The Plant Cell, Vol. 22: 2184–2200, July 2010, www.plantcell.org

Natural Variation of Transcriptional Auxin Response Networks in *Arabidopsis thaliana* [C][W][OA]

Carolin Delker,[a,1] Yvonne Pöschl,[b,1] Anja Raschke,[a] Kristian Ullrich,[a] Stefan Ettingshausen,[a] Valeska Hauptmann,[a] Ivo Grosse,[b] and Marcel Quint[a,2]

[a] Leibniz Institute of Plant Biochemistry, Independent Junior Research Group, 06120 Halle (Saale), Germany
[b] Institute of Computer Science, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany

Natural variation has been observed for various traits in *Arabidopsis thaliana*. Here, we investigated natural variation in the context of physiological and transcriptional responses to the phytohormone auxin, a key regulator of plant development. A survey of the general extent of natural variation to auxin stimuli revealed significant physiological variation among 20 genetically diverse natural accessions. Moreover, we observed dramatic variation on the global transcriptome level after induction of auxin responses in seven accessions. Although we detect isolated cases of major-effect polymorphisms, sequencing of signaling genes revealed sequence conservation, making selective pressures that favor functionally different protein variants among accessions unlikely. However, coexpression analyses of a priori defined auxin signaling networks identified variations in the transcriptional equilibrium of signaling components. In agreement with this, cluster analyses of genome-wide expression profiles followed by analyses of a posteriori defined gene networks revealed accession-specific auxin responses. We hypothesize that quantitative distortions in the ratios of interacting signaling components contribute to the detected transcriptional variation, resulting in physiological variation of auxin responses among accessions.

INTRODUCTION

Naturally occurring genetic variation has been reported for numerous phenotypes in *Arabidopsis thaliana*. In addition to various developmental traits, response phenotypes that are primarily correlated with adaptations to natural environments have been under investigation. The stimuli triggering the respective responses ranged from pathogens or effectors to different light conditions, abiotic stress, and a variety of other environmental perturbations (reviewed in Alonso-Blanco et al., 2009).

The translation of a stimulus into cellular responses is often mediated by plant hormones. Auxin in particular is known to be a potent regulator of various aspects of plant development (Delker et al., 2008). At the cellular level, auxin responses are initiated by altering the expression of a multitude of genes, which requires the proteolytic degradation of transcriptional repressors by the 26S proteasome (Quint and Gray, 2006). In the absence of auxin, AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins repress auxin signaling by heterodimerization with transcription factors of the AUXIN RESPONSE FACTOR (ARF) family (Tiwari et al., 2003). With increasing auxin levels, the Aux/IAA proteins bind to the auxin receptors. These consist of a small family of F-box proteins (TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN [TIR1/AFB]) that integrate into functional S-phase kinase-associated protein, Cullin, F-box ($SCF^{TIR1/AFB}$) complexes (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005; Parry and Estelle, 2006) and confer substrate specificity to the complex. Aux/IAA proteins are recruited for polyubiquitination and are subsequently degraded by the proteasome (Ramos et al., 2001; Zenser et al., 2001). This allows the ARF transcription factors to initiate downstream auxin responses by regulating the expression of auxin-responsive genes (Guilfoyle et al., 1998; Ulmasov et al., 1999). Such auxin responses can be summarized as cell division, cell differentiation, and cell elongation: essential cellular processes that can translate into an array of different physiological phenotypes.

Many plant developmental events and reactions in response to environmental cues are tightly regulated by auxin and other phytohormones. Natural variation in hormone responses, however, has not been studied in detail as yet (Maloof et al., 2001; Delker et al., 2008). Phytohormones usually act via extensive reprogramming of expression patterns for a unique cassette of genes (Nemhauser et al., 2006). Up to now, the intraspecific variation in phytohormone-induced transcriptional responses has only been assessed for salicylic acid (SA; van Leeuwen et al., 2007). For other phytohormones (e.g., auxins), the impact or even presence of natural variation has hardly been approached experimentally at all. While it is obvious that natural variation should exist for pathways that specifically regulate adaptation to certain natural environment perturbations, it is uncertain whether this is also true for essential conserved messenger systems that transduce multiple environmental or developmental signals into specific responses. As such, the auxin

[1] These authors contributed equally to this work.
[2] Address correspondence to mquint@ipb-halle.de.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Marcel Quint (mquint@ipb-halle.de).
[C] Some figures in this article are displayed in color online but in black and white in the print edition.
[W] Online version contains Web-only data.
[OA] Open Access articles can be viewed online without a subscription.
www.plantcell.org/cgi/doi/10.1105/tpc.110.073957

signaling pathway is an ideal model to study naturally occurring genetic variation of essential messenger systems.

We have investigated the natural variation in auxin responses and signaling at the physiological, population genetic, and transcriptional levels. First, classic physiological auxin response assays were used to assess the general extent of natural variation. Second, nucleotide diversities were estimated for early auxin signaling elements to determine potential differences in the signaling ability of natural accessions. Third, network analyses of ATH1-based transcriptional profiles were used to investigate the variation and outcomes in global transcriptome changes of seven accessions in response to an auxin stimulus. Finally, based on our data, we present a model to explain the observed variation in various response levels.

RESULTS

Natural Variation of Physiological Auxin Responses

The high degree of natural variation observed for numerous physiological traits prompted us to study the physiological responses to auxin in 20 different accessions, which represent a maximal degree of genetic diversity (Clark et al., 2007). We performed standard bioassays to quantify root inhibition and hypocotyl elongation in response to auxin and found significant differences between accessions with respect to absolute root length and growth responses (Figure 1; see Supplemental Figures 1–3 online). Phenotypic variation in root growth was higher in response to the synthetic auxins naphthylacetic acid (NAA) and 2,4-D than to the natural auxin IAA (Figures 1A–1C). This phenomenon is likely attributable to a slower removal via catabolization of the synthetic auxins, whereas a large excess of IAA is usually rapidly removed by conjugation to amino acids, sugars, or direct oxidation (Delker et al., 2008). High temperatures promote auxin-mediated hypocotyl elongation by increasing endogenous auxin contents (Gray et al., 1998; Stavang et al., 2009). To analyze potential variations in the response to resulting increased endogenous auxin levels, plants were grown at elevated temperatures (29°C) and the increase in hypocotyl elongation was quantified for each accession and found to differ significantly in many pair-wise comparisons (Figure 1D; see Supplemental Figure 4 online). Remarkably, individual accessions varied in their responses depending on the specific auxin and type of assay (root versus hypocotyl assays). One can assume, therefore, that the mechanisms underlying the variations in response to different auxins are not uniformly regulated but rather result from complex mechanisms in a tissue-specific manner.

Additional evidence for intraspecific variation in auxin responses was obtained by analysis of the activation of the synthetic auxin reporter construct *DR5:GUS* in three accessions that differed significantly in their response to IAA-induced root growth inhibition (see Supplemental Figure 3 online). The analysis of several independent and homozygous T3 lines revealed considerable differences among Fei-0, Sha, and Col-0 in histochemical β-glucuronidase (GUS) assays (Figure 1E; see Supplemental Figure 5 online). The extent of DR5 promoter activation was determined by quantitative (q)RT-PCR of *GUS* expression after mock treatment or treatment with three different IAA concentrations. Col-0 showed the strongest response in auxin-induced expression changes, whereas the levels in Sha were significantly lower. Fei-0 exhibited *GUS* expression responses intermediate to Col-0 and Sha (Figure 1F). In addition, we analyzed two known endogenous auxin-responsive genes, *GH3.1* and *IAA2*, in the transgenic *DR5:GUS* lines. The expression response of *GH3.1* showed similar results to those already detected for the *GUS* gene. Even although the accession-specific differences in the expression response of *IAA2* were not quite as distinct, the general trend in expression responses was confirmed. Here, too, significant differences between Col-0 and the other two accessions were detectable (Figure 1F).

Alterations in the expression responses could be the result of differences in endogenous auxin concentrations causing hypersensitive/hyposensitive reactions to an additional exogenous auxin stimulus. Therefore, we quantified free IAA levels in 7-d-old seedlings and were unable to identify significant differences among the seven accessions that were further analyzed in this study (see Supplemental Figure 6 online). Thus, auxin responsiveness is most likely not affected by endogenous IAA levels in these accessions.

Arabidopsis Accessions Differ in Auxin-Induced Transcriptional Changes

The expression data of the *DR5:GUS* transgenic lines suggested that differences in auxin sensitivity and expression responses might contribute to the observed variation. To gain a more global insight into the differential auxin responses on a transcriptional level, we performed ATH1-based expression profiling of auxin responses with a set of 7 of the 20 accessions that differed in their phenotypic auxin response (Figure 1). To avoid potential secondary effects, we performed a time-course analysis that focused on the early transcriptional changes induced by auxin. Seven-day-old *Arabidopsis* seedlings were treated with 1 μM IAA, and samples were taken before induction (0 h) and at 0.5, 1, and 3 h post induction (hpi). Auxin-induced transcriptional changes were detectable in all seven accessions, with an average of 651 genes that showed a significant (Benjamini-Hochberg–corrected $P < 0.05$) auxin response of at least twofold change in expression levels at 3 hpi. Surprisingly, many of these genes are differentially expressed in three or fewer accessions, whereas only ~100 genes showed a twofold or higher expression change in all seven accessions (Figure 2A). Auxin-induced transcriptional responses of 17 arbitrary genes of the latter group were independently reexamined across all time points by qRT-PCR. The relatively high correlation coefficient of $r_s = 0.8$ (Spearman correlation coefficient) between both data sets offered further validation of the microarray data (see Supplemental Figure 7 online) and indicated the robustness of the expression levels detected by microarray analysis (Czechowski et al., 2004).

While the total number of genes with an auxin-induced transcriptional response was similar for 0.5 and 1 hpi, the numbers of differentially expressed genes increased notably 3 h after the auxin stimulus. This is in agreement with previously published data (Goda et al., 2008) and most likely denotes the establishment of secondary responses following an auxin treatment.

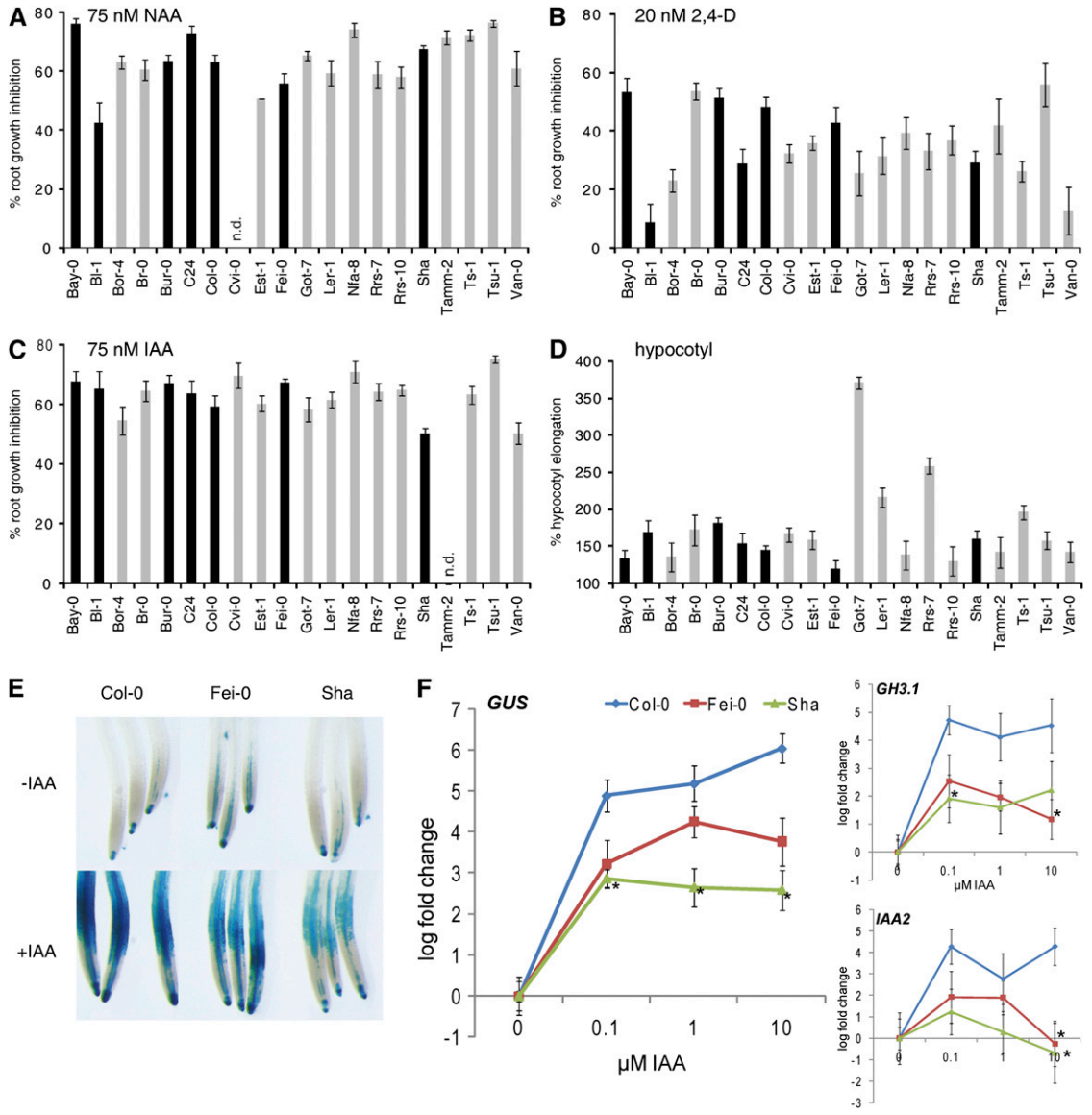


Figure 1. Natural Variation in Physiological Auxin Responses.

(A) to **(D)** Physiological auxin responses of 20 *Arabidopsis* accessions were determined in root growth inhibition and hypocotyl elongation assays of 8- and 10-d-old seedlings (n = 12), respectively. Black bars highlight accessions that were subsequently analyzed for whole genome transcriptome changes. Error bars show SD. Experiments were repeated twice with similar results. Data of absolute root and hypocotyl lengths and statistical analyses are shown in Supplemental Figures 1 to 4 online.

(A) to **(C)** Bars represent mean root length of treated roots as a percentage of untreated roots.

(D) Hypocotyl elongation of seedlings grown at 29°C is given in percentage relative to seedlings grown at 20°C.

(E) Histochemical detection of GUS activity after 3 h of mock treatment (−IAA) or treatment with 1 μM IAA (+IAA). Three seedlings of a single representative T3 line are shown for each accession. All independent T3 lines for each accession are shown in Supplemental Figure 5 online.

(F) Quantification of *GUS*, *IAA2*, and *GH3.1* expression by qRT-PCR at 1 hpi with 0.1, 1, and 10 μM IAA, respectively. Mean log fold changes (treatment versus mock) in expression were determined by analysis of eight, six, and seven independent T3 lines for Fei-0, Sha, and Col-0, respectively. Error bars denote SE. Significant differences from Col-0 expression responses were assessed by two-way ANOVA and are marked by asterisks.

To obtain further insight into the apparent diversity of the transcriptome, we compared the differentially expressed genes between all analyzed accessions. The overlap of individual accessions with Col-0 ranged from only 34% (Sha; 3 hpi) up to 77% (Fei-0; 0.5 hpi). Hence, a relatively large proportion of genes showed an auxin-induced expression change in one or more accessions other than Col-0 or were specifically induced in a single accession (Figure 2B).

The variation in differentially expressed genes could be indicative of hypersensitive and hyposensitive auxin responses on the

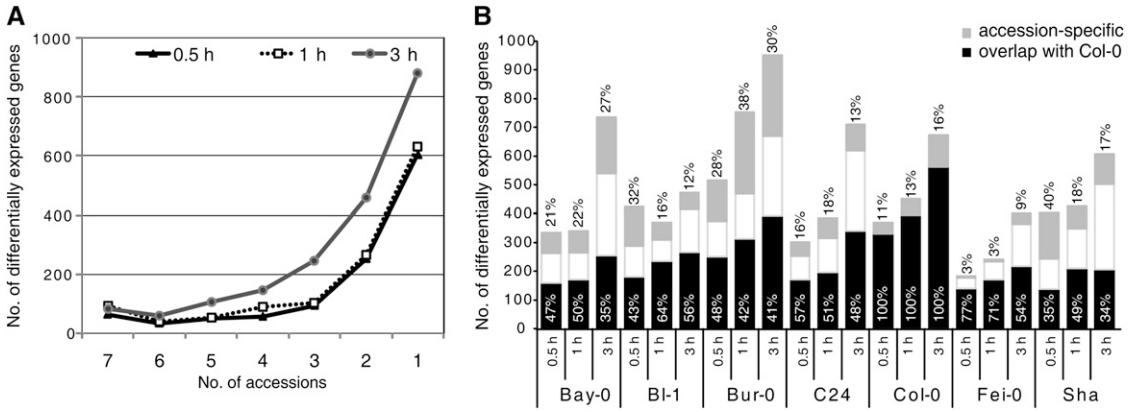


Figure 2. Accession-Specific Differences in Auxin-Induced Transcriptional Changes.

(A) Differentially expressed genes with a significant ($P < 0.05$; Benjamini-Hochberg corrected) expression change of at least twofold (i.e., $\Delta\log_2 > 1$) compared with untreated plants were categorized by the number of accessions in which they were differentially expressed.

(B) Bar plots show the number of differentially expressed genes in individual expression profiles. The fraction of genes that is specifically regulated in an individual accession is indicated in gray (black numbers), whereas the fraction of genes also differentially expressed in Col-0 is marked in black (white numbers).

expression level. To address this hypothesis, we compared the number of differentially expressed genes as well as the respective amplitudes of expression changes between all accessions. In both cases, significant differences were observed (see Supplemental Figure 8 online). However, no clear correlation between the number of differentially induced genes and median fold changes in expression was observed; thus, based on this criterion, we could not justify the classification in truly hyperresponsive or hyporesponsive accessions. As such, the variation in the total number of genes as well as the different degrees of accession specificity and Col-0 overlap can serve only as general indicators for a high variability in auxin-induced transcriptional changes in different *Arabidopsis* accessions.

Intraspecific Variation of Whole Genome Responses

Whole genome expression profiles of all accessions at individual time points were compared to further assess the degree of natural variation. Identification of common patterns in such complex data sets is usually complicated by the multidimensional nature of the data. Thus, we used the Local Context Finder (LCF; Katagiri and Glazebrook, 2003), a nonlinear dimensionality reduction method for pattern recognition. In contrast to other coexpression algorithms, an important advantage of the LCF is the translation of multidimensional relationships between expression profiles into a two-dimensional network that makes complex interactions more intelligible. To reduce the effect of possible noise and to filter for robust coexpressions, we applied a bootstrapping procedure as suggested by Katagiri and Glazebrook (2003). Expression profiles are presented as nodes within the LCF-generated networks, and interconnections between them are presented as directed edges.

LCF analysis of whole genome transcriptome profiles separated the seven analyzed accessions into three groups (Figure 3A). Bay-0 and Sha represent one isolated group, and C24 and Fei-0 constitute another. The third group is formed by Col-0 and Bur-0. Bl-1 shows no clear affiliation with a specific group, and Bl-1 nodes share edges with all accessions except Fei-0 (Figure 3). While edges within each group were quite frequent, considerably fewer edges connect nodes of one group with nodes of another. In general, all nodes of an accession are tightly linked to each other regardless of the time point. Edges between nodes of different accessions can only be detected for identical time points (Figure 3). This illustrates a tight temporal regulation of auxin responses and argues against delays or shifts in the kinetics of auxin responses as the cause for the observed variation. In summary, global auxin-induced expression changes among *Arabidopsis* accessions differ considerably in comparison with each other as well as with the reference accession Col-0, illustrating the large potential for variation in the regulation of diverse auxin-regulated processes.

Sequence Diversity of Auxin Signaling Genes

The $SCF^{TIR1/AFB}$-dependent signaling pathway regulates the expression of auxin response genes (Quint and Gray, 2006). A possible cause for the above-described natural variation in the transcriptional and subsequent physiological auxin responses, therefore, may be variations at the level of early signaling events. It is likely that slight changes in the function or the equilibrium of signaling components would contribute to the dramatic differences we observed in the transcriptional response downstream of the initial signaling events. Genes encoding signaling elements may display accession-specific differences at the sequence level, possibly resulting in signaling components with altered biochemical properties between accessions. To test this hypothesis, we analyzed the sequence diversity of auxin signaling genes for 19 of the accessions used in this study. Three gene families were considered: (1) the *TIR1/AFB* auxin receptors, (2) the *Aux/IAA* repressors, and (3) the *ARF* transcription factors.

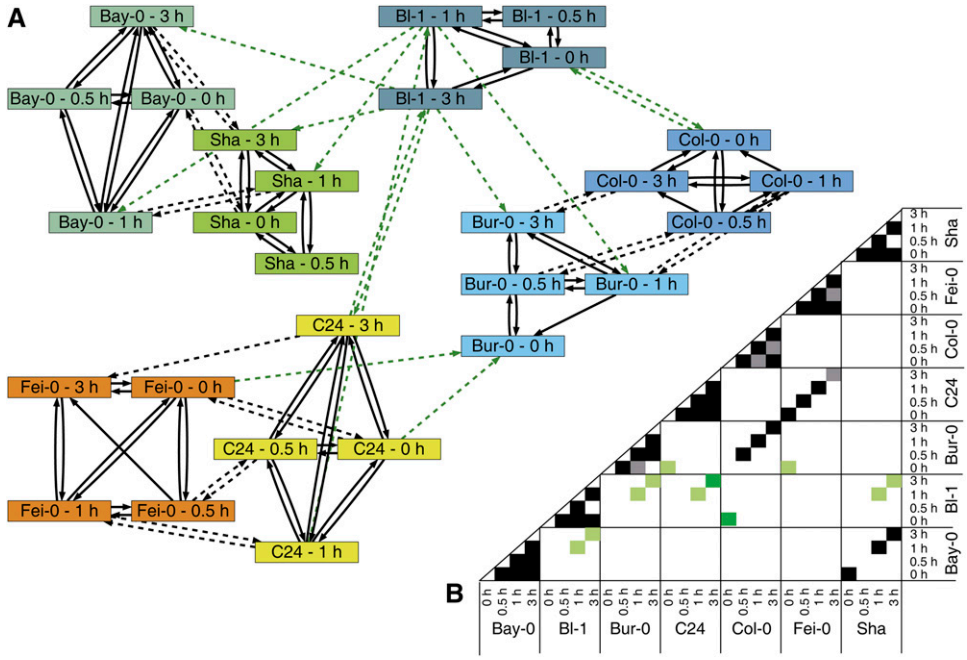


Figure 3. Intraspecific Variation in Whole Genome Transcriptome Profiles.

(A) Profiles for individual accessions were compared by LCF. Similarities in profiles of an individual accession at different time points post induction are indicated by solid lines; dashed lines represent similarities between different accessions at similar time points. Edge colors specify similarities between accessions within a subgroup (black) or between accessions of another subgroup (green).

(B) Tabular presentation of edges detected within the LCF network. Black/dark green and gray/light green squares denote the presence of two edges and one edge between nodes, respectively; black/gray squares represent edges within the same subgroup; green squares represent edges between different subgroups.

We sequenced ~1-kb fragments and identified two major-effect changes with potential functional consequences. First, *IAA11* contained a splice site specific to Col-0 and L*er*-1, which results in a different splice variant than in the other 17 accessions. Second, *ARF13* contains premature stop codons or alternative splice variants in Sha, Tsu-1, Tamm-2, and Bay-0. However, *ARF13* generally does not have the ARF–Aux/IAA interaction domains III and IV (Okushima et al., 2005); therefore, it is unlikely that these mutations are of functional significance.

Taking a molecular population genetic approach, we analyzed whether patterns of selection resulting in sequence diversification would favor the possibility that functional differences in signaling genes contribute to the transcriptional and physiological variation in response to an auxin signal. On the other hand, selective constraints resulting in sequence conservation might argue against such a hypothesis. As a control data set for the population genetic approach, we used an empirical distribution of genome-wide polymorphisms as suggested previously by Kreitman (2000) and Nordborg et al. (2005). For the analysis of the empirical distribution, we took advantage of 876 equally spaced fragments sequenced from a panel of 96 accessions (which include all our analyzed accessions except for Bl-1) generated by Nordborg et al. (2005). We then calculated nucleotide diversities for the coding sequences of the auxin signaling genes and compared them with the empirical distribution (i.e., control genes). The nucleotide diversity π can be used to measure the degree of polymorphism within a population (Nei and Li, 1979). We measured π for all sites and for synonymous (π_s) and nonsynonymous (π_a) sites separately. Figure 4 depicts the nucleotide diversities for the auxin signaling gene families and the control genes in a bar plot. The underlying gene-wise summary statistics are shown in Supplemental Data Set 1 online.

The summary statistics showed no significant deviations from the control genes. For the auxin receptors, however, we found evidence for lower values for π ($P = 0.15$) and π_a ($P = 0.06$). Auxin receptors are members of the superfamily of F-box genes that belong, together with nucleotide-binding-Leu-rich repeat genes, to the most diverse and rapidly evolving gene families in the *Arabidopsis* genome (Clark et al., 2007). Hence, if these data were compared with those of the F-box gene superfamily instead of the empirical distribution, significant differences indicating some degree of purifying selection could be expected. Likewise, we detected lower values for the assayed nucleotide diversities for *Aux/IAA*s and *ARF*s (Figure 4). However, although we identified genes with no amino acid substitutions in each gene family (see Supplemental Table 1 online), it has to be kept in mind that, theoretically, a single nonsynonymous mutation at a functional residue might result in functional variation at the protein level. In summary, although not statistically significant, these results demonstrate that sequence diversity among the accessions tested was rather low for auxin signaling genes.

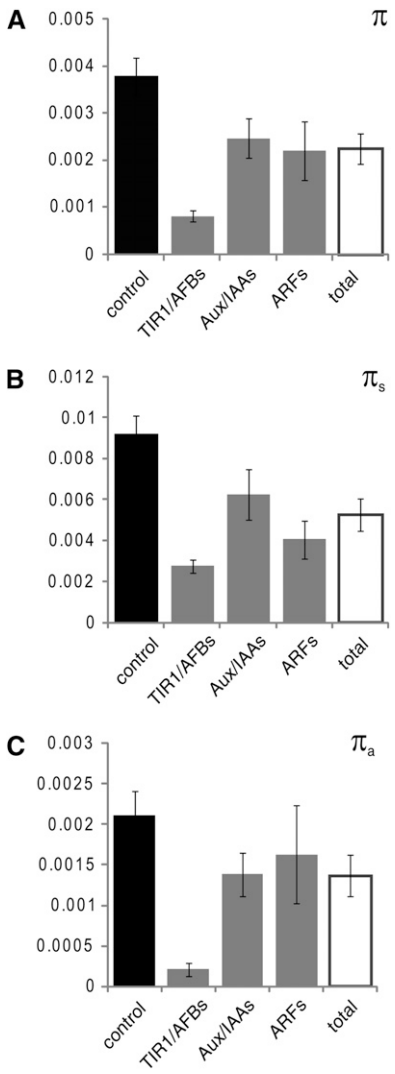


Figure 4. Nucleotide Diversity of Auxin Signaling Genes.

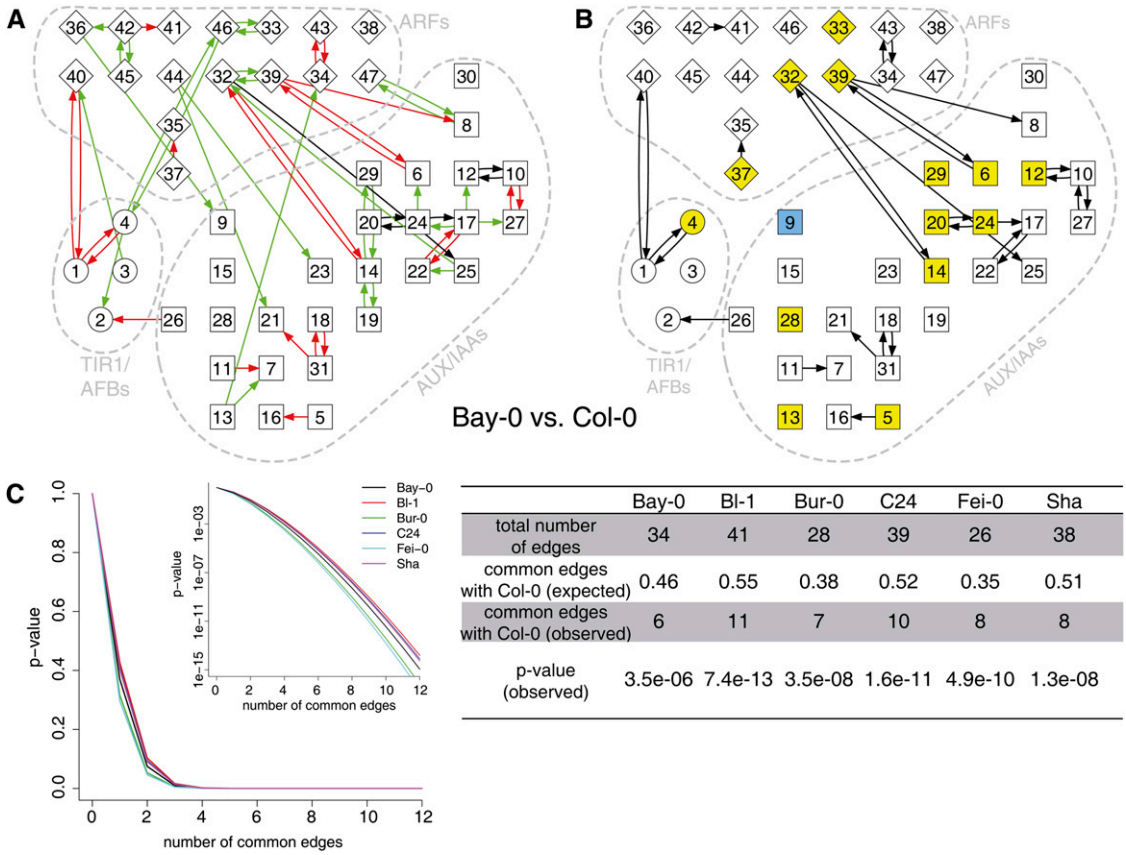
Nucleotide diversity (π) was determined for all sites **(A)**, synonymous sites **(B)**, and nonsynonymous sites **(C)** by comparing ~1-kb fragments in 19 accessions. Results were summarized for control genes ($n = 236$) as well as for the three gene families of receptors ($n = 4$), *Aux/IAA*s ($n = 29$), and *ARF*s ($n = 16$) separately and combined (total, $n = 49$). Error bars denote SE. Summary statistics for individual genes are presented in Supplemental Data Set 1 online.

Coexpression Networks of Auxin Signaling Genes

While the conservation of auxin signaling genes at the sequence level indicates a possible conservation of functional protein properties, differences in the transcriptional regulation of signaling genes may directly influence auxin responses by causing changes in specific TIR1/AFB–Aux/IAA and/or Aux/IAA–ARF interactions. This would in turn contribute to the diversity in downstream transcriptional responses of different accessions. To address this hypothesis, we used the LCF to inspect the transcriptional coregulation of signaling genes. This a priori defined network represented the same three gene families as above: (1) *TIR1/AFB*s, (2) *Aux/IAA*s, and (3) *ARF*s (see Supplemental Table 2 online for gene list). After LCF analysis of individual accessions, including bootstrapping of the signaling gene expression profiles, we performed pair-wise comparisons between networks of Col-0 and the other accessions. This revealed remarkable differences between the individual network structures (Figure 5A; see Supplemental Figure 10 online). As in every coexpression analysis, the edges detected by LCF do not necessarily reflect a true functional or physical interaction between linked neighbors. Nevertheless, the results bear functional significance for the comparison of different accessions, as the LCF provides characteristic pattern information or fingerprints for each individual complex data set. The overlap of edges detected in the networks of Col-0 and the respective other accessions ranged from 18% for Bay-0 to 31% for Fei-0 (Figures 5A and 5C; see Supplemental Figure 10 online). The majority of edges seemed to be specific for the network of an individual accession, which indicates that the individual expression profiles of genes differ considerably between accessions. Since *Aux/IAA* genes are known to be auxin-inducible themselves, it is not surprising that connections in individual networks were most prevalent among *Aux/IAA* gene family members, which confirms the validity of this approach. Most of these connections, however, seem to be specific for the expression set of a single accession, whereas only a few appear to be more conserved and can be detected in several of the analyzed comparisons, such as the edges connecting genes 20 (*IAA2*) and 24 (*IAA1/AXR5*).

To ensure that the detected common edges between Col-0 and the respective edges in other accessions represent robust congruencies and did not result by chance, we computed P values based on the hypergeometric distribution of the number of expected common edges, which ranged from 0.35 to 0.55 (Figure 5C). For each accession, the number of common edges is significantly higher than expected (Figure 5C). The probability of the six common edges between Bay-0 and Col-0 occurring by chance was found to be 3.5×10^{-6}. This probability was even lower for the 11 common edges detected between Bl-1 and Col-0 (7.4×10^{-13}; Figure 5C). Hence, the detected common edges in our pair-wise comparisons are highly significant and represent true overlaps in the coexpression networks of the respective accessions.

The overall low numbers of common edges with Col-0 indicate considerable deviations in the transcriptional equilibrium of signaling components. Since the LCF procedure only considers the shapes of expression profiles but fails to provide information about the respective amplitudes, we performed a modified *t* test for small sample sizes (Opgen-Rhein and Strimmer, 2007) to compare the degree of expression changes induced by the auxin stimulus. For all three time points, significant differences were detected for various genes in pair-wise comparisons with Col-0 (Figure 5B; see Supplemental Figure 11 online). Highest variations in expression levels were detected for genes of the *Aux/IAA* family, whereas expression changes in *ARF* genes varied considerably less. Almost no variation was detectable in the transcriptional changes of the auxin receptor family (see Supplemental Figure 11 online).



	Bay-0	Bl-1	Bur-0	C24	Fei-0	Sha
total number of edges	34	41	28	39	26	38
common edges with Col-0 (expected)	0.46	0.55	0.38	0.52	0.35	0.51
common edges with Col-0 (observed)	6	11	7	10	8	8
p-value (observed)	3.5e-06	7.4e-13	3.5e-08	1.6e-11	4.9e-10	1.3e-08

Figure 5. Coexpression Analyses of Auxin-Induced Transcriptional Changes in Signaling Genes.

(A) Coregulation across time points of genes encoding TIR1/AFB auxin receptors (1–4, circles), AUX/IAA proteins (5–31, squares), and ARFs (32–47, diamonds) was analyzed by LCF. Red edges indicate connections detected specifically in the network of Col-0, green edges are specific for Bay-0, and black edges represent connections detected in both networks.

(B) Differences in auxin-induced changes for signaling genes at 1 hpi are highlighted in blue and yellow for significantly lower or higher transcriptional responses, respectively, in Bay-0 compared with Col-0 ($P < 0.05$, Benjamini-Hochberg corrected). A complete summary of pair-wise comparisons of LCF networks and time point–specific expression changes of all accessions is available as Supplemental Figures 9 to 11 online.

(C) Probability of the number of common edges between Col-0 (29 edges) and all other accessions. The plot shows probabilities of common edges, the inset shows identical data on a logarithmic scale, and the tabulated data are a summary of the results.

Cluster Analysis

The significant differences in the coexpression networks and expression levels of signaling genes suggested a more detailed analysis of the global transcriptional responses. To reduce the complexity of the expression data sets, we first performed cluster analysis using the Col-0 expression data. This resulted in the identification of 112 clusters, many of which contained only a single gene. We chose a minimum cutoff of six genes per cluster, which reduced the cluster number to 51. These were further reduced to 46 by only considering clusters that showed a significant transcriptional response in at least one of the analyzed accessions at least at one time point.

Inspection of heat maps of the mean expression changes seen at 0.5, 1, and 3 hpi illustrates that some clusters (e.g., clusters 91, 97, and 100) showed relatively strong differences among accessions, whereas others (e.g., clusters 99 and 101) showed a relatively uniform response, with more subtle differences between accessions (Figure 6A). A dendrogram based on hierarchical clustering of all three time points separates the accessions into different groups (Figure 6B). Bay-0 and Sha as well as Fei-0 and C24 form distinct groups from Bl-1, Col-0, and Bur-0, confirming the pattern in expression variation between individual accessions detected by LCF analysis of whole genome expression data (Figure 3A).

For a more detailed analysis of the expression differences, we (1) compared the coexpression of clusters by LCF and (2) inspected the mean expression levels of clusters at individual time points post induction. In both cases, pair-wise comparisons between Col-0 and the other accessions were performed.

For the coexpression analysis, the mean expression response profiles of genes within a cluster were generated and subjected to LCF analysis, resulting in accession-specific coexpression networks. These were subjected to pair-wise comparisons of

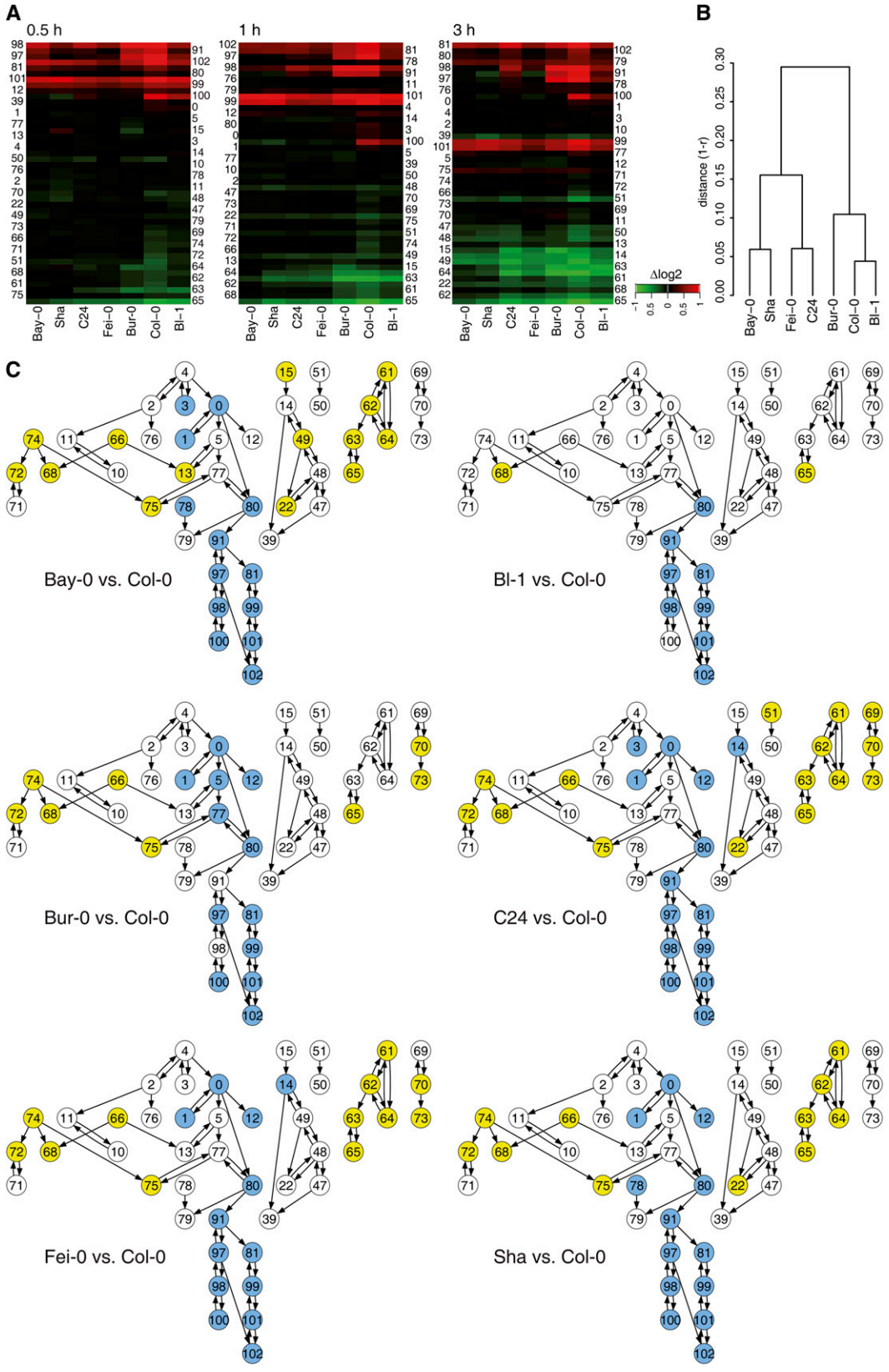


Figure 6. Accession-Specific Differences in the Expression Response of Gene Clusters.

individual accessions and Col-0, which again revealed a high degree of diversity between the individual LCF networks, in agreement with the high degree of diversity observed for the signaling genes (Figure 5A; see Supplemental Figures 10 and 12 online).

To assess the extent to which the mean cluster expression changes differ among accessions, we inspected auxin-induced expression changes at individual time points after auxin induction. The network structure and connections detected by LCF for the Col-0 data were selected for convenient visualization. The significant differences in the mean expression changes between an accession and Col-0 are indicated by colored nodes (Figure 6C). Numerous alterations from the Col-0 expression levels were detected for several clusters, and some of them were accession-specific. Interestingly, some general patterns of congruency can be observed as well, since many clusters with significantly altered expression levels were detectable as such in almost all pair-wise comparisons (Figure 6C). In this respect, the group formed by clusters 81 to 102 is of special interest. In most cases, the expression changes of these clusters are significantly lower than in Col-0 with only a few exceptions (e.g., cluster 100, 1 hpi; Figure 6C; see Supplemental Figure 13 online). This group of clusters is also highlighted by the LCF coexpression analysis (see Supplemental Figure 12 online). First, the expression profiles of clusters within this group showed strong responses to the auxin treatment (see Supplemental Data Set 2 online). Second, since common edges between Col-0 and other accessions appeared in many of the pair-wise comparisons, auxin responsiveness seemed to be rather conserved within this group. Although accession-specific edges also occurred among this group of clusters, the number of edges that overlapped with Col-0 was exceptionally high (see Supplemental Figure 12 online). Last, many genes that have been shown to be auxin-responsive in a broad-scale microarray data-mining approach (Paponov et al., 2008) were found within these clusters (see Supplemental Data Set 3 online). Among them were several *GH3* and *Aux/IAA* genes, transport-associated genes (e.g., *PIN3*), and representatives of the LOB domain (LBD) transcription factor family (e.g., *LBD16*).

Similarly, expression changes of several clusters seemed to be uniformly higher in the six accessions than in Col-0 (e.g., 61–65; Figure 6C), indicating extensive deviations from the transcriptional response observed in Col-0.

Accession-Specific Expression Differences in Selected Clusters

To demonstrate an example of accession-specific differences, we further investigated the transcriptional responses of the 100 individual genes of cluster 100. A close-up view of the expression changes of individual genes 1 hpi within this cluster demonstrated that the majority of genes indeed showed almost contrary transcriptional responses between several accessions and Col-0 (Figure 7; see Supplemental Figure 14 online). Expression changes in Bl-1 and Col-0 genes were similar, and Bl-1 and Col-0 were thus grouped into a clade separated from the five other accessions. This grouping was caused mainly by a large block of ~60 genes that were upregulated in Col-0 and Bl-1 and downregulated in the other five accessions (Figure 7). To verify the differential response detected in the microarray data, we reexamined the transcriptional responses for a subset of 10 of the total 100 genes by qRT-PCR. For 8 out of 10 genes, auxin-induced transcriptional responses detected by microarray analyses were reproducible by qRT-PCR (Figure 7), which confirms that accession-specific regulation of gene clusters does occur in response to auxin stimuli.

The high degree of accession specificity observed on the level of gene clusters complicates a direct identification of a single cluster or a few clusters of genes, which are potentially responsible for the observed natural variation of physiological auxin responses. Therefore, we performed a second analysis to correlate the expression and physiological IAA responses on the level of individual genes. The expression profiles of the genes with highest and lowest Pearson r values mirror almost perfectly the variation observed in the physiological assay (see Supplemental Figures 15A and 15C online). Among the 230 genes with $r > 0.8$, a significant enrichment of Gene Ontology terms related to hormone responses and transcription factor activity was detected (see Supplemental Figure 15B online). Notably, one member of the *Aux/IAA* gene family, *IAA5* (*AT1G15580*), was highly correlated, with an r value of 0.94. However, determination of the functional relevance of these genes for the variation in downstream auxin responses needs to be further investigated, and these results can only be seen as indicators of potential factors that contribute to the extensive variation found in auxin responses.

DISCUSSION

In plants, many traits exhibit a high degree of interspecific as well as intraspecific variation, and many of these traits have been extensively analyzed in the reference plant species *A. thaliana*. The vast majority of traits investigated from the perspective of natural variation are studied for their proposed roles in adaptations to natural environments. Less is known about integrative signaling pathways essential for the coordination of a multitude of specific environmental and/or developmental

Figure 6. (continued).

(A) Mean expression changes of all genes within individual clusters were calculated for each time point post induction and are presented as heat maps.
(B) Dendrogram based on the hierarchical clustering of expression data of all four time points. $1 - r$ (Pearson) was used as a distance measure of the agglomerative hierarchical clustering with complete linkage.
(C) Pair-wise comparison of cluster expression levels at 1 hpi ($P < 0.05$, Benjamini-Hochberg corrected). Deviations from Col-0 expression changes are highlighted in blue and yellow for significantly lower and higher expression changes, respectively. Network structure was obtained by LCF analysis of Col-0 cluster expression data (see Supplemental Figure 12 online).

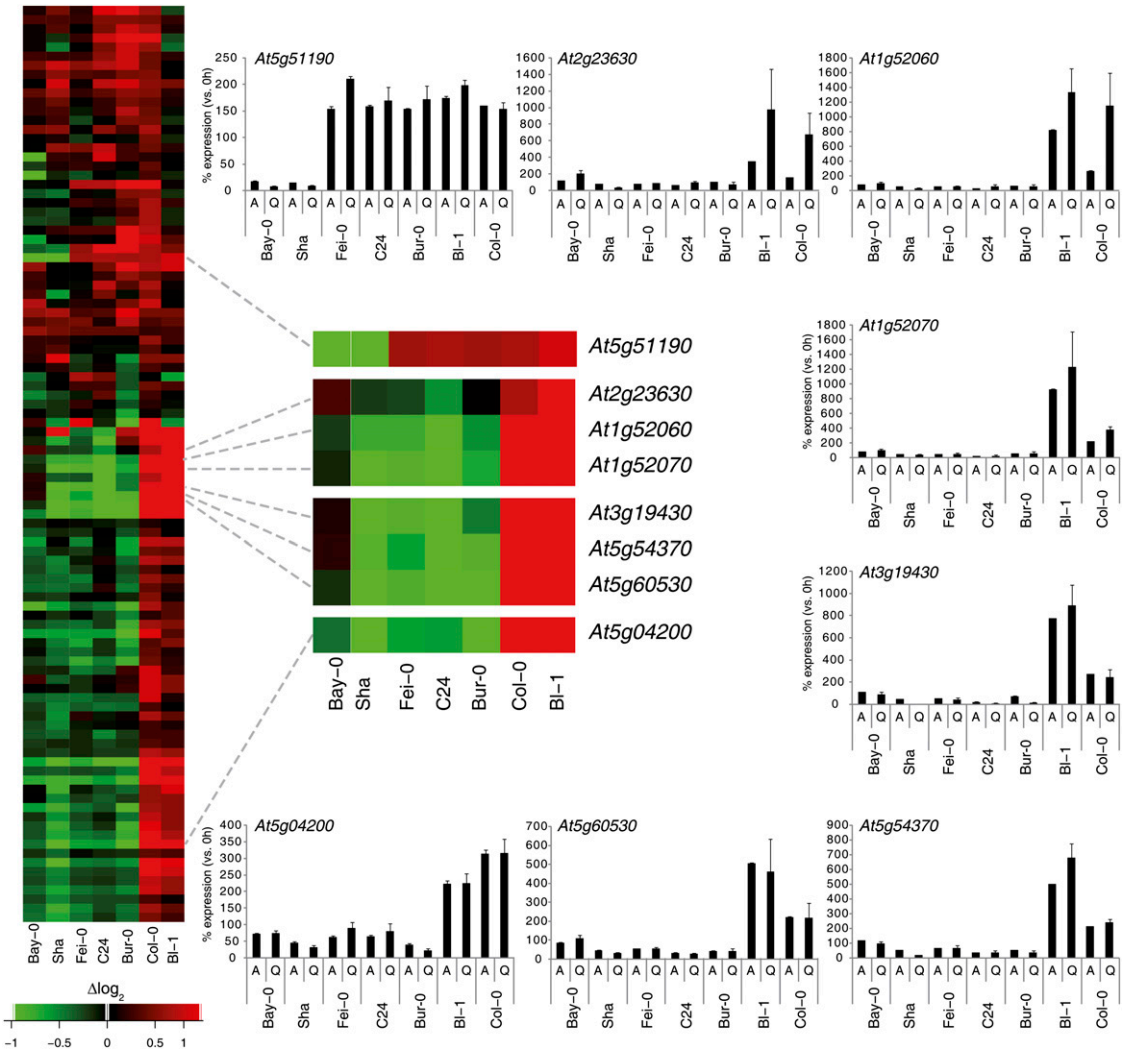


Figure 7. Accession-Specific Expression Differences at 1 hpi within Cluster 100.

Microarray data (A) were validated with qRT-PCR (Q) for eight selected genes. Error bars represent SE (n = 3). For a higher resolution of the cluster 100 heat map, see Supplemental Figure 14 online.

stimuli. Therefore, we investigated the genetic variation of auxin responses in natural accessions of *Arabidopsis* on the physiological, molecular population genetic, and transcriptional levels.

Natural Variation of Physiological and Transcriptional Auxin Responses

We first demonstrated for a set of 20 genetically diverse accessions that variations in physiological auxin responses were evident (Figure 1). Auxin responses were, at least in part, tissue-specific, and they depended on the auxin compound applied. Differences observed on the physiological level were also reflected in variations in a *DR5:GUS* reporter assay (Figures 1E and 1F). In agreement with this, we detected extensive variation in auxin-induced transcriptional responses in seven of the phenotyped accessions (Figure 2). The actual degree of accession specificity measured was surprisingly high. However, similar or even higher accession-specific differences were previously described for transcriptional changes induced by SA (Kliebenstein et al., 2006; van Leeuwen et al., 2007) or between parental strains of recombinant inbred line populations used in expression quantitative trait locus (eQTL) studies (Keurentjes et al., 2007). Likewise, the presence of intraspecific variation for pathogen responses in *Arabidopsis* has been demonstrated for various pathogens (van Poecke et al., 2007; Rowe and Kliebenstein, 2008; Narusaka et al., 2009). In this respect, natural variation in SA responses might likely aid adaptation processes related to plant defense mechanisms. While the known SA functions are restricted, one would assume a much tighter regulation of responses to a signaling molecule that is known to translate a multitude of stimuli into diverse responses, such as auxin.

Therefore, the degree of variation we detected might be considered unexpected.

Global Auxin Response Networks

LCF analysis enabled us to detect and visualize patterns of congruency and variation in global auxin-induced transcriptomes between accessions (Figure 3). The results divided the accessions into different subgroups (Figure 3A). This classification was largely confirmed by hierarchical clustering of the expression data, which indicates robust differences between accessions (Figure 6B). Congruencies were detected most frequently for the transcriptional profiles of an individual accession at different time points (Figure 3B). This can be expected, since only ~1 to 3% of the genes were differentially regulated by auxin. The strong similarities of transcriptomes, therefore, are most likely due to the remaining ~97 to 99% nonresponsive genes whose expression levels are constant within the same accession. However, the finding that transcriptional profiles of different accessions are only coregulated at similar time points (Figure 3) indicates a tight temporal regulation of auxin responses. This argues against accession-specific time shifts in transcriptional regulation as a possible cause for the observed variation.

Sequence Conservation of Auxin Signaling Genes

Mechanisms known to be involved in the manifestation of natural variation must be encoded genetically or epigenetically. Evidence for extensive sequence variation among *Arabidopsis* accessions has been obtained in several studies (Borevitz et al., 2007; Clark et al., 2007; Zhang et al., 2008). We considered genetic variation in the *TIR1/AFB*, *Aux/IAA*, and *ARF* gene families. All signaling genes known from the Col-0 reference genome were present in the other tested accessions. Therefore, we can exclude the absence of signaling genes in accessions other than Col-0 as a cause for variation, although we cannot rule out the presence of additional family members in these accessions that are absent in Col-0. We identified two isolated events of major-effect polymorphisms, but their functional significance is questionable and restricted to the affected accessions. On a population level, auxin signaling genes seemed more conserved than the control genes (Figure 4; see Supplemental Table 1 online). This argues against selective pressures that favor the existence of multiple protein variants with different functions among accessions as the primary cause for the downstream transcriptional variation.

Transcriptional Networks of Auxin Signaling and Response Genes

Alternatively, sequence polymorphisms in *cis*-regulatory regions or *trans*-acting factors can affect the regulation of gene expression, which will influence the spatiotemporal conditions and amounts in which the resulting gene product is present to exert its function. The regulation of auxin responses requires direct physical interactions between signaling components, including (1) TIR1/AFBs recruiting Aux/IAAs for proteasomal degradation, (2) heterodimerization of Aux/IAAs and ARFs, and (3) heterodimerization and homodimerization of ARFs. All three signaling components are encoded by gene families in *Arabidopsis*. It has been hypothesized that a major key to auxin's ability to regulate such a wealth of diverse processes is constituted by the multitude of putatively different interactions of this tripartite signaling ensemble (Lokerse and Weijers, 2009). In agreement with this hypothesis, individual members of each gene family are at least partially expressed in a temporal- and tissue-specific manner (Dharmasiri et al., 2005b; Teale et al., 2006). Analyses of loss- and gain-of-function mutants have provided further evidence for functional specificities as well as redundancies of TIR1/AFBs, ARFS, and Aux/IAAs, respectively (Chapman and Estelle, 2009; Parry et al., 2009). While several *Aux/IAA* loss-of-function mutants do not show obvious phenotypes in the Col-0 background (Overvoorde et al., 2005), others do exhibit auxin-related defects, as in the case of *SHORT HYPOCOTYL2* (*SHY2/IAA3*; Tian and Reed, 1999). Interestingly, *SHY2/IAA3*, among others, shows accession-specific differences in coexpression patterns and expression levels in several pair-wise comparisons with Col-0 (Figure 6C; see Supplemental Figures 12 and 13 online). This does not necessarily prove a relevant role for *SHY2/IAA3* in the observed variation in downstream auxin responses. However, it shows that we find alterations for signaling components with known specificity. Taken together, selected *Aux/IAA*s may be sensitive to transcriptional downregulation (such as *SHY2/IAA3*), while others most likely are not. Furthermore, there are several examples of phenotypes resulting from overexpression alleles of individual *Aux/IAA*s (Nagpal et al., 2000; Ploense et al., 2009). Therefore, *Aux/IAA*s may be more prone to cause downstream natural variation by transcriptional upregulation in certain accessions.

In agreement with these findings, it has been shown for Col-0 that modifications in expression patterns that ultimately change the protein abundance of signaling components can have a profound effect on auxin signaling. Changes in perception of the auxin stimulus to more or less receptive can be modulated by increasing or decreasing the levels of auxin receptors (Ruegger et al., 1998; Navarro et al., 2006; Perez-Torres et al., 2008). If such differences in receptor levels were genetically fixed between accessions, auxin responses would likewise differ. However, we have detected only minimal variation in expression profiles of receptor-encoding genes. This argues against a general hyperresponse or hyporesponse of an accession, even though this might be the case in specific tissues or could be mediated by posttranscriptional processes. Alterations in transcriptional responses and coexpression networks were more frequent among *ARF*s and *Aux/IAA*s than between receptor and ARF- or Aux/IAA-coding genes, respectively (Figures 5A and 5B; see Supplemental Figures 10 and 11 online), which suggests that the accession-specific equilibria of *Aux/IAA*s and *ARF*s show considerable variation, at least in comparison with the reference strain Col-0. Here, posttranscriptional processes such as modifications or turnover rates might further affect the equilibrium of signaling components. For instance, both Aux/IAAs and ARFs are subjected to proteasomal degradation. In contrast to Aux/IAAs, the degradation of ARFs seems to be auxin- and TIR1-independent, as shown in the case of ARF1 (Salmon et al., 2008). In this respect, auxin-independent factors could also contribute to the observed natural variation in expression profiles.

Based on the assumption that specific compositions of available signaling components trigger specific auxin responses, we hypothesize that the accession-specific alterations in the equilibria of available signaling components contribute considerably to the variation in downstream transcriptional responses. As a consequence, accession-specific clusters of coregulated response genes could be expected. These were indeed observed and validated by qRT-PCR (Figures 6 and 7). Ultimately, this may then translate into quantitative differences in physiological responses to the auxin stimulus.

In a highly simplified scheme, the basal auxin response is caused by a balanced equilibrium between Aux/IAA and ARF proteins that function together in the regulation of a particular trait, such as root growth inhibition (Figure 8). In response to an auxin stimulus, a shift in the composition and relative amount of Aux/IAA and ARF proteins enables downstream responses. The strength of the triggered response in different accessions depends on the equilibria of the activating and repressing signaling components involved in the specific process. Since our data set is based on whole seedlings, functional analysis and dissection of putative changes in Aux/IAA–ARF compositions and subsequent correlation to specific physiological processes such as root growth are not easily achieved. This would require tissue-specific or even cell type–specific analysis of transcriptional auxin responses, which would be essential to circumvent dilution effects caused by the mixture of different tissue types and/or the simultaneous exhibition of different auxin responses at the transcriptional level (Teale et al., 2006; Paponov et al., 2008).

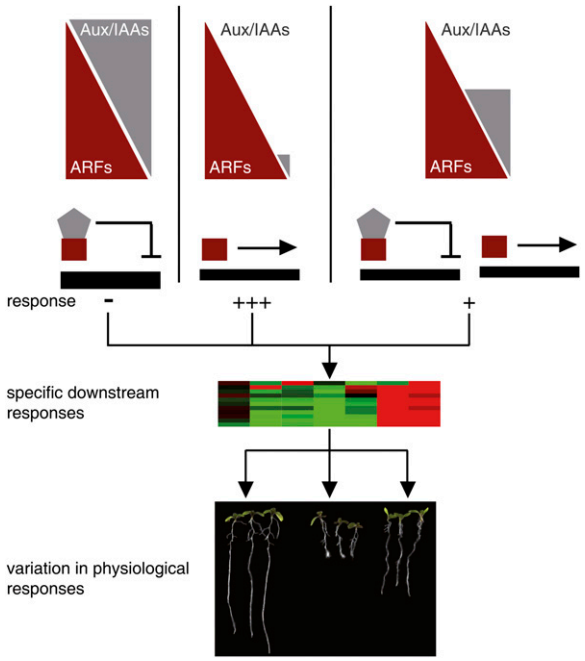


Figure 8. Model of the Putative Impact of Different Aux/IAA–ARF Equilibria on Downstream Transcriptional and Physiological Responses.

ARFs (red square) are known to regulate the activity of primary auxin response genes. The direct interaction with Aux/IAA proteins (gray pentagon), however, inhibits ARF function. Therefore, differences in the equilibria of interacting Aux/IAAs and ARFs are likely to trigger alterations in downstream transcriptional responses that might ultimately contribute to variations at the physiological level.
[See online article for color version of this figure.]

Identification of Specific Factors Involved in the Natural Variation of Auxin Responses

Our model suggests that quantitative distortions in signaling element compositions contribute to the downstream variation in auxin responses. This raises two major questions: (1) what influences/regulates the variation at the level of signaling genes? and (2) what are the relevant downstream factors that are actually involved in the regulation of auxin responses at the physiological level? Auxin biology is influenced by several major processes, such as biosynthesis, metabolism, transport, and signaling. Natural variation in signaling gene expression and subsequent responses could, in principle, be caused by variation in all of these processes or by different sensitivities to stimuli that have been shown to influence them, such as other phytohormones or circadian rhythms (Covington and Harmer, 2007). While extensive accession-specific differences in expression patterns have been observed in several studies, it is also evident that most likely only a fraction of the detectable differences will actually contribute to the variation seen on a phenotypic level. This effect of genetic buffering has also been assessed in *Arabidopsis*, and it was shown that 16% of the transcriptional variation detected between Col-0 and L*er* can be attributed to as few as six QTL "hot spot" regions (Fu et al., 2009). Based on our data, we can make no estimation on how many factors are actually involved in the regulation of the observed expression level polymorphisms (ELPs). Some ongoing experiments in our laboratory based on accession intercrosses and QTL analysis favor quantitative genetics versus Mendelian inheritance. Hence, we assume that the causative factors for the observed natural variation in auxin responses are regulated and inherited in a quantitative genetic manner. Future studies, such as eQTL analysis, need to be employed to unravel the cause of the detected ELPs. Even though we were able to identify genes whose variations in expression profiles correlate with the physiological responses (see Supplemental Figure 15 online), their actual relevance in the observed variation in auxin responses needs to be verified in tissue-specific approaches.

In summary, we found that natural variation does not only exist for traits and pathways that display obvious ecological relevance in terms of adaptive advantages/specializations, but natural variation is similarly present in a pathway that is essential for the integration of numerous developmental and environmental stimuli. The extensive accession-specific variations in auxin responses add yet another fundamental set of data to the startlingly complex picture of intraspecific variation. The data obtained in this study suggest that the temporal response to auxin stimuli is tightly regulated and conserved across accessions. Furthermore, natural *Arabidopsis* accessions generally possess the same set of signaling genes. Although isolated polymorphisms resulting in functional variation on the protein level cannot be ruled out, auxin signaling genes seem to be highly conserved at the population level. However, ELPs within these

gene families seem to contribute considerably to the variation in downstream responses. This may ultimately impact the observed natural variation in physiological responses and, thereby, potentially contribute to adaptation processes. Future approaches will need to focus on specific physiological responses or tissue-specific assays in order to facilitate the association of phenotypes to specific genes or to relevant expression profiles in causative tissues.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana accessions were obtained from the Nottingham Arabidopsis Stock Centre and the Arabidopsis Biological Resource Center (see Supplemental Table 3 online for stock numbers). Seeds were surface-sterilized and imbibed in deionized H_2O for 3 d at 4°C before sowing. Seedlings were germinated and grown under sterile conditions on Arabidopsis thaliana solution (ATS) nutrient medium (Lincoln et al., 1990).

For root growth assays, seedlings were cultivated vertically on unsupplemented ATS for 3 d (IAA) or 5 d (2,4-D and NAA) before transfer to plates supplemented with IAA, 2,4-D, or NAA at the indicated concentrations. Root lengths were quantified after an additional 5 d (IAA) or 3 d (2,4-D and NAA). Hypocotyl growth was quantified in seedlings cultivated for 10 d under long-day lighting conditions at 29°C. Root and hypocotyl lengths of hormone- and heat-treated seedlings, respectively, were determined in relation to seedlings grown on unsupplemented ATS medium at 20°C.

For expression studies, seedlings of the seven accessions were germinated and cultivated in liquid ATS under continuous illumination to minimize potential circadian effects. After 7 d, ATS was supplemented with 1 μM IAA for 0, 0.5, 1, and 3 h. Yellow long-pass filters were applied in all IAA treatment experiments to prevent photodegradation of IAA.

Statistical Analysis of Physiological Data

After $\log_2$ transformation of the four physiological growth response data sets (IAA, 2,4-D, NAA, and hypocotyl), the following two variants of analysis of variance (ANOVA) were performed. (1) Treated and untreated samples were separated for each of the data sets. For both sets of 20 treated and untreated samples, a one-way ANOVA with 20 groups corresponding to the 20 accessions studied was performed. Subsequently, the Tukey post hoc test was conducted to identify the pairs of accessions that are significantly different among the 20 treated samples and among the 20 untreated samples. (2) A two-way ANOVA with 2×2 groups was conducted for each of the 190 pairs of the 20 accessions, testing which pairs of accessions show a significantly different response to auxin. The resulting P values were corrected for multiple testing using the Benjamini-Hochberg correction method.

Microarray Experiments and qRT-PCR Analyses

RNA was extracted from the homogenized plant material of whole seedlings (7 d) of seven accessions grown in liquid culture in three biological replicates for each time point (with the exception of only two replicates for the 3-hpi Fei-0 sample) using the Qiagen RNeasy Plant Mini Kit with an on-column DNase treatment. Further processing of purified RNA and hybridization to whole genome Affymetrix ATH1 GeneChip microarrays was performed by the Nottingham Arabidopsis Stock Centre's International Affymetrix Service (http://affymetrix.arabidopsis.info/). Processing of plant material and RNA purification for qRT-PCR were performed similarly. One microgram of total RNA was subjected to reverse transcription by SuperScript III reverse transcriptase (Invitrogen). Power SYBR Green PCR Master Mix (Applied Biosystems) was used for subsequent quantitative real-time PCR analyses. The *PP2A* catalytic subunit gene *At1g13320* served as the constitutively expressed reference gene (Czechowski et al., 2005). Comparative expression levels for the respective genes of interest were calculated as $2^{(\text{Ct reference gene} - \text{Ct gene of interest})}$. For oligonucleotide sequences and a complete list of analyzed genes, see Supplemental Table 3 online.

DR5:GUS Cloning, Plant Transformation, and Histochemical Glucuronidase Staining

The *DR5:GUS* construct (Ulmasov et al., 1997) was transferred into the binary vector pGWB1 (Nakagawa et al., 2007) by Gateway cloning via the entry vector pDONR221 (Invitrogen). *Agrobacterium tumefaciens*–mediated (GV3101) transformation of *Arabidopsis* accessions was performed by floral dip (Clough and Bent, 1998), and transgenic seedlings were selected as described previously (Quint et al., 2009). For the histochemical glucuronidase assays, seedlings of eight (Fei-0), six (Sha), and seven (Col-0) independent and homozygous T3 lines were mock treated (0.1% ethanol in liquid ATS) or treated with 1 μM IAA in liquid ATS medium for 3 h and stained overnight at 37°C, as described previously (Stomp, 1991). *GUS* expression in seedlings of the same transgenic lines mock treated or treated with 0.1, 1, or 10 μM IAA for 1 h was quantified by qRT-PCR as described above. For statistical analysis of the *GUS* expression response, a two-way ANOVA was performed on the log-transformed comparative expression level data as described for the analysis of the physiological data (see above).

Quantitation of Free IAA

Five hundred milligrams of plant material of 7-d-old seedlings was homogenized with 10 mL of methanol and 100 ng of $[^{13}C_6]$IAA as internal standard. The homogenate was filtered and placed on a 3-mL DEAE-Sephadex A25 column (Amersham Pharmacia Biotech) followed by three subsequent wash steps with 0.1, 1, and 1.5 N acetic acid in methanol. Elution with 3 mL of 3 N acetic acid in methanol was repeated three times. The solvent of the eluate was evaporated, and the residue was resuspended in 110 μL of 50% methanol followed by HPLC using a Eurospher 100-C18 column. Appropriate fractions were evaporated, resuspended in 100 μL of methanol, and incubated with 400 μL of diazomethane for 10 min. After evaporation of the solvent, samples were resuspended in 60 μL of acetonitrile and analyzed by gas chromatography–mass spectrometry (PolarisQ; Thermo-Finnigan).

Statistical Analyses

Statistical analyses were performed using the software R. The hopach package (2.4.0), multtest package (2.0.0), and simpleaffy package (2.20.0) were obtained from www.bioconductor.org. The fdrtool package (1.2.5), gplots package (2.6.0), st package (1.1.1), and stats package (2.9.0) were downloaded from www.r-project.org.

Processing of Microarray Data

The simpleaffy package was used to obtain robust multi-chip average-normalized $\log_2$ expression levels using default settings. A quality control analysis was conducted using the simpleaffy package to verify that the arrays had similar hybridization efficiencies and background intensities for all accessions.

Defining Gene Clusters

Genes were classified according to their expression profile patterns in Col-0 using a hierarchical clustering algorithm named HOPACH (van der Laan and Pollard, 2003) from the hopach package with Pearson correlation coefficient ($1 - r$) as a distance measure. In total, 112 clusters were identified. The number of genes per cluster ranged from 1 to 1301, with an average of 203.1 genes. Only clusters with at least six genes were retained, which resulted in 51 clusters. The gene-to-cluster mapping of the Col-0 clustering was also used for the other six accessions. The mean expression profile of each of the clusters was computed for each of the seven accessions as the arithmetic mean of the expression values of the genes contained in the cluster. Each mean expression profile has 12 values corresponding to the four time points and the three biological replicates (two replicates for Fei-0, 3 hpi) for each time point. Complete lists of cluster expression profiles and gene cluster identities are presented in Supplemental Data Sets 2 and 3 online, respectively. Each mean expression profile was tested for significant changes in expression levels to retain only the relevant clusters that respond significantly to auxin treatment in at least one accession and at least one time point. To this end, we conducted a one-way ANOVA with four groups (corresponding to the four time points) for each of the seven accessions using the stats package. The obtained P values were adjusted for multiple testing using the Benjamini-Hochberg procedure from the multtest package. Five of the 51 clusters did not reach a Benjamini-Hochberg–corrected P value below 0.05 and were eliminated, yielding a final set of 46 clusters. The initial cluster numbers according to the HOPACH clustering result were maintained ranging from 0 to 111.

Coexpression Network Analysis by LCF

Network analyses were conducted on the expression data using LCF following the procedure described by Katagiri and Glazebrook (2003), with a maximum number of seven possible neighbors (k = 7). The visualization of the graphs was done using Graphviz version 2.20.3 (http://www.graphviz.org) with neato as layout algorithm.

Three data sets were subjected to LCF analysis: (1) whole transcriptome data sets of all accessions, (2) expression profiles of 47 auxin signaling genes analyzed separately for each accession, and (3) mean expression profiles of 46 predefined clusters also analyzed separately for each accession. To filter for robust edges in the LCF networks, we used the bootstrapping approach (Katagiri and Glazebrook, 2003) by keeping genes and drawing with replacement experiment vectors. For each of the 1000 surrogate data sets generated by the bootstrapping approach, one LCF network was computed.

For analysis of variations in whole genome transcriptomes, the averaged $\log_2$ expression values of the three replicates of each of the four time points for each of the seven accessions were analyzed by LCF. All of the 28 analyzed transcriptome profiles consisted of 22,746 genes. For analysis of auxin signaling elements, all genes coding for TIR1/AFBs, Aux/IAAs, or ARFs were selected that are represented by a single, unique probe on the ATH1 microarray, resulting in 47 genes (see Supplemental Table 2 online). LCF and bootstrapping were performed for each accession individually based on the expression profiles consisting of all replicates at all time points. Subsequently, the Col-0 network was compared with the networks of the other six accessions by determining common edges of both networks. To determine the robustness and significance of the number of common edges, 1000 surrogate data sets were generated by bootstrapping for each of the accessions.

LCF analysis and bootstrapping of cluster coexpressions was also performed separately for each accession based on the averaged expression profiles, each consisting of 12 values of the 46 predefined clusters. Individual networks were subsequently compared with the Col-0 network.

Expression Level Analysis

Variations in auxin-induced expression changes of a gene or cluster at individual time points post induction were analyzed by using the modified *t* test of the st package from Opgen-Rhein and Strimmer (2007). The mean $\Delta\log_2$ expression values of a gene between each treatment time point and the 0-h control were computed for each accession. The resulting P values were Benjamini-Hochberg corrected for multiple testing. The results were projected on the Col-0 LCF network structures defined above. Significant differences ($P < 0.05$) in expression changes of a gene or a cluster are denoted by colored nodes for each accession. Nodes were colored blue and yellow if the corresponding *t* value was lower or higher than 0, respectively.

Heat Maps

Heat maps were generated using the heatmap.2 function of the gplots package. The Pearson correlation coefficient ($1 - r$) was used as a distance measure of the agglomerative hierarchical clustering with complete linkage. Heat maps of mean $\Delta\log_2$ values were generated for clusters and signaling genes (see Supplemental Figure 9 online). Data sets were separated into three parts based on the time point post induction. The dendrogram of the accessions was computed based on the mean $\Delta\log_2$ cluster data of all three time points also using agglomerative hierarchical clustering with complete linkage and $1 - r$ as a distance measure.

Correlation Analysis of Physiological and Expression Data

The $\Delta\log_2$ levels of the physiological IAA responses were computed for each of the seven accessions used for the expression studies, resulting in a profile consisting of seven values. For each of the 22,810 genes in the expression data set, the $\Delta\log_2$ values of the treated (mean of expression values of all replicates for 0.5 and 1 h) and untreated (0 h) samples for each ecotype were computed, resulting in 22,810 profiles consisting of seven values each. The Pearson *r* value between each of the $\Delta\log_2$ profiles of the 22,810 genes and the $\Delta\log_2$ profile of the physiological IAA responses was computed. The $\Delta\log_2$ profiles of the 10 genes with highest and lowest correlation coefficients are presented in Supplemental Figure 15 online. Genes with $r > 0.8$ and $r < -0.8$ were considered to be positively and negatively correlated, respectively. Both groups were subjected to Gene Ontology term enrichment analysis using the AmiGO tool (http://amigo.geneontology.org/cgi-bin/amigo/term_enrichment).

Sequence Analysis of Signaling Genes

For sequence analysis of auxin signaling genes, the respective gene fragments from the 18 accessions used in the physiological growth assays were sequenced (all except Van-0). The available Col-0 reference sequence was also included in all subsequent analyses. DNA was extracted from leaf tissue. Primers were designed on the basis of the Col-0 reference genome (see Supplemental Table 1 online) for *TIR1/AFB*s, *Aux/IAA*s, or *ARF*s, resulting in 49 genes (see Supplemental Table 1 online). Sequences of ~1-kb PCR products were generated on an ABI 3730 XL (Applied Biosystems) automated sequencer in collaboration with the Leibniz Institute of Plant Genetics and Crop Research in Gatersleben, Germany. Fragments were sequenced in both directions. All sequences and polymorphisms were validated by visual inspection of the chromatograms and edited where appropriate. Alignments were performed with BioEdit version 7.0.5 software (Hall, 1999). Nucleotide diversity for all sites (π), synonymous sites (π_s), and nonsynonymous sites (π_a) was calculated in DnaSP 5.1 (Librado and Rozas, 2009) after Nei (1987). Heterozygous sites were treated as missing data. The 19 accessions included in this study are part of a set of 96 accessions that have

been extensively characterized for polymorphism in 876 genomic fragments (Nordborg et al., 2005). The sequences of 236 fragments were extracted with a minimum of 400-bp coding sequence from this data set for the 19 accessions. This empirical distribution was aligned and analyzed in exactly the same manner as the auxin signaling genes. Distributions of nucleotide diversity summary statistics calculated for the auxin signaling genes were then compared with the empirical distributions of the control genes by performing Mann-Whitney *U* tests in R.

Accession Numbers

All microarray data from this article are publicly available at the Gene Expression Omnibus under accession number GSE18975. Sequence data from this article have been submitted to GenBank (accession numbers GU348425–GU348653 and HM487319–HM487971). Arabidopsis Genome Initiative locus identifiers of individual genes analyzed in this article are listed in Supplemental Tables 1, 2, and 4 online.

Supplemental Data

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

Supplemental Figure 1. NAA Root Growth Assay (Data and Statistics).

Supplemental Figure 2. 2,4-D Root Growth Assay (Data and Statistics).

Supplemental Figure 3. IAA Root Growth Assay (Data and Statistics).

Supplemental Figure 4. Temperature-Induced Hypocotyl Elongation Assay (Data and Statistics).

Supplemental Figure 5. Histochemical Detection of DR5:GUS Activity (Individual Lines).

Supplemental Figure 6. IAA Quantitation.

Supplemental Figure 7. Correlation of qRT-PCR and Microarray Data.

Supplemental Figure 8. Identification of Putative Hyperresponsive and Hyporesponsive Accessions.

Supplemental Figure 9. Heat Maps of Signaling Gene Expression.

Supplemental Figure 10. Pair-Wise Comparison of LCF Networks for Signaling Genes.

Supplemental Figure 11. Pair-Wise Comparison of Signaling Gene Expression.

Supplemental Figure 12. Pair-Wise Comparison of LCF Networks of Cluster Genes.

Supplemental Figure 13. Pair-Wise Comparison of Cluster Gene Expression.

Supplemental Figure 14. Detailed Heat Map Presentation of Mean Expression Changes ($\Delta\log_2$) of All Genes within Cluster 100 (1 hpi).

Supplemental Figure 15. Correlation between Phenotypic and Expression Responses to IAA.

Supplemental Table 1. Signaling Genes Selected for LCF Analyses.

Supplemental Table 2. *A. thaliana* Accession Numbers.

Supplemental Table 3. qRT-PCR Primers.

Supplemental Data Set 1. Nucleotide Diversity of Auxin Signaling Genes.

Supplemental Data Set 2. Cluster Expression Profiles.

Supplemental Data Set 3. Cluster Gene Identities.

ACKNOWLEDGMENTS

We are grateful to Tom Guilfoyle for providing the *DR5:GUS* construct, Renate Schmidt for sequencing support, Kathrin Denk for technical assistance, and Jan Grau, Jens Keilwagen, and Steffen Neumann for valuable discussions. We thank Claus Wasternack and Jerry Cohen for critical reading of the manuscript and anonymous reviewers for constructive comments. Furthermore, M.Q. thanks Bill Gray for support in the initial phase of the project. Our work was supported by a grant from the Exzellenznetzwerk Biowissenschaften "Structures and Mechanisms of Biological Information Processing" funded by the Federal State of Sachsen-Anhalt to M.Q.

Received January 13, 2010; revised May 19, 2010; accepted June 18, 2010; published July 9, 2010.

REFERENCES

Alonso-Blanco, C., Aarts, M.G.M., Bentsink, L., Keurentjes, J.J.B., Reymond, M., Vreugdenhil, D., and Koornneef, M. (2009). What has natural variation taught us about plant development, physiology, and adaptation? Plant Cell **21:** 1877–1896.

Borevitz, J.O., Hazen, S.P., Michael, T.P., Morris, G.P., Baxter, I.R., Hu, T.T., Chen, H., Werner, J.D., Nordborg, M., Salt, D.E., Kay, S.A., Chory, J., et al. (2007). Genome-wide patterns of single-feature polymorphism in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **104:** 12057–12062.

Chapman, E.J., and Estelle, M. (2009). Mechanism of auxin-regulated gene expression in plants. Annu. Rev. Genet. **43:** 265–285.

Clark, R.M., Schweikert, G., Toomajian, C., Ossowski, S., Zeller, G., Shinn, P., Warthmann, N., Hu, T.T., Fu, G., Hinds, D.A., Chen, H., Frazer, K.A., et al. (2007). Common sequence polymorphisms shaping genetic diversity in *Arabidopsis thaliana*. Science **317:** 338–342.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Covington, M.F., and Harmer, S.L. (2007). The circadian clock regulates auxin signaling and responses in Arabidopsis. PLoS Biol. **5:** e222.

Czechowski, T., Bari, R.P., Stitt, M., Scheible, W.-R., and Udvardi, M.K. (2004). Real-time RT-PCR profiling of over 1400 *Arabidopsis* transcription factors: Unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J. **38:** 366–379.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.-R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. **139:** 5–17.

Delker, C., Raschke, A., and Quint, M. (2008). Auxin dynamics: The dazzling complexity of a small molecule's message. Planta **227:** 929–941.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005a). The F-box protein TIR1 is an auxin receptor. Nature **435:** 441–445.

Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L., Ehrismann, J.S., Jürgens, G., and Estelle, M. (2005b). Plant development is regulated by a family of auxin receptor F box proteins. Dev. Cell **9:** 109–119.

Fu, J., Keurentjes, J.J.B., Bouwmeester, H., America, T., Verstappen, F.W.A., Ward, J.L., Beale, M.H., de Vos, R.C.H., Dijkstra, M., Scheltema, R.A., Johannes, F., Koornneef, M., et al. (2009). System-wide molecular evidence for phenotypic buffering in Arabidopsis. Nat. Genet. **41:** 166–167.

Goda, H., Sasaki, E., Akiyama, K., Maruyama-Nakashita, A., Nakabayashi, K., Li, W., Ogawa, M., Yamauchi, Y., Preston, J., Aoki, K., Kiba, T., Takatsuto, S., et al. (2008). The AtGenExpress hormone and chemical treatment data set: Experimental design, data evaluation, model data analysis and data access. Plant J. **55:** 526–542.

Gray, W.M., Östin, A., Sandberg, G., Romano, C.P., and Estelle, M. (1998). High temperature promotes auxin-mediated hypocotyl elongation in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95:** 7197–7202.

Guilfoyle, T., Ulmasov, T., and Hagen, G. (1998). The ARF family of transcription factors and their role in plant hormone-responsive transcription. Cell. Mol. Life Sci. **54:** 619–627.

Hall, T. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:** 95–98.

Katagiri, F., and Glazebrook, J. (2003). Local Context Finder (LCF) reveals multidimensional relationships among mRNA expression profiles of *Arabidopsis* responding to pathogen infection. Proc. Natl. Acad. Sci. USA **100:** 10842–10847.

Kepinski, S., and Leyser, O. (2005). The *Arabidopsis* F-box protein TIR1 is an auxin receptor. Nature **435:** 446–451.

Keurentjes, J.J.B., Fu, J., Terpstra, I.R., Garcia, J.M., van den Ackerveken, G., Snoek, L.B., Peeters, A.J.M., Vreugdenhil, D., Koornneef, M., and Jansen, R.C. (2007). Regulatory network construction in Arabidopsis by using genome-wide gene expression quantitative trait loci. Proc. Natl. Acad. Sci. USA **104:** 1708–1713.

Kliebenstein, D.J., West, M.A.L., van Leeuwen, H., Kim, K., Doerge, R.W., Michelmore, R.W., and St. Clair, D.A. (2006). Genomic survey of gene expression diversity in Arabidopsis thaliana. Genetics **172:** 1179–1189.

Kreitman, M. (2000). Methods to detect selection in populations with applications to the human. Annu. Rev. Genomics Hum. Genet. **1:** 539–559.

Librado, P., and Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics **25:** 1451–1452.

Lincoln, C., Britton, J., and Estelle, M. (1990). Growth and development of the *axr1* mutants of *Arabidopsis*. Plant Cell **2:** 1071–1080.

Lokerse, A.S., and Weijers, D. (2009). Auxin enters the matrix—Assembly of response machineries for specific outputs. Curr. Opin. Plant Biol. **12:** 1–7.

Maloof, J.N., Borevitz, J.O., Dabi, T., Lutes, J., Nehring, R.B., Redfern, J.L., Trainer, G.T., Wilson, J.M., Asami, T., Berry, C.C., Weigel, D., and Chory, J. (2001). Natural variation in light sensitivity of *Arabidopsis*. Nat. Genet. **29:** 441–446.

Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C., Estelle, M., and Reed, J.W. (2000). AXR2 encodes a member of the Aux/IAA protein family. Plant Physiol. **123:** 563–574.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., and Kimura, T. (2007). Development of series of Gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J. Biosci. Bioeng. **104:** 34–41.

Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M., and Narusaka, Y. (2009). *RRS1* and *RPS4* provide a dual resistance-gene system against fungal and bacterial pathogens. Plant J. **60:** 218–226.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D.G. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science **312:** 436–439.

Nei, M. (1987). Molecular Evolutionary Genetics. (New York: Columbia University Press).

Nei, M., and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76:** 5269–5273.

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

Nordborg, M., Hu, T.T., Ishino, Y., Jhaveri, J., Toomajian, C., Zheng, H., Bakker, E., Calabrese, P., Gladstone, J., Goyal, R., Jakobsson, M., Kim, S., et al. (2005). The pattern of polymorphism in *Arabidopsis thaliana*. PLoS Biol. **3:** e196.

Okushima, Y., Overvoorde, P.J., Arima, K., Alonso, J.M., Chan, A., Chang, C., Ecker, J.R., Hughes, B., Lui, A., Nguyen, D., Onodera, C., Quach, H., et al. (2005). Functional genomic analysis of the *AUXIN RESPONSE FACTOR* gene family members in *Arabidopsis thaliana*: Unique and overlapping functions of ARF7 and ARF19. Plant Cell **17:** 444–463.

Opgen-Rhein, R., and Strimmer, K. (2007). Accurate ranking of differentially expressed genes by a distribution-free shrinkage approach. Stat. Appl. Genet. Mol. Biol. **6:** Article 9.

Overvoorde, P.J., Okushima, Y., Alonso, J.M., Chan, A., Chang, C., Ecker, J.R., Hughes, B., Liu, A., Onodera, C., Quach, H., Smith, A., Yu, G., et al. (2005). Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in *Arabidopsis thaliana*. Plant Cell **17:** 3282–3300.

Paponov, I.A., Paponov, M., Teale, W., Menges, M., Chakrabortee, S., Murray, J.A.H., and Palme, K. (2008). Comprehensive transcriptome analysis of auxin responses in Arabidopsis. Mol. Plant **1:** 321–337.

Parry, G., Calderon-Villalobos, L.I., Prigge, M., Peret, B., Dharmasiri, S., Itoh, H., Lechner, E., Gray, W.M., Bennett, M., and Estelle, M. (2009). Complex regulation of the TIR1/AFB family of auxin receptors. Proc. Natl. Acad. Sci. USA **106:** 22540–22545.

Parry, G., and Estelle, M. (2006). Auxin receptors: A new role for F-box proteins. Curr. Opin. Cell Biol. **18:** 152–156.

Perez-Torres, C.-A., Lopez-Bucio, J., Cruz-Ramirez, A., Ibarra-Laclette, E., Dharmasiri, S., Estelle, M., and Herrera-Estrella, L. (2008). Phosphate availability alters lateral root development in *Arabidopsis* by modulating auxin sensitivity via a mechanism involving the TIR1 auxin receptor. Plant Cell **20:** 3258–3272.

Ploense, S.E., Wu, M.-F., Nagpal, P., and Reed, J.W. (2009). A gain-of-function mutation in IAA18 alters Arabidopsis embryonic apical patterning. Development **136:** 1509–1517.

Quint, M., Barkawi, L.S., Fan, K.-T., Cohen, J.D., and Gray, W.M. (2009). Arabidopsis IAR4 modulates auxin response by regulating auxin homeostasis. Plant Physiol. **150:** 748–758.

Quint, M., and Gray, W.M. (2006). Auxin signaling. Curr. Opin. Plant Biol. **9:** 448–453.

Ramos, J.A., Zenser, N., Leyser, O., and Callis, J. (2001). Rapid degradation of Auxin/Indoleacetic Acid proteins requires conserved amino acids of domain II and is proteasome dependent. Plant Cell **13:** 2349–2360.

Rowe, H.C., and Kliebenstein, D.J. (2008). Complex genetics control natural variation in *Arabidopsis thaliana* resistance to *Botrytis cinerea*. Genetics **180:** 2237–2250.

Ruegger, M., Dewey, E., Gray, W.M., Hobbie, L., Turner, J., and Estelle, M. (1998). The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast Grr1p. Genes Dev. **12:** 198–207.

Salmon, J., Ramos, J., and Callis, J. (2008). Degradation of the auxin response factor ARF1. Plant J. **54:** 118–128.

Stavang, J.A., Gallego-Bartolomé, J., Gómez, M.D., Yoshida, S., Asami, T., Olsen, J.E., García-Martínez, J.L., Alabadí, D., and Blázquez, M.A. (2009). Hormonal regulation of temperature-induced growth in *Arabidopsis*. Plant J. **60:** 589–601.

Stomp, A.-M. (1991). Histochemical localization of β-glucuronidase. In GUS Protocols, S.R. Gallagher, ed (London: Academic Press), pp. 103–113.

Teale, W.D., Paponov, I.A., and Palme, K. (2006). Auxin in action: Signalling, transport and the control of plant growth and development. Nat. Rev. Mol. Cell Biol. **7:** 847–859.

Tian, Q., and Reed, J.W. (1999). Control of auxin-regulated root development by the Arabidopsis thaliana SHY2/IAA3 gene. Development **126:** 711–721.

Tiwari, S., Hagen, G., and Guilfoyle, T. (2003). The roles of auxin response factor domains in auxin-responsive transcription. Plant Cell **15:** 533–543.

Ulmasov, T., Hagen, G., and Guilfoyle, T. (1999). Activation and repression of transcription by auxin-response factors. Proc. Natl. Acad. Sci. USA **96:** 5844–5849.

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9:** 1963–1971.

van der Laan, M.J., and Pollard, K.S. (2003). A new algorithm for hybrid hierarchical clustering with visualization and the bootstrap. J. Statist. Plann. Inference **117:** 275–303.

van Leeuwen, H., Kliebenstein, D.J., West, M.A.L., Kim, K., van Poecke, R., Katagiri, F., Michelmore, R.W., Doerge, R.W., and St. Clair, D.A. (2007). Natural variation among *Arabidopsis thaliana* accessions for transcriptome response to exogenous salicylic acid. Plant Cell **19:** 2099–2110.

van Poecke, R.M.P., Sato, M., Lenarz-Wyatt, L., Weisberg, S., and Katagiri, F. (2007). Natural variation in RPS2-mediated resistance among *Arabidopsis* accessions: Correlation between gene expression profiles and phenotypic responses. Plant Cell **19:** 4046–4060.

Zenser, N., Ellsmore, A., Leasure, C., and Callis, J. (2001). Auxin modulates the degradation rate of Aux/IAA proteins. Proc. Natl. Acad. Sci. USA **98:** 11795–11800.

Zhang, X., Shiu, S., Cal, A., and Borevitz, J.O. (2008). Global analysis of genetic, epigenetic and transcriptional olymorphisms in *Arabidopsis thaliana* using whole genome tiling arrays. PLoS Genet. **4:** e1000032.