Figures 2B and 4A). These patterns include early (e.g., DE clusters 5, 7, and 10) and late responding clusters (e.g., DE clusters 3, 4, and 8), transient (e.g., DE cluster 5) and sustained responses (e.g., DE cluster 10), and clusters that show both up- and downregulated responses at different times (e.g., DE cluster 9). DE cluster 10 is unique both for the large magnitude and sustained increases in transcript accumulation throughout the time course. Most of the clusters become active mid-phase and return to baseline by 24 h. A few exceptions include DE clusters 3 and 8, which are upregulated late (8 h) and sustained through 24 h. Analysis of the average time profiles of the largest 10 consensus clusters revealed that several pairs were mirror images of each other (similar colors, top panel, Figure 4A). DE clusters 6 and 7 are rapidly regulated, while DE clusters 1 and 2 reach peak up- or downregulation at 4 h, respectively. Similarly, genes in cluster 3 are downregulated slightly followed by sustained upregulation after 8 h; cluster 4 genes behave inversely. The behavior of individual genes in two sets of mirror image clusters is also shown in Figure 4B, illustrating the consistency of the transcriptional changes within each cluster.

Annotation analysis of the top 10 consensus clusters using the Database for Annotation, Visualization and Integrated Discovery (DAVID; Huang et al., 2009a, 2009b) identifies overrepresented function annotations within each cluster with statistically significant annotations (P < 0.05) reported in Table 1. Early upregulated clusters (cluster 5, 7, and 10) are enriched with auxin response–related annotations, which coincide with the initial induction of many previously described primary auxin response genes, including AUX/IAA, GH3, and SAUR genes (Chapman and Estelle, 2009).
Figure 2. A Variety of Temporal Profiles Are Observed with the Majority of Transcriptional Changes Occurring between 2 and 8 h after Auxin Treatment.

(A) The gene expression distribution for each time point was calculated for the filtered list of 1246 transcripts. Data for each replicate are graphed individually.

(B) The average gene expression at each time point was calculated for all 60 DE consensus clusters numbered from the largest (DE cluster 1) to the smallest (DE cluster 60). Columns contain the unique cluster ID (Cluster Number), number of transcripts included in each cluster (Gene Count), and the average gene expression for the respective cluster at each time point, color-coded where red is upregulation, blue is downregulation, and white indicates no change from control on average (see color bar). Profiles were manually ordered to place similar clusters next to each other, and those highlighted in yellow refer to the largest 10 clusters.
and allow the identification of specific gene products that function in this capacity.

Comparison of These Results with Previously Published Microarray Results

To provide information on the overlap in transcripts identified in this detailed time-course analysis of auxin-responsive genes with those identified by other published microarray experiments, we performed a meta-analysis. We identified four experiments using Affymetrix ATH1 arrays with data available in Affymetrix CEL data files deposited at the Gene Expression Omnibus (GEO) (Edgar et al., 2002), with three of these studies examining whole seedlings treated with 5 µM IAA for 2 h (Okushima et al., 2005a, 2005b; Overvoorde et al., 2005). The fourth data set used root tissues treated with the auxin transport inhibitor, naphthalphthalamic acid, followed by treatment with the synthetic auxin, 1-naphthaleneacetic acid (NAA), at 10 µM for 0, 2, or 6 h to synchronize lateral root initiation (Vanneste et al., 2005). Our filtering analysis (excluding consistency filters, which require multiple time points) was applied to raw data from these experiments and the results compared with our set of 1246 genes (see Supplemental Table 1 online). The most striking result is the small overlap between the IAA treatments of whole seedlings with our root data: only 7 to 21% of our genes were detected in those three data sets. These differences in auxin transcriptional responses in roots and shoots are consistent with the often opposite growth and developmental responses to auxin in these two tissues.

Comparison of our time-course transcript data set with our filtered list of the Vanneste et al. (2005) data set found more similarities. Sixty-seven percent of our auxin-regulated DE transcripts were identified in the root-specific Vanneste data set. We asked where the overlapping genes fit into our kinetic DE clusters, as our time profile contained time points both earlier and later than these published reports. We calculated the number and percentage of significantly expressed genes from our filtered list of genes in our largest 10 clusters (Table 1; see Supplemental Table 1 online). On a DE cluster by cluster comparison, the number of genes also in the Vanneste data set ranged between 20 and 77% of the transcripts. For example, in cluster 10, a cluster with a significantly enriched auxin response annotation, 77% of the genes were also identified by Vanneste et al. (2005). This cluster is upregulated at 30 min and sustained during the entire time course, consistent with these genes being elevated at the 2- and 6-h time points in the Vanneste data set. Three of our clusters (3, 4, and 8) were differentially expressed later in the process (8 to 24 h) with only 14 to 32% of these genes also identified by Vanneste, consistent with the later time points included in our data set. Interestingly, only 20% of the 129 genes in our cluster 2 are also identified by Vanneste, which, on the surface, seems odd, since the cluster is downregulated from 1 to 8 h, times which overlap with those of the Vanneste study. However, observation of the actual cluster 2 time profile (Figure 4A) shows that the strongest downregulation of these genes occurs at 4 h, a time point not covered by Vanneste et al. (2005). This observation demonstrates that additional information on transcriptional changes can be obtained through a time-course analysis, suggesting this data set will complement existing genome-wide data sets.

Figure 3. Clusters with Highly Connected Cores Contain Transcripts with Very Similar Temporal Profiles.

Network nodes represent genes and edges represent cluster membership. Gene regulation at 0.5, 2, 8, and 24 h after auxin treatment is denoted by the node shape where circular nodes indicate positive regulation (SLR > 0) and arrowhead shaped nodes indicate negative regulation (SLR < 0). Nodes are also colored based on gene expression to convey the magnitude of regulation with red indicating upregulation, blue downregulation, and white no change from control (see color bar). The largest 10 clusters are indicated by the numbers 1 to 10.
Additional Well-Characterized Auxin Response Genes Are Identified in a Complementary Present-Absent Filtering Analysis

We analyzed in detail one class of well-characterized primary auxin response genes, the AUX/IAA gene family, to illustrate the information gained and that which might be missed by stringent analysis of this kinetic microarray data set. Figure 5A illustrates the relative expression change of these gene family members. Some of these transcripts are rapidly induced, with substantial SLR change at the earliest time point after treatment, including IAA1, IAA2, and IAA13, found in auxin-annotated clusters 5, 7, and 10. By contrast, several others, including IAA27, IAA8, and IAA9, are found in more slowly induced clusters. The transcript analysis of this kinetic microarray data set. Figure 5A illustrates the relative expression change of these gene family members. Some of these transcripts are rapidly induced, with substantial SLR change at the earliest time point after treatment, including IAA1, IAA2, and IAA13, found in auxin-annotated clusters 5, 7, and 10. By contrast, several others, including IAA27, IAA8, and IAA9, are found in more slowly induced clusters. The transcript

Table 1. Summary of Annotations and Kinetics of the 10 Most Populated DE Clusters

| DE Cluster No. | Gene Count | Cluster Regulation | Overrepresented Cluster Annotations | Transcript Changes with SLR ≥ 0.5 | Vanneste Gene Count (%)a |
|---------------|------------|--------------------|-------------------------------------|----------------------------------|--------------------------|
| 5             | 57         | Up                 | Response to auxin stimulus          | 30 min to 4 h                    | 27 (47%)                 |
| 10            | 13         | Up                 | Response to auxin stimulus          | 30 min to 24 h                   | 10 (77%)                 |
| 7             | 52         | Up                 | Response to auxin stimulus          | 1 to 24 h                        | 13 (25%)                 |
| 1             | 270        | Up                 | RNA processing, RNA polymerase activity, DNA replication, RNA degradation | 2 to 4 h                          | 141 (52%)                |
| 6             | 57         | Down               | Cell wall organization, cell wall biogenesis/degradation | 2 to 24 h                          | 18 (32%)                 |
| 2             | 129        | Down               | Transmembrane region, response to osmotic stress | 2 to 4 h                          | 26 (20%)                 |
| 9             | 22         | Down and up        | Intrinsic to membrane, oxidoreductase | Complex                          | 17 (77%)                 |
| 8             | 40         | Up                 | Signal peptide, extracellular region | 8 to 24 h                        | 14 (35%)                 |
| 3             | 116        | Up                 | Regulation of cell cycle, cell division | 8 to 24 h                        | 32 (28%)                 |
| 4             | 90         | Down               | Phosphorylation, protein kinase activity | 8 to 24 h                        | 28 (31%)                 |

aThe percentage was calculated by dividing the number of genes in the Vanneste data set in each DE cluster by the total gene count in each cluster.
with the greatest abundance changes is IAA19, which exhibits an SLR of 3.76, a 13.5-fold increase; however, it was not in the list of 1246 filtered genes because of inconsistency in the magnitude of the change across the three replicates. IAA7 was removed because the SLR magnitude change did not reach 1.4-fold at any time point. IAA17 and IAA28 were filtered out because of inconsistent patterns of expression change. Most transcripts were eliminated in the stringent detection P value filtering step, which required the detection P value to be significant in all replicates at all time points in the control and auxin-treated samples (see Supplemental Figure 2 online). Transcripts not passing this filter, such as IAA5 and IAA29, usually exhibited no detectable expression in one or more control treatments, consistent with no expression in the absence of IAA.

A second analysis was thus performed to identify such genes, which exhibited an interesting IAA response but were not significantly detected on the chip over background in all time points (i.e., detection P value was absent, or $P > 0.06$, for one or more replicates in IAA or control treatments). A complementary low-resolution analysis (Klink et al., 2010) was performed on each time point independently to identify transcripts with a consistent absent-present gene expression pattern in one or more time points across all replicates. The expression pattern for genes identified by the present/absent (PA) analysis is marked as P-A (present in control and absent under auxin treatment) or A-P (absent in control and present under auxin treatment).

The PA analysis identified 497 transcripts exhibiting a consistent P-A or A-P pattern across all replicates for any one time point (see Supplemental Data Set 2 online). The A-P and P-A

### Table 1

| Gene Symbol | Cluster # / Filtered Filter | 0.0 hr | 0.5 hr | 1 hr | 2 hr | 4 hr | 8 hr | 12 hr | 24 hr |
|-------------|-----------------------------|--------|--------|------|------|------|------|-------|-------|
| IAA1 (AXR5)| 10                          | -0.09  | 2.23   | 1.94 | 2.12 | 2.30 | 2.62 | 2.40  | 1.98  |
| IAA2        | 10                          | -0.24  | 2.07   | 1.79 | 1.97 | 2.16 | 2.32 | 1.84  | 1.54  |
| IAA14 (SLR)| 265                         | -0.12  | 0.70   | 1.01 | 1.30 | 1.63 | 1.61 | 2.04  | 0.93  |
| IAA18       | 7                           | -0.24  | 0.36   | 0.28 | 1.18 | 1.50 | 1.70 | 1.49  | 0.54  |
| IAA13       | 45                          | -0.14  | 1.93   | 1.81 | 2.21 | 2.18 | 1.66 | 1.04  | 0.32  |
| IAA9        | 7                           | 0.00   | 0.53   | 0.74 | 1.17 | 1.33 | 1.21 | 1.07  | 0.28  |
| IAA10       | 40                          | -0.13  | 0.72   | 0.23 | 0.36 | 0.41 | 0.37 | -0.01 | 0.26  |
| IAA27 (PAP2)| 192                         | -0.11  | -0.20  | -0.28 | 0.05 | 0.27 | 0.90 | 0.86  | 0.23  |
| IAA8        | 76                          | 0.06   | 0.13   | 0.28 | 0.53 | 0.80 | 1.03 | 0.94  | 0.23  |
| IAA16       | 139                         | -0.11  | 0.69   | 0.35 | 0.20 | 0.09 | 0.02 | 0.08  | 0.18  |
| IAA12 (BD1)| 5                           | -0.06  | 0.68   | 0.82 | 0.03 | 0.64 | 0.14 | 0.01  | -0.07 |
| IAA26 (PAP1)| ED                         | -0.08  | 0.48   | 0.78 | 1.40 | 2.21 | 2.62 | 2.46  | 1.53  |
| IAA19 (MSG2)| ED                         | -0.03  | 2.53   | 2.83 | 3.11 | 3.76 | 3.56 | 2.72  | 1.48  |
| IAA11       | ED                          | 0.05   | 2.63   | 2.54 | 2.67 | 2.55 | 2.20 | 1.64  | 0.46  |
| IAA4 (ATAX2)| ED                          | 0.05   | 1.18   | 1.21 | 1.40 | 1.38 | 1.26 | 0.96  | 0.30  |
| IAA3 (SHY2) | ED                          | -0.06  | 1.36   | 1.10 | 1.06 | 1.81 | 2.13 | 2.05  | 0.24  |
| IAA17 (AXR3)| PCC                        | 0.01   | 0.00   | 0.10 | -0.16 | 0.18 | 0.50 | 0.61  | 0.32  |
| IAA28 (AR2) | PCC                        | 0.23   | -0.60  | -0.13 | -0.28 | -0.37 | -0.06 | 0.16  | -0.02 |
| IAA7 (AXR2)| SLR                        | 0.01   | 0.16   | 0.42 | 0.23 | 0.44 | 0.52 | 0.35  | 0.46  |

(A) The distribution of the transcripts encoding the Aux/IAA family in the DE filtering indicates the cluster in which these transcripts partition and revealed many transcripts that were removed by the filtering as noted in the second column. The transcript abundance for each AUX/IAA transcript is shown to the right, and boxes are colored based on the average SLR at each time point, with red indicating upregulation, blue downregulation, and white no change from control (see color bar). Asterisks indicate genes whose SLR is an estimate, since the control values did not pass the detection P value filter. Genes highlighted in yellow were identified in the PA analysis.

(B) A selection of clusters from the PA analysis reveals a variety of present-absent (P-A) or absent-present (A-P) patterns. The magnitude of the normalized signal intensity is shown as a color gradient, with white indicating zero and red indicating intensity level 12, for those signals that had a present detection P value ($P < 0.04$) in all three replicates for any given time point. Gray indicates at least one replicate had a marginal or absent P value; thus, the transcript abundance values were not reliable.

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**Figure 5.** The PA Analysis Identifies Additional Members of the Aux/IAA Family.
patterns were found in 315 genes (64%) and 178 genes (36%), respectively, for one or more time points. Additionally, four genes were found to have both the P-A and A-P pattern, each occurring at different time points. The majority of PA filtered transcripts (356) were detectable in only one time point (see Supplemental Figure 4 online, blue bars); however, some (141) were detectable in two or more time points (see Supplemental Figure 4 online, red bars). The dominant A-P pattern contained transcripts whose signal was not detectable until after auxin treatment. This list included several known auxin-induced genes (IAA5, IAA6, IAA29, and SAUR68), as well as transcripts encoding transcription factors (WRKY54, MYB108, ETHYLENE RESPONSE FACTOR12 (ERF12), ERF12, and ATNAVAC2), kinases, and phosphatases (PINOID and genes encoding two putative protein phosphatase 2C subunits), and proteins regulating ethylene synthesis (1-aminocyclopropane-1-carboxylic acid (ACC) synthase 8 (ACS8), ACS11, and ETHYLENE OVERPRODUCER3). Four IAA gene transcripts were recovered, including the rapidly and strongly induced IAA5, and more slowly, but even more strongly induced IAA29 transcript.

The 497 PA transcripts were clustered (Figure 5B) to identify those with a similar P-A or A-P temporal profile. Because relative transcript abundance could not be reliably calculated for all time points, only the normalized signal values marked as present in all replicates were used for clustering. Absent or marginal signals were assigned a value of −1 (colored gray in Figure 5B) for clustering purposes. Annotation analysis indicated that PA cluster 4 is strongly enriched in known auxin-responsive genes, which is a common functional annotation and similar kinetics to DE clusters 5, 7, and 10, suggesting that DE and PA analyses identify complementary sets of transcripts. Cell wall biogenesis/degradation and cell wall modification were overrepresented terms in PA clusters 2 and 3, respectively, as well as DE cluster 6, suggesting that auxin-regulated cell wall–related proteins are relevant candidates for reverse genetic analysis.

Reverse-Genetic Analysis Reveals Mediators of Auxin Response and Auxin-Mediated Growth and Development

A primary goal of this analysis was to identify genes whose protein products functioned in regulating growth and developmental responses to auxin. We used the annotation of the 10 major DE and PA clusters to hypothesize specific biological processes that were regulated by auxin. For example, genes with cell wall synthesis/remodeling annotations are significantly overrepresented in DE cluster 6, a group of 57 transcripts exhibiting decreased expression (relative to control) from 0.5 to 24 h, with maximum downregulation at 8 h (Figure 4B). This time profile is consistent with both inhibition of primary root elongation and promotion of lateral root development. Thus, we conducted a reverse-genetic analysis of Arabidopsis mutants with insertions in genes whose transcript changes passed either the DE or PA analysis and that were predicted to encode cell wall–modifying enzymes, identifying homologous insertion lines in 72 of the 78 genes. A list of these 72 genes and the publicly available T-DNA insertion lines examined for altered growth, development, and auxin response are listed in Supplemental Data Set 3 online. This data set also includes the publicly available annotation of the biological function of the protein product of targeted genes and a summary of their kinetic response to IAA treatment, including cluster membership. For all these insertion mutants, the presence of T-DNA inserts in these genes was verified by PCR analysis and the position of insertion was verified by DNA sequence analysis for lrx2-2 and cel3/gh9b3-1.

To explore growth and development in these mutant lines, an initial analysis was performed where developmentally matched 6-d-old mutant and wild-type seedlings were treated with or without 1 µM IAA for 3 d. Primary root length, lateral root number, and root gravitropic curvature were quantified in two sequential generations. We identified eight insertion mutant lines with reproducible phenotypes (Table 2), found in a variety of DE and PA clusters. All transcripts are detected in Arabidopsis roots in publicly available data sets, as shown using Genevestigator (Zimmermann et al., 2004) (see Supplemental Figure 5 online). Some show very high transcript abundance (EXGT-A1), while others are much lower (EXPA6). The expression of the two genes CEL3/GH9B3 and LRX2 are graphed separately, as plants with insertions in these two genes had the most striking lateral root development phenotypes. CEL3/GH9B3 is expressed in all root tissues, but with the highest expression levels in root cap, columella, and lateral root cap protoplasts and much lower expression in the root stele, cortex, and epidermal protoplasts. In lateral root primordia and protoplasts derived from them or pericycle cells that give rise to them, CEL3/GH9B3 is expressed at intermediate levels. By contrast, LRX2 transcripts show highest accumulation in the cortex, epidermis, and lateral root cap, with much lower transcript abundance in pericycle and lateral root primordia protoplasts. The longitudinal and cell type–specific expression patterns of these genes in a previously published data set were also examined (Brady et al., 2007); LRX2 was maximally expressed in the maturation zone where root hairs and lateral roots form.

We examined second alleles of the interesting cel3/gh9b3 and lrx2 mutants, with insertion positions illustrated in Supplemental Figure 6 online. Transcript abundance of CEL3/GH9B3 and LRX2 was examined by quantitative RT-PCR (qRT-PCR) to confirm and extend the results from the microarray. Comparison of transcript levels quantified by qRT-PCR and microarray analysis identifies similar kinematic responses of CEL3/GH9B3 and LRX2 to IAA, although the magnitude of the change in transcript abundance was greater when measured by qRT-PCR (Figure 6A). qRT-PCR was also used to identify the range of IAA concentrations that affect CEL3/GH9B3 and LRX2 transcript abundance after 24 h of treatment. At IAA concentrations below 100 nM, there is little change in abundance of these transcripts, but both transcripts are maximally induced at concentrations at and above 500 nM, consistent with profound lateral root developmental responses at these IAA concentrations (Figure 6C). Finally, we used a LRX2p::GUS (for β-glucuronidase) reporter line (Baumberger et al., 2003) to examine LRX2 expression. We found a striking increase in LRX2p::GUS product accumulation after treatment with 1 µM IAA, both in root and shoot tissues (Figure 6D).

The CEL3/GH9B3 gene encodes a protein that is a member of the glycoside hydrolase 9 family in the class B subfamily. Many GH9 enzymes catalyze the breakdown of cellulose microfibrils, which results in cell wall loosening (Gilbert, 2010). Proteins in the class B family lack a cellulose binding domain (Urbanowicz et al., 2007) and have unknown biochemical activity. The mutant
alleles that we examined, cell3-1/gh9b3-1 (Salk_057689) and cell3-2/gh9b3-2 (Sail_71_C12), both possess T-DNA insertions in the fifth exon of at1g71380, which were confirmed by PCR and DNA sequence analysis. CEL3/GH9B3 transcript abundance was measured in these mutants and, as expected, these exon insertions decreased message abundance to below 1% of wild-type levels (Figure 6B). These mutants form fewer roots than the wild type on either control or IAA-containing media, using mal- achite green staining to visualize primordia at stage 3 or later (Figure 7A). cell3/gh9b3 exhibits defects in all phases of auxin-induced lateral root development, with significant reductions in unemerged primordia and emerged lateral roots at 72 h after mock or IAA treatment (P < 0.05) (Figure 7B).

To examine the structure of developing cell3/gh9b3-1 roots, we bent primary roots to induce lateral root formation at the position of the bend and then examined the structure of roots that formed using the endogenous autofluorescence of cell walls upon excitation with a 504-nm laser. Under these conditions, 67% of the bent roots formed morphologically normal lateral root primordia, while 33% of the bent roots formed no stage 2 or later primordia (Figure 8A). By contrast, over 95% of wild-type roots form primordia at the site of the bend. The lower root primordia phenotype in this mutant is consistent with its rapid transcript increases after IAA treatment that coincide with the timing of lateral root initiation (DE cluster 5; Figure 3B).

LRX2 is a member of the leucine-rich repeat extensin family, which has a canonical extensin domain and a protein/protein or protein/ligand interaction domain with leu-rich repeats (Baumberger et al., 2001). The extensin domain allows insolubilization of LRX1 protein in the cell wall and is required for protein function (Ringli, 2010). An lrx1 mutant shows defective cell walls and a profound root hair phenotype (Ringli, 2010). The lnx2-2 allele (Salk_029225) and lnx2-3 allele (Sail-1303-D07) both contain T-DNA insertions in the third exon of at1g62440, which were confirmed by PCR and DNA sequencing. Expression of LRX2 in lnx2-2 and lnx2-3 was <1% of wild-type levels (Figure 6B). Both lnx2 alleles formed fewer emerged lateral roots in the presence of IAA, at 72 h after treatment (Figure 7C). This reduction in emerged lateral roots is accompanied by increased numbers of unemerged primordia, leaving the total number of lateral roots (of all developmental stages) unchanged. We also examined the lnx1 lnx2 double mutant (Baumberger et al., 2003) and found that it had an even more striking lateral root phenotype than lnx2 mutants, with increased magnitude of lnx2 mutant phenotype in the presence and absence of auxin. Interestingly, the addition of the lnx1 mutation to the lnx2 background changes the nature of the phenotype, reducing the number of initiated primordia, as well as emerged primordia. We also analyzed the lnx1 single mutant and found no defect in any phase of lateral root development, in the presence or absence of auxin (see Supplemental Figure 7 online), though we did observe the previously characterized root hair defect that is found in lnx1 and the lnx1 lnx2 double, but absent in the lnx2 single mutant (Baumberger et al., 2003). The lnx2 mutants have a defect linked to lateral root emergence, as evidenced by their increased number of primordia and decreased number of emerged lateral roots. The specificity of this phenotype along with the timing of accumulation of LRX2 transcript at the latest points in our microarray analysis is consistent with LRX2 affecting the later stages of lateral root development.

The morphology of developing lnx2 lateral roots showed defects in lateral root emergence through the cortical and epidermal layers of the primary root (Figure 8A). This result is consistent with LRX2 expression in the outer layers of the root through which primordia must emerge. To test this hypothesis, we examined the expression of a LRX2p:GUS reporter line (Baumberger et al., 2003). This line shows very little GUS expression in the lateral root primordia, pericycle, and stele, but it is expressed in outer cell layers (Figure 8B), mirroring the previously reported microarray results (see Supplemental Figure 5 online). This contrasts with the expression pattern of LRX1, which is much lower in cortical cell protoplasts than LRX2. What is even more striking is the difference in longitudinal expression patterns of GUS reporters for these two genes. LRX2 is expressed along much of the root, while LRX1 expression is concentrated in the epidermal cells in the root differentiation zone (Baumberger et al., 2001). Since lateral roots emerge in more basal regions of the root, the expression of LRX1 may not be in the region of the root from which lateral root initiate in wild-type plants, consistent with the absence of a lateral root phenotype in the lnx1 single mutant.

### DISCUSSION

Genome-wide transcriptional analyses provide a wealth of information on transcripts whose abundance changes in response to a perturbation and which may encode proteins that play a role in the resulting changes in growth and developmental processes. This hypothesis-generating approach has identified numerous interesting transcripts whose abundance changes in response to

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Table 2. Summary of IAA-Regulated Genes in Which Mutants Had Phenotypes

| Cluster | Gene Name | Description | Phenotype |
|---------|-----------|-------------|-----------|
| 0       | at1g71380 | CEL3/GH9B3 cellulase3/glycosylhydroxylase 9B3 | Reduced IAA-induced root gravitropism |
| 5       | at1g62440 | LRX2 leucine-rich repeat/extensin 2 | Reduced IAA-stimulated lateral root emergence |
| 24 h (A-P) | at1g62440 | LRX2 leucine-rich repeat/extensin 2 | Reduced IAA-stimulated lateral root emergence |
| 8       | at1g62850 | EXGT-A1 endoxylanoglcucan transferase | Reduced primary root elongation |
| 8 h (P-A) | at1g62850 | EXGT-A1 endoxylanoglcucan transferase | Reduced primary root elongation |
| 2       | at1g18780 | CESA8 cellulose synthase 8 | Reduced IAA-induced root gravitropism |
| 3       | at2g28950 | EXP16 expansin A6 | Reduced IAA inhibition of primary root elongation |

*P, absent-present; P-A, present-absent. Blank under Gene Name indicates there is no name available.
elevated levels of the plant hormone auxin (Paponov et al., 2008). However, many of these studies have been done in whole seedlings (Okushima et al., 2005a, 2005b; Overvoorde et al., 2005), which complicates the analyses, as auxin oppositely regulates growth and development in shoots and roots (Thimann, 1977). In shoots, auxin predominantly stimulates elongation and inhibits branching, while in roots, this hormone inhibits elongation and stimulates branching. Previous studies have employed different auxins including endogenous auxins, such as IAA, and synthetic molecules, such as NAA (Himanen et al., 2004; Redman et al., 2004; Vanneste et al., 2005), which have distinct, albeit overlapping physiological actions (Delbarre et al., 1998; Yamamoto and Yamamoto, 1998; Lewis et al., 2011a). Additionally, prior studies have rarely considered auxin-dependent transcript changes over an extended time course, examining both the rapidly induced transcripts and the induction of downstream targets that mediate growth and developmental responses. This study was designed to uncover a different set of auxin-regulated genes, specifically in root tissue, whose transcript abundance changes overlap with the physiological response timeline to complement previously published genome-wide transcript abundance data sets.

This study observes the kinetic transcriptional response to IAA specifically in roots and uses the results as a basis for a reverse-genetic screen for mediators of auxin response. Roots are an ideal tissue for this analysis, as previous studies have provided detailed information on the transcriptome of distinct cell types and along the longitudinal axis of the root (Brady et al., 2007), which can then be overlaid upon auxin-dependent transcriptional changes. We selected a time course optimized to explore transcript abundance...
changes and physiological responses to auxin in roots in parallel, including the rapid inhibition of primary root growth and the slower stimulation of lateral root development, selecting eight time points from 0.5 to 24 h following transfer to either control or IAA-containing growth media. The DE analysis identified 1246 transcripts with reproducible and significant expression changes.

We identified temporal correlations between changes in transcript abundance observed in clusters of these 1246 transcripts and growth and developmental responses. For example, cluster 3 shows increases in transcripts with changes becoming profound at 8 h after IAA treatment, mirroring IAA-dependent increases in lateral root formation. Consistent with this physiological response, cluster 3 contains a set of genes significantly enriched with cell division and lateral root formation annotations, including four genes encoding cyclins and several genes in which mutations have been shown to result in lateral root phenotypes. Also present in this cluster are genes not originally linked to lateral root development, laying a foundation for future mechanistic studies.

We performed a meta-analysis to ask if there are transcripts identified by the combination of our filtering and kinetic analysis of transcript abundance changes that differed from the previously reported transcripts that we examined. We find limited overlap between our DE data set (<21% of genes from this data set) and three data sets performed with whole seedlings and treated with 5 μM IAA (Okushima et al., 2005a, 2005b; Overvoorde et al., 2005). A more comparable data set focused on roots harvested at 0, 2, and 6 h after treatment with naphthalphthalamic acid then 10 μM NAA to synchronize the induction of lateral root initiation. Our DE data set displayed 67% overlap with this analysis, but yielded an additional 411 auxin targets. Our cluster 10 exhibits the most significant overlap with all four data sets. By contrast, most genes in DE clusters 3, 4, and 8, with transcript abundance changes at later time points than used in the other experiments examined, were unique to our data set. One interesting data set, which used a custom microarray, could not be included in our meta-analysis (Laskowski et al., 2006). This data set has a 12-h time point and identified six cell wall–related transcripts with significant and slow responses (out of 80 total genes in their final data set). One of those genes, EXPAl, encoding an expansin, was also found in our data set, suggesting that a late time course might identify more auxin-responsive cell wall genes. This analysis provides strong evidence that the broad time course of our microarray allows identification of additional auxin-regulated genes beyond those in the previously reported microarrays that we examined.

One challenge of the DE analysis was that some genes expected to be present in this data set were eliminated by the

Figure 7. Lateral Root Phenotypes in cel3/gh9b3 and lnx2 Mutants.

(A) The phenotype of the wild type (WT) is compared with cel3/gh9b3, lnx2, or a lnx1 lnx2 double mutant 3 d after transfer to control media (left) or 1 μM IAA (right). The black mark on the plate indicates the position of the root tip at time of transfer to new control or IAA-containing medium. Bar = 1 mm. (B) and (C) The average ± se number of lateral roots in multiple alleles of cel3/gh9b3 and lnx2 and in the lnx1 lnx2 double mutant with n = 9. Lateral roots were separately quantified as unemerged primordia and emerged mature lateral roots. Asterisks indicate significant difference in number of primordia or emerged roots between mutant and the wild type within treatment (P < 0.05).
stringent P value filtering step. Even though some of these genes were strongly auxin responsive, one or more of their data points did not pass the P value filters, most frequently in the control samples, so accurate calculation of the SLR change by auxin treatment was not possible. Genes that are not expressed until auxin is elevated above endogenous levels were included in this group, and transcripts in this category are of particular interest because they are completely auxin-dependent. Our analysis of the transcript abundance of the AUX/IAA genes highlighted this result. The PA analysis identified transcripts that were absent at some time points (or nondetectable based on the P value filter), but present, or above that threshold, at other time points. These transcripts were also clustered, and like the DE clusters, the PA clusters include groups that show rapid increase with strong annotation of auxin signaling (PA cluster 1). Furthermore, two oppositely regulated clusters (PA clusters 2 and 3) contained annotations linked to cell wall synthesis or remodeling. The opposite regulation of these clusters is consistent with the presence of genes whose products are predicted to encode both cell wall loosening and stiffening and with auxin-inhibiting elongation growth and stimulating lateral root initiation and emergence.

The average SLR graphs shown in Figure 4A highlight the magnitude of transcription changes and the kinetics of the response revealing three sets of clusters that show mirror image responses. DE cluster 7 is annotated with primary auxin response genes, while its mirror image (DE cluster 6) shows opposite, but equally rapid inhibition, with a significant enrichment of genes with annotations linked to cell wall remodeling or growth. Specifically, DE cluster 6 contained a cellulose synthase (CESA2) and several genes encoding pectin-modifying enzymes (including a pectinase, a pectin [methyl] esterase, and a galacturonosyl transferase). There is strong evidence that these classes of enzymes actively contribute to the regulation of cell expansion (Jiang et al., 2005; Chu et al., 2007; Ellis et al., 2010) and as such make attractive candidates for mediators of auxin action. Downregulation of their expression during the auxin response suggests that their molecular function may involve cell wall rigidification, but during lateral root development and growth, expression may be downregulated to allow cell wall loosening. An additional 68 transcripts in other clusters with similar annotations were identified through the DE and PA analyses, providing the foundation for significant future work to understand the molecular mechanisms underlying cell wall modification during lateral root formation and growth.

The significant enrichment of cell wall–related gene products in clusters with interesting kinetic responses suggested the testable hypothesis that auxin may affect development by modulating cell wall mechanics or composition. We performed a reverse-genetic analysis by examining the publicly available insertion lines for 72 of the 78 auxin-regulated genes with annotation suggesting that they might encode enzymes that build or modify the cell wall, from the DE and PA analyses. These genotyped, homozygous mutants were examined for altered root elongation, number of lateral roots, and root gravitropism over two generations in the presence and/or absence of IAA. We performed this analysis in an unbiased fashion, disregarding the magnitude or direction of the transcript abundance changes and information on the tissue or developmental expression patterns. Eight of these lines displayed consistent and reproducible root growth or developmental phenotypes (Table 2). The high phenotype discovery rate, 11% in this analysis versus 3.5% in one undirected screen (McElver et al., 2001), demonstrated the value of developing hypotheses based on biological functions enriched at strategic time points in the kinetic microarray experiment.

We developed hypotheses for the molecular mechanisms of CEL3/GH9B3 and LRX2 function in auxin-mediated growth and development based upon their transcriptional response to auxin,
their predicted biological function, their transcript localization within the root, and their phenotype. Two cel3/gh9b3 mutant alleles display a defect in lateral root initiation in both the presence and absence of auxin. The GH9 family of cellulases in plants is predicted to have a much broader substrate specificities than those of bacteria that cleave crystalline cellulose (Urbanowicz et al., 2007). The protein product of the CEL3/GH9B3 gene lacks a cellulose binding domain (Urbanowicz et al., 2007) and has an unknown effect on cell wall biochemistry. The induction of CEL3/GH9B3 by auxin coincides with the earliest events in lateral root initiation and is consistent with the role of this gene product in facilitating wall loosening for lateral root initiation. This result is particularly interesting given the function of CEL5, a paralog of CEL3/GH9B3, which facilitates separation of the root cap (del Campillo et al., 2004). Additionally, the identification of other glycosyl hydrolase family members that are responsive to auxin in other species (Catala et al., 1997) suggests that this protein family may play a unique role in hormonal regulation of cell wall remodeling. Taken together, these results suggest cell wall loosening, resulting from hydrolysis of cell wall polymers, promotes lateral root development much earlier than lateral root emergence.

The impact of the lnx2 mutations on lateral root emergence is well coordinated with the timing of auxin induction and localization of LRX2 expression. The lnx2 mutant plants show accumulation of early stage primordia, with reduced numbers of emerged primordia. The LRX2 transcripts accumulate slowly, peaking at 24 h after IAA treatment, which is the time window where IAA-induced lateral root primordia are emerging (see Supplemental Figure 1B online). Examination of the expression patterns of LRX2 both in publically available microarray data sets and in a proLRX2:GUS fusion line are consistent with this gene being induced in the cells surrounding, but not within, lateral root primordia. Our results indicate that LRX2 did not specifically accumulate near emerging lateral root primordia. We therefore hypothesize that there is a general change in the cell wall along the root that alters the ability of roots to emerge through this layer of cells. Our results are consistent with auxin-regulated initiation of lateral roots at local positions in the root and a more ubiquitous auxin regulation of cell wall structure that affects the ability of roots with previously specified positions to emerge. Analysis of the lnx1 lnx2 double mutant revealed a more severe lateral root development phenotype that differed in one key aspect from the lnx2 single mutant. While the number of emerged lateral roots is reduced further in the double mutant, there is also a significant reduction in early stage primordia.

What is particularly intriguing about this synergistic effect in the lnx1 lnx2 double mutant is the previously reported amplification of a root hair defect (Baumberger et al., 2003). In that case, the root hair phenotype is present only in lnx1 and the double mutant. Our results indicate that different stages of lateral root development are regulated by the CEL3/GH9B3 and LRX2 gene products, consistent with the timing of their induction by auxin.

This study provides a diversity of auxin-regulated genes with distinct temporal patterns of response that can be used to develop additional hypotheses for gene products that regulate root development. The experiments described here provide support to the hypothesis that auxin transcriptionally regulates genes encoding proteins that modify cell wall structure and/or extensibility. Specifically, we identify a role of CEL3/GH9B3 and LRX2 in auxin-mediated lateral root development.

METHODS

Plant Growth and Arabidopsis thaliana Genotypes

Columbia-0 seeds of Arabidopsis were purchased from Lehigh seeds. DR5:GFP was used previously (Lewis et al., 2011a), and T-DNA insertion alleles were ordered from the ABRC (Ohio State University, Columbus, OH), which were identified from the Salk Institute, Syngenta/TMRI, or the Wisconsin Ds/Lox populations using T-DNA express (http://signal.salk.edu/cgi-bin/tdnaexpress) (Salk Institute Genomic Analysis Laboratory, San Diego, CA). Seeds from the ABRC were planted, and homozygous individuals were selected using a medium throughput PCR-based genotyping procedure described below. The lnx1, lnx1 lnx2 double mutants, and the proLRX2:GUS transgenic line were generously provided by Christoph Ringli, University of Zurich (Baumberger et al., 2003).

Plants were grown on 1 X Murashige and Skoog medium (Caisson Labs), pH 5.6, Murashige and Skoog vitamins, and 0.8% agar, buffered with 0.05% MES (Sigma-Aldrich) and supplemented with 1.0% Suc. After stratification for 48 h at 4°C, plants were grown under 100 µmol m−2 s−1 continuous cool white light. For phenotypic analyses, wild-type and mutant plants were grown on control medium for 5 d, then transferred to control and 1 µM IAA containing media. Three days later, seedling images were captured with a scanner. Primary root growth was measured using Image J (NIH) and lateral root number was manually quantified. Phenotypes that were consistent over multiple trials and at least two generations are listed in Table 2, and a subset of these mutants was selected for high-resolution analysis of lateral root developmental stages. All lateral root phenotypic data are from representative experiments containing between seven and 20 seedlings, which were replicated in three or more experiments.

Confocal Microscopy

Images were captured with a Zeiss 710 confocal laser scanning microscope. For the examination of DR5:GFP fluorescence, plants were grown on control medium for 6 d, then transferred to medium containing 1 µM IAA. For all confocal microscopy, roots were stained with 25 µg/mL propidium iodide. Tiled, single-plane micrographs were then collected at the indicated times after treatment using a large (2.5AU) pinhole diameter to create an optical slice that encapsulated the whole root in the Z dimension. Gain and pinhole settings were held constant for comparisons, and images were captured under conditions where the signal was not saturating, although in some image sets, the brightness has been adjusted for publication clarity. Although representative images are shown for each GFP reporter, more than six seedlings were examined for each time point.

For the quantification of fluorescence intensity in DR5:GFP, differently sized regions of interest were drawn at set distances from the root apices and average pixel intensity was calculated within these regions. The root tip region was defined as 0 to 500 µm from the root apex and the mature or lateral root-forming region was defined as 4.75 to 5.25 mm from the apex. Lateral root primordia number was quantified by examining the DR5:GFP micrographs used to create Figure 1B and scoring local GFP accumulations that were accompanied by a primordia in the associated bright field image. For identification of early stage primordia in mutants without crossing to reporters, we stained roots with 0.1% Malachite Green (Sigma-Aldrich) in water for 2 min. Malachite green preferentially stains lateral root primordia, which increased the accuracy of lateral root developmental analysis over bright-field analysis alone. Roots were imaged using 488-nm excitation and 520- to 650-nm emission settings. The addition of this contrast agent allowed us to reliably detect stage 2 and later primordia for quantification of numbers in Figure 7. For the images in Figure 8A, cell wall...
autofluorescence was captured with 405-nm excitation and 415- to 740-nm emission settings. The images in Figure 8A represent a maximum intensity projection of a Z-stack.

IAA Treatments and RNA Isolation
RNA was isolated from seedlings grown on a nylon filter (03-100/32 Sefar Filtration) as described previously (Levesque et al., 2006). Plants were stratified and subsequently germinated on a filter pressed tightly against control medium with ~100 seedlings per plate. On the fifth day after germination, the screen was transferred to growth medium with and without 1 µM IAA for the indicated times. At the end of the treatment period, the roots were aligned and excised, carefully excluding the root/shoot junction. Samples were promptly frozen in liquid nitrogen and stored at ~80°C until RNA isolation.

Frozen samples were ground in liquid nitrogen, and RNA isolation was performed according to the Qiagen plant RNeasy kit protocol, followed by glycoblue addition (Ambion/Life Technologies), ethanol precipitation, and resuspension. After DNase treatment (Promega), RNA samples were quantified by absorbance at 260 nm using a Nanodrop spectrophotometer (Nanodrop Technologies). RNA concentrations were standardized to 150 ng/µL ≥ 0.1% by the addition of 10 mM Tri-HCl, pH 8.0. Each sample yielded ~4.5 µg of RNA.

Microarray Analyses
RNA samples were sent to the Wake Forest University Comprehensive Cancer Center Microarray Shared Resource Center and were repurified on Qiagen RNeasy columns. The samples were analyzed on an Agilent Bioanalyzer and Eppendorf BioPhotometer for RNA integrity and concentration. Samples with RNA integrity values >8.0 were carried forward for cDNA synthesis, labeling, and fragmentation. The samples were hybridized to the arrays and washed, and Affymetrix AGCC software was used to process the chips and perform image capturing. The resulting data in Affymetrix CEL data file format were analyzed for quality assurance using internal Affymetrix parameters and custom signal distribution analyses developed in house.

Raw data were normalized by the microarray facility using systematic variation normalization as described previously (Chou et al., 2005), and the log2 of the signal intensity was reported along with the detection P value calculated by the Affymetrix software. Relative expression (SLR) was calculated as the time-matched log2 ratio of the signal intensity (for each transcript individually) to the average intensity of the control replicates for a given time point because control and experimental data sets were not paired; therefore, averaging the control provided a consistent baseline for replicate comparisons.

In response to IAA (SLR ≥ 0.06, and the SLR value for at least one time point was ≤ ~0.5 or ≥ 0.5 (roughly a 1.4-fold change). The list of transcripts that passed the detection P value and SLR filters independently in all replicates (overlap filter) was filtered for consistent response to auxin over time using Pearson’s correlation coefficient (PCC) and Euclidean distance (ED) (consistency filter). PCC and ED scores were calculated for each transcript’s time profile to measure the level of pattern consistency and magnitude (ED score) agreement between each replicate over time (Olex et al., 2010). All pairwise combinations of replicates were compared resulting in three PCC and three ED scores for each transcript. Only two of the three PCC and ED scores were required to meet the following cutoffs: PCC ≥ 0.7 and ED ≥ 1.09. The PCC cutoff was determined based on statistical reasoning where any two data sets with a correlation >0.7 were considered as highly correlated. The ED cutoff was chosen to be the median ED score over all three sets of scores for the entire filtered data set. Transcripts meeting or exceeding all filtering criteria were considered to be significantly and consistently expressed.

Consensus Clustering
Prior to clustering, a figure of merit (FOM) analysis was performed on the set of transcripts meeting the filtering criteria to identify the inherent number of clusters present in each replicate data set. The FOM also determined which clustering algorithm generated the most homogeneous clusters with respect to the Euclidean distance metric (k-means and hierarchical agglomerative clustering were compared) (Yeung et al., 2001; Olex et al., 2007). The DE filtered set of transcripts were clustered using the consensus clustering option provided by SC2ATmd (Olex and Fetrow, 2011), which was updated to allow users to specify a custom consensus threshold, where transcripts are included in a consensus cluster if they are grouped together >100% of the time. The calculated consensus matrix (Monti et al., 2003; Olex et al., 2010) is filtered based on the chosen threshold and converted to an adjacency matrix where all values passing the threshold are changed to a “1” and all other values are changed to a “0.” The adjacency matrix is then searched using MATLAB’s “graphconncomp” function, which uses a depth-first-search algorithm to identify consensus clusters. Consensus clustering was run using k-means and Euclidean distance with 10 starting clusters (parameters determined by the FOM analysis). A consensus threshold of 90%, where transcripts found in the same cluster 90% of the time would be included in the same consensus cluster, was chosen as it returned clusters with consistent expression kinetics as well as a limited number of singletons compared with other thresholds. Ten clustering iterations were performed per replicate.

Meta-Analysis of Published Microarray Data Sets
All data sets for comparison were .CEL files downloaded from GEO (Edgar et al., 2002) and were previously published (Okushima et al., 2005a, 2005b; Overvoorde et al., 2005). GEO accession numbers for the data sets are GSE3350 (Okushima et al., 2005a), GSE631 (Okushima et al., 2005b), GSE627 (Overvoorde et al., 2005), and GSE629. Data were normalized using the RNA normalization method in R (Bolstad et al., 2003) followed by P value calculation with the Affymetrix package in R. Within each data set, each of the two experimental replicate expression values was compared with the averaged control to obtain signal-to-log ratios for each gene. To remain consistent with our filtering process, probes were deemed differentially expressed as long as the SLR was >0.5 or ~0.5 (1.4-fold change) for each replicate of a sample. Probes only had to be differentially expressed in response to hormone treatment or in mutant genotypes (but not both) to remain in the comparison. Overlap between data sets was determined using a Python 3.2.3 program written in house.

PA Filtering and Clustering
PA filtering was performed as previously described (Klink et al., 2010) with some alterations. A pattern of present/absent (P-A) was assigned if all three control replicates for a given time point had a detection P value ≥0.04 (Affymetrix Present call) (Affymetrix) and all IAA-treated replicates for the same time point had a detection P value ≥ 0.06 (Affymetrix Absent call) (Affymetrix). Similarly, a pattern of absent/present (A-P) was assigned if the inverse filter was met (control P value ≤ 0.06 and auxin P value ≤ 0.04). Detection P values in the marginal range (0.06 > P > 0.04) were not
considered, as those genes would have been included in the initial filtering analysis. A full list of genes passing the P-A or A-P filter for at least one time point was constructed.

The PA genes were clustered to identify groups with similar patterns over time. For each time point and condition (control or IAA) of a given gene, all three replicate signals were set to a −1 if any one of the replicates was absent or marginal. Otherwise, if all three replicates were present (i.e., had detection P values ≤0.04) then the signals were left intact for clustering. This modified data matrix was imported into the tool SC²ATmd for a FOM analysis. The standard clustering tab in SC²ATmd (Olex and Fetrow, 2011) was then used to cluster these data into six clusters using k-means and Euclidean distance, as determined by the FOM analysis.

Annotation Analysis of Microarray Data
Identification of significantly overrepresented annotations in each DE and PA cluster was performed using the Functional Annotation Clustering tool provided by DAVID (Huang et al., 2009a, 2009b). The genes for each cluster were imported into DAVID using the Affymetrix probe identification number with the Affymetrix ATH1-121501 Arabidopsis genome chosen as background; all other options were left at their default setting. Annotation groups with an enrichment score ≥1.3 (equivalent to a P value ≤0.05) were identified as significantly overrepresented.

Genotypic Analysis of Mutant Lines
Twenty-four-leaf seeds from each line were sown individually in soil, and mature tissue was excised and placed in a 96-well deep well plate. DNA extraction buffer (100 mM LiCl, 10 mM Na₂EDTA, 100 mM Tris-HCl, and 1% [w/v] SDS, pH 8.0) and a 0.177-cal steel bead were added and agitated by attachment to a pneumatic paint shaker (Northern Tools) at 60 p.s.i. for 5 min until tissue was adequately disrupted, followed by centrifugation at 5000 rpm for 5 min. Genomic DNA in the supernatant was precipitated with isopropanol, resuspended, and stored at −20°C until further use. PCR using primers designed by primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was performed using a set of gene-specific primers as well as a set of one gene specific primer and one T-DNA-specific primer to identify homozygous individuals for propagation. PCR products were mixed with SYBR Green (Invitrogen) and analyzed by melt curve analysis on an ABI 7600 fast quantitative PCR machine (Applied Biosystems). Positive PCR reactions were distinguishable from negative and nonspecific PCR reactions when greater than 80°C temperature was needed to denature the double stranded DNA and eliminate SYBR green fluorescence. For confirmation of T-DNA position in cel3-1/gsh9b3-1 and lnx2-2, PCR reactions using a left border primer and a gene-specific primer were sent to a sequencing facility, along with a nested sequencing primer.

qRT-PCR
Samples containing 900 ng of RNA were used for CDNA synthesis with a 1:1 mixture of oligo(dT) and random hexamer primers and SuperScript III enzyme (Invitrogen). After digestion with RNase (Invitrogen), the A₂₆₀ was measured using a Nanodrop spectrophotometer (Thermo Scientific) to ensure equal efficiency in the CDNA synthesis reactions between samples. qRT-PCR analysis using this cDNA was performed on an Applied Biosystems 7600-fast thermal cycler using SYBR Green detection chemistry. Each reported value is the average of three biological replicates each containing three technical replicates.

Accession Numbers
Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are at5g51500 (PE60), at3g58790 (GAUT15), at1g71380 (CEL3/GH9B3), at1g62440 (LRX2), at2g06850 (EXGT-A1), at1g10770 (put. PMI), at4g18780 (CES8), and at2g28950 (EXPA6). These .CEL files are posted with the GEO accession series GSE42007. GEO accession numbers for the data sets that we used in our meta-analysis are GSE3350 (Okushima et al., 2005a), GSE631 (Okushima et al., 2005b), GSE627 (Overyevoorde et al., 2005), and GSE629.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. IAA Rapidly Inhibits Primary Root Elongation and More Slowly Stimulates Lateral Root Initiation.

Supplemental Figure 2. Flow Chart of the Filtering Process.

Supplemental Figure 3. Consensus Cluster Networks for the 1246 Auxin-Responsive Genes.

Supplemental Figure 4. The Majority of PA-Filtered Transcripts Appeared in Only One Time Point.

Supplemental Figure 5. Genevestigator Report of Expression Profile of Genes That, When Mutated, Confers a Phenotype.

Supplemental Figure 6. Description of cel3/gsh9b3 and lnx2 Alleles Used in This Study.

Supplemental Figure 7. The lnx1 mutant forms wild-type numbers of lateral roots.

Supplemental Table 1. Overlap Analysis with Other Published Auxin Transcriptome Data Sets.

Supplemental Data Set 1. The List of 1246 DE-Filtered Transcripts.

Supplemental Data Set 2. The List of 497 PA-Filtered Transcripts.

Supplemental Data Set 3. The List of Cell Wall Mutants Used for Functional Analysis.

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AUTHOR CONTRIBUTIONS
D.R.L. designed and performed experiments and participated in the drafting and editing of this article. A.L.O. developed and performed the analyses of the microarray data and participated in the drafting and editing of this article. S.R.L. wrote and executed the program to perform the meta-analysis of microarray data sets and edited the article. W.H.T. assisted with the development of the clustering approaches and the meta-analysis and edited the article. J.S.F. participated in microarray experimental design.
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A Kinetic Analysis of the Auxin Transcriptome Reveals Cell Wall Remodeling Proteins That Modulate Lateral Root Development in *Arabidopsis*

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