

# Gibberellins Regulate Lateral Root Formation in *Populus* through Interactions with Auxin and Other Hormones <sup>©|W</sup>

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**The role of gibberellins (GAs) in regulation of lateral root development is poorly understood. We show that GA-deficient (*35S:PgA2ox1*) and GA-insensitive (*35S:rg1*) transgenic *Populus* exhibited increased lateral root proliferation and elongation under in vitro and greenhouse conditions, and these effects were reversed by exogenous GA treatment. In addition, RNA interference suppression of two poplar GA 2-oxidases predominantly expressed in roots also decreased lateral root formation. GAs negatively affected lateral root formation by inhibiting lateral root primordium initiation. A whole-genome microarray analysis of root development in GA-modified transgenic plants revealed 2069 genes with significantly altered expression. The expression of 1178 genes, including genes that promote cell proliferation, growth, and cell wall loosening, corresponded to the phenotypic severity of the root traits when transgenic events with differential phenotypic expression were compared. The array data and direct hormone measurements suggested crosstalk of GA signaling with other hormone pathways, including auxin and abscisic acid. Transgenic modification of a differentially expressed gene encoding an auxin efflux carrier suggests that GA modulation of lateral root development is at least partly imparted by polar auxin transport modification. These results suggest a mechanism for GA-regulated modulation of lateral root proliferation associated with regulation of plant allometry during the stress response.**

## INTRODUCTION

In addition to physical support, the root system of plants enables the absorption and transport of nutrients and water (McCully and Canny, 1988; Varney and Canny, 1993). Lateral roots (LRs) are the most dynamic and physiologically active part of the root system. The developmental plasticity of LR formation (Robinson, 1994) allows the plant to explore the highly heterogeneous soil environment and to adapt to changing nutrient and water availability. Because of its significance to agronomic traits, such as stress tolerance and nutrient and water use efficiency, the mechanisms of LR formation have been intensively studied (reviewed in Osmont et al., 2007).

LRs are initiated in the differentiation zone of primary roots from pericycle founder cells that are adjacent to the protoxylem poles. A series of cell divisions in the founder cells result in formation of a primordium and subsequent root emergence (Bhalerao et al., 2002). LR formation is regulated by an intrinsic

developmental program and environmental signals, such as nutrient concentrations (Zhang and Forde, 2000; Malamy and Ryan, 2001; Osmont et al., 2007). Auxin has a major role in almost all steps of LR initiation and development (Himanen et al., 2004; Aloni et al., 2006). However, other phytohormones that include ethylene, cytokinin, brassinosteroid, and abscisic acid (ABA) can also regulate the process, usually in an auxin-dependent manner (De Smet et al., 2003; Aloni et al., 2006; Stepanova et al., 2007). By contrast, very little is known about what role, if any, gibberellins (GAs) have in LR formation (Osmont et al., 2007; Fukaki and Tasaka, 2009).

GAs are phytohormones that regulate a wide range of developmental processes, including seed germination, leaf expansion, stem elongation, flowering, and fruit and seed development (Sun and Gubler, 2004; Swain and Singh, 2005). Because of their important role(s) in plant development, and because they played a major part in the green revolution (Hedden, 2003), GA metabolic and signaling pathways have been intensively dissected (Olszewski et al., 2002). GA biosynthesis proceeds through three main stages with specific intracellular localizations (Olszewski et al., 2002). The flux of bioactive GAs is regulated by three dioxygenase enzymes, including GA 20-, GA 3-, and GA 2-oxidases. GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox) catalyze the final steps in the synthesis of bioactive GAs, whereas GA 2-oxidase (GA2ox) is the major GA deactivation enzyme (Yamaguchi, 2008). These enzymes are encoded by small gene families with distinct spatiotemporal

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expression patterns. In poplar (species of genus *Populus* spp), GA 20ox and GA 2ox, but not GA 3ox, appear to regulate the level of bioactive GAs (Eriksson et al., 2000; Eriksson and Moritz, 2002; Busov et al., 2003, 2006).

Components of the GA signal transduction cascade, including the receptor and several positive and negative regulators, have also been reported (Sun and Gubler, 2004; Ueguchi-Tanaka et al., 2005). DELLA proteins play a central role in the GA response and appear to be a crosstalk point with other signals (Achard et al., 2006; Nemhauser et al., 2006). They act as negative regulators, and their proteolytic degradation in the presence of GA leads to activation of GA-mediated responses (Sun and Gubler, 2004; Zentella et al., 2007). Deletions or nonsynonymous mutations in the conserved DELLA domain render the protein insensitive to degradation and constitutively block the GA response. In addition to mediating GA responses, recent studies suggest that DELLAs may mediate the response to ABA, ethylene, auxin, and abiotic stress (Achard et al., 2003, 2006; Fu and Harberd, 2003; Weiss and Ori, 2007).

GA's role in root development is poorly understood (Osmont et al., 2007; Fukaki and Tasaka, 2009). Several studies have suggested a role for GAs in primary root elongation. GA appears to affect cell expansion in the root elongation zone via destabilizing DELLA proteins like Repressor of GA1 (RGA) (Fu and Harberd, 2003). Lesions in auxin transport or signaling lead to delay of DELLA destabilization, suggesting crosstalk with auxin during root elongation (Fu and Harberd, 2003). Similarly, ethylene-induced inhibition of root elongation was found to be mediated by DELLA (GA Insensitive-GAI and RGA) proteins (Achard et al., 2006). Most recently, GAI was found to play a role in endodermal cell expansion in the elongation zone of *Arabidopsis thaliana* primary roots (Ubeda-Tomas et al., 2008). Microarray analysis showed that the expression pattern of GA-related genes did not conform to classically defined tissue boundaries, suggesting a novel function of GAs in generating organizing centers of root development (Birnbaum et al., 2003).

The role of GAs in LR formation is almost completely unknown. Several lines of evidence suggest that active GAs may inhibit LR formation. Studies in tomato (*Solanum lycopersicum*) (Berova and Zlatev, 2000), pepper (*Capsicum chinense*) (Grossi et al., 2005), and several trees species (Chaney, 2003; Watson, 2004) suggest that inhibitors of GAs biosynthesis, such as paclobutrazol, can stimulate LR formation. Blockage of GA signaling via heterologous expression of DELLA-less versions of GAI and RGL1 in *Populus* elicited an increase in root biomass, likely via LR proliferation (Busov et al., 2006). By contrast, GA-overproducing mutations and exogenous GA applications in aspen (*Populus tremula*) led to suppression of lateral and adventitious root formation (Eriksson et al., 2000). Similarly, in rice (*Oryza sativa*), a deficiency of GA increased adventitious root formation, while exogenous application of GA<sub>3</sub> suppressed it (Lo et al., 2008). Several recent studies have examined GA responses using microarray transcription profiling, focusing on flowers and seeds (Cao et al., 2006; Zentella et al., 2007; Hou et al., 2008). However, none of these have studied the root transcriptome. Here, we demonstrate a major role for GA in LR formation in *Populus* using transgenic and physiological experiments and provide insight into the transcriptional mechanisms of its effects.

## RESULTS AND DISCUSSION

### Altered GA Synthesis and Signaling Affected Later Root Development in *Populus*

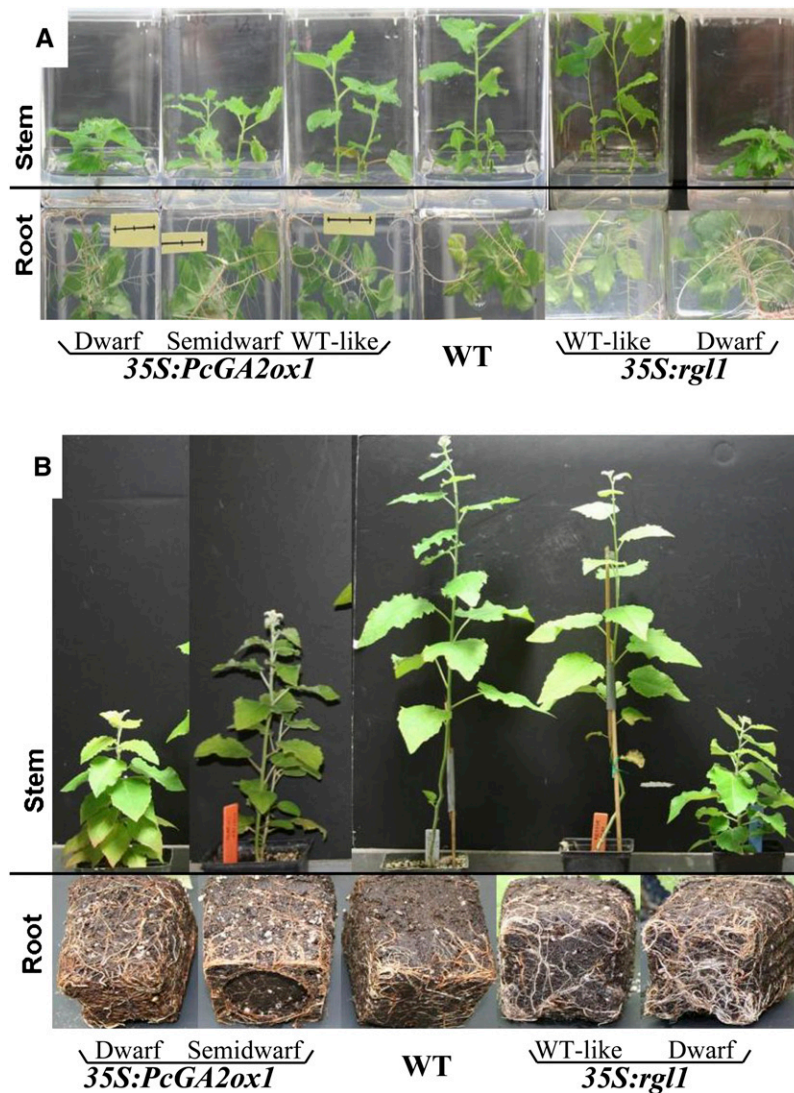
We investigated the role of GAs in LR development using GA-deficient and GA-insensitive transgenic *Populus* overexpressing Pc GA2ox1 and *rgl1* genes, respectively, under the constitutive cauliflower mosaic virus 35S promoter. Generation and initial characterization were described elsewhere (Busov et al., 2003, 2006). Among the multiple (20+) independent events for each of the two transgenic types, we selected events that display different levels of dwarfism: dwarf, semidwarf, and wild type like. Because *rgl1* caused a more severe phenotype, no semidwarf events were recovered (Figure 1). All events stably displayed their phenotypes for 3 years under in vitro (Figure 1A), greenhouse (Figure 1B), and field conditions.

Preliminary greenhouse observations indicated that dwarfing in both transgenic types was accompanied by increased root biomass (Figure 1). Quantitative analysis of LR density and elongation under in vitro conditions showed that the degree of dwarfism in both GA-deficient and GA-insensitive plants was positively correlated with the extent of LR formation and elongation (Figures 2A to 2D). The most severely dwarfed events had two to three times more, as well as longer, LRs than the wild-type control. By contrast, primary root length in transgenics was unaffected (Figures 2E and 2F). Greenhouse-grown dwarf and semidwarf plants of both transgenic types displayed a significant reduction in aerial biomass and an increase in belowground biomass, leading to a significant reduction in the shoot-to-root ratio relative to the wild-type control (Figure 3).

### Microarray Analysis Revealed Crosstalk of Several Hormonal Pathways in the Transgenic Roots

To gain insight into the molecular mechanisms underpinning the observed root phenotypes, we studied the transcriptome changes in transgenic roots using the Affymetrix GeneChip Poplar Genome Array. The expression of 3707 and 3144 genes was found to differ significantly from the control in the 35S: PcGA2ox1 and 35S:*rgl1* transgenics, respectively, and 2069 genes were significant between the control and the dwarf plants of both transgenic types (see Supplemental Data Set 1A online). To validate the microarray data, we performed RT-PCR expression analyses for a subset of 12 differentially expressed (six upregulated and six downregulated) genes. We used the same RNA employed for the microarray hybridizations as well as an additional independent RNA isolation. All of the 12 selected genes displayed expression patterns consistent with the microarray results (Figure 4).

Gene ontology (GO) enrichment analysis showed that among the top 10 most enriched categories, nine were the same in the two transgenic types (Table 1). Notably, the most highly enriched category in both transgenic types was response to hormone stimulus. This suggests a significant crosstalk with other hormones (further discussed below). Half of the top 10 categories were associated with metabolism of amino acid, phenylpropanoid, and steroid and biosynthesis of glucan and lignin. This is



**Figure 1.** Aerial and Root Phenotypes of Different *35S:PcGA2ox1* and *35S:rgl1* Transgenic Lines under in Vitro and Greenhouse Conditions.

**(A)** Four-week-old in vitro-grown plants. Top and bottom panels represent photos of the same plants taken from the side and bottom of the Magenta box. Bars = 10 mm.

**(B)** Two-month-old greenhouse-grown plants. Top and bottom panels show photos of the stem and roots of the same plants, respectively. The root plug was carefully removed from the pot prior to taking the photograph. All photos were taken at the same time and are representative of multiple ramets (+15) of the same transgenic events.

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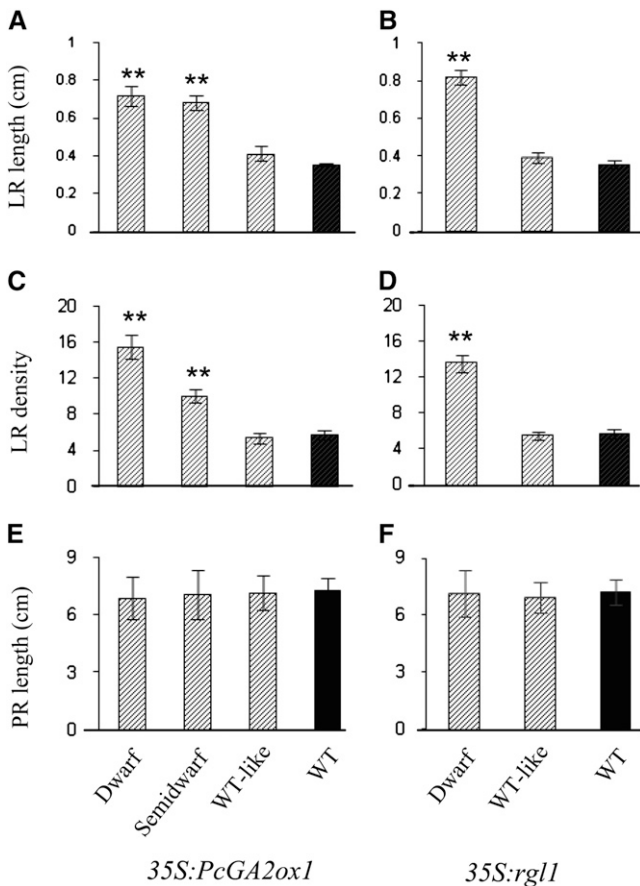
consistent with our previous findings of significant changes in primary and secondary metabolism in the same transgenic poplars (Busov et al., 2006).

We hypothesized that genes that are involved in the observed root phenotypes would (1) show significant expression changes in both transgenic types and (2) be correlated either positively or negatively with the degree of root morphological alterations. Of the 2069 differentially expressed genes common to the two transgenic types (see Supplemental Data Set 1A online), we found 1178 whose expression significantly correlated with the severity of the root phenotype (Figure 5; see Supplemental Data

Set 1B online). Among these, 493 were of unknown function and therefore only 685 genes were informative with respect to putative role(s) in root development. We therefore focused on these 685 genes for further analysis.

#### Cell Wall Modification

One of the responses among the 685 genes' set was the upregulation of genes whose putative functions facilitate cell growth and proliferation (see Supplemental Data Sets 1C and 1D online). This is consistent with the increased LR elongation and



**Figure 2.** Root Development in *35S:PcGA2ox1* and *35S:rgl1* Transgenic Lines Grown In Vitro.

Top is the LR length in *35S:PcGA2ox1* (A) and *35S:rgl1* (B) transgenics. Middle is the LR density in *35S:PcGA2ox1* (C) and *35S:rgl1* (D) transgenics. Bottom is the primary root (PR) length in *35S:PcGA2ox1* (E) and *35S:rgl1* (F) transgenics. Bars show means and SE over three independent experiments where each experiment was performed with at least six ramets per event. \*\*, Significance determined by Student's *t* test ( $P < 0.01$ ). Events in each phenotypic class are as shown in Figure 1. Bars with diagonal stripes indicate transgenics, and black bars are wild-type control plants. Measurements were taken at the same time as described in Methods.

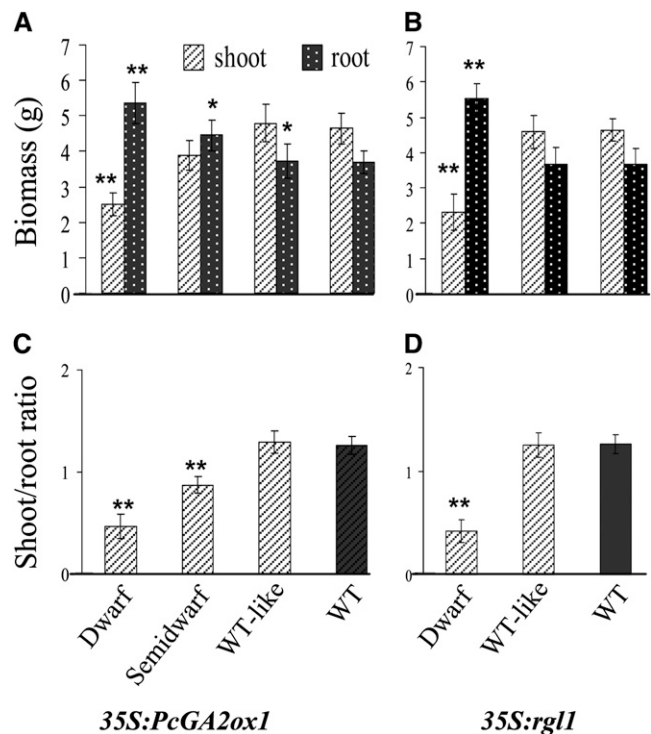
formation that was observed in GA-deficient and GA-insensitive plants. For example, 21 genes encoding various glycosyl hydrolases were upregulated. These enzymes are primarily involved in cleaving the xyloglucan hemicellulose chains cross-linking the cellulose microfibrils, thus leading to cell wall loosening (Cosgrove, 2000). Meanwhile, three UDP-glucose glucosyltransferases that form the xyloglucan hemicellulose chains (Hayashi, 1989; Herrero et al., 2004) were downregulated. Other genes encoding modifiers of cell wall extensibility included two expansins (Cosgrove, 2000) and 15 pectin esterases (Derbyshire et al., 2007). Modification of cell wall rigidity is a prerequisite for both cell elongation and division, as well as for organ growth (Kutschera, 1990). Recently, it was reported that induction of cell

wall remodeling enzymes facilitates the cell separation in front of the emerging LR (Swarup et al., 2008).

Genes encoding enzymes such as cellulose synthase and cellulose synthase-like proteins that are involved in synthesis of polysaccharides for the newly formed cells were well represented in the upregulated category (see Supplemental Data Set 1D online). Likewise, two UDP-glucose dehydrogenase (UGD) genes that encode important enzymes in the synthesis of pectins and hemicelluloses were also activated in the transgenic plants. Expression of UGD genes is typically restricted to growing cells (Johansson et al., 2002; Klinghammer and Tenhaken, 2007).

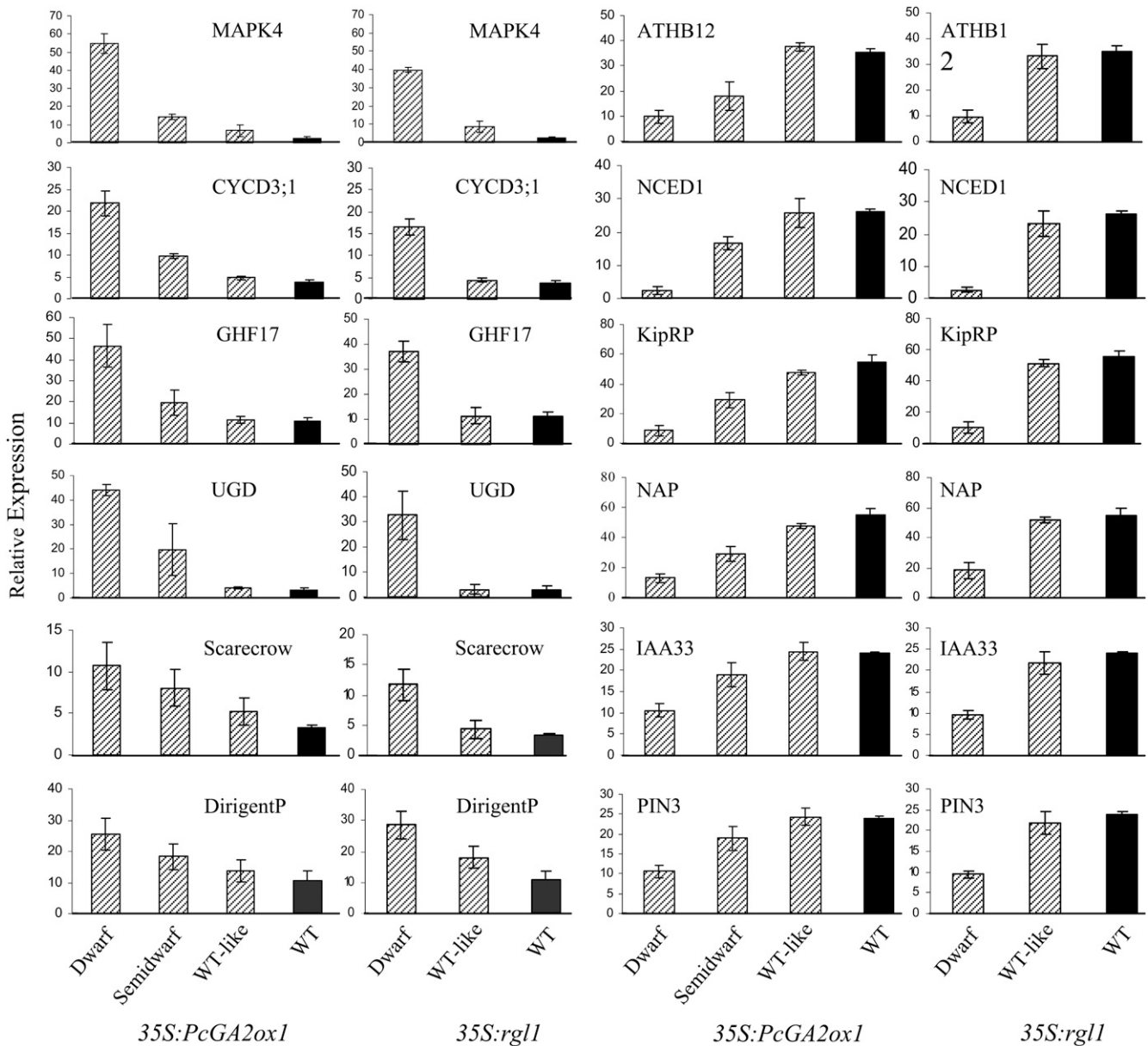
### Cell Division and Cell Cycle Regulation

LR initiation and emergence is preceded by a series of cellular divisions (Bhalerao et al., 2002). Therefore, it is not surprising that we found increased expression of eight genes encoding Cyclins A, B, and D and Cyclin-Dependent Kinase B (see Supplemental Data Set 1D online) that are known to promote both G1 → S and G2 → M transitions. Four other genes encoding proteins involved



**Figure 3.** Root and Shoot Biomass (Including Both Stem and Leaves in the Latter) under Greenhouse Conditions.

Top panel shows the fresh biomass of shoots and roots in *35S:PcGA2ox1* (A) and *35S:rgl1* (B) transgenics. Bottom is the shoot/root ratio in *35S:PcGA2ox1* (C) and *35S:rgl1* (D) transgenics. Bars indicate means and SE of two independent experiments. Each experiment was performed with at least five ramets per event. Biomass was determined as described in Methods on 3-month-old greenhouse-grown plants. \*\* and \* indicate significance as determined by Student's *t* test at  $P < 0.01$  and  $P < 0.05$ , respectively.



**Figure 4.** RT-PCR Verification of Differential Gene Expression.

The left two columns show upregulated genes, and the right two columns show downregulated genes. Bars show means and SE over three independent biological replications. Abbreviations used in the figure correspond to the names used in Supplemental Data Set 1K online.

in cell cycle regulation were also found (see Supplemental Data Set 1D online). For example, Kip-related protein (KipRP) is a CDKA-specific inhibitor that prevents cells from entering the G1→S transition and cell cycle reentry (Sorrell et al., 2001). These proteins play essential roles during LR primordium initiation in *Arabidopsis*, acting during the reactivation of the pericycle founder cells (Himanen et al., 2002). As expected, we found downregulation of *KipRP* poplar putative orthologs in transgenic roots (see Supplemental Data Set 1D online), while other genes encoding proteins promoting various aspects of cell prolifera-

tion, such as *MCM5* (Lake et al., 2007) and *SCARECROW* (Heidstra et al., 2004), were upregulated.

#### Hormone Metabolism and Response

Major shifts in the expression of genes associated with hormone metabolism, signaling, and response were observed (see Supplemental Data Sets 1E to 1H online), due to the regulatory role of hormones in LR formation (Osmont et al., 2007). Consistent with earlier observations showing dramatic changes in GAs

**Table 1.** Overview of Significantly ( $P < 0.05$ ) Enriched Functional GO Categories

Transgene	Category	Percentage of Genes in List	Adjusted P Value
35S:PcGA2ox1	GO:9725: Response to hormone stimulus	3.034	0.000262
	GO:6575: Amino acid derivative metabolism	2.131	3.47E-06
	GO:9698: Phenylpropanoid metabolism	1.481	3.61E-05
	GO:9415: Response to water	1.445	5.41E-12
	GO:9737: Response to ABA stimulus	1.102	9.17E-06
	GO:8202: Steroid metabolism	0.993	1.47E-07
	GO:42545: Cell wall modification	0.903	2.74E-09
	GO:9250: Glucan biosynthesis	0.722	0.000725
	GO:9809: Lignin biosynthesis	0.650	6.43E-05
	GO:9828: Cell wall loosening	0.433	2.03E-09
	35S:rgl1	GO:9725: Response to hormone stimulus	2.358
GO:6575: Amino acid derivative metabolism		2.351	0.00951
GO:9698: Phenylpropanoid metabolism		1.692	4.16E-06
GO:9415: Response to water		1.307	2.00E-06
GO:42545: Cell wall modification		1.051	1.64E-09
GO:8202: Steroid metabolism		0.974	3.05E-05
GO:9250: Glucan biosynthesis		0.897	2.70E-05
GO:9809: Lignin biosynthesis		0.769	1.43E-05
GO:30244: Cellulose biosynthesis		0.692	7.65E-05
GO:9828: Cell wall loosening		0.487	3.44E-08

A hypergeometric test was used to test enrichment significance (Rivals et al., 2007).

concentrations in these transgenics (Busov et al., 2003, 2006), microarray data showed a downregulation of a *GA2ox* gene encoding the main GA catabolic (inactivating) enzymes, while a *GA2ox* (involved in GA biosynthesis) was significantly upregulated (see Supplemental Data Set 1E online). This suggests feedback/feedforward regulation of the GA metabolic pathway. Similar changes were reported in GA mutants of other species and reflect distinct metabolic and catabolic adjustments to altered GA concentration or signaling (Fujioka et al., 1988; Talon et al., 1990; Tonkinson et al., 1997; Chandler et al., 2002). Five genes associated with the GA signal transduction pathway were downregulated, with the exception of *PHOR1* (for photoperiod-responsive protein 1). The GA response mainly involves negative regulators, and *PHOR1* is one of the few GA positive regulators (Amador et al., 2001; Thomas and Sun, 2004). The lack of bioactive GAs or GA response may have caused upregulation of the positive regulators and muting of the repressive circuitry of the pathway.

Several genes involved in the phytohormone (e.g., ABA, ethylene, and auxin) transcriptional circuits were differentially regulated (see Supplemental Data Sets 1F to 1H online). Genes involved in ABA biosynthesis and signaling were all downregulated. These included major biosynthetic enzymes and a battery of signaling and downstream response genes (see Supplemental Data Set 1F online). By contrast, ethylene biosynthetic, signaling, and response genes showed much more diverse expression changes (see Supplemental Data Set 1G online). For example, three key ethylene biosynthetic genes were upregulated but two were downregulated. Similarly, six ethylene response genes showed higher transcript abundance, while another six were lower than in the wild type. Genes encoding components of auxin signaling pathways showed complex patterns (see Supplemental Data Set 1H online). For example, we identified three auxin

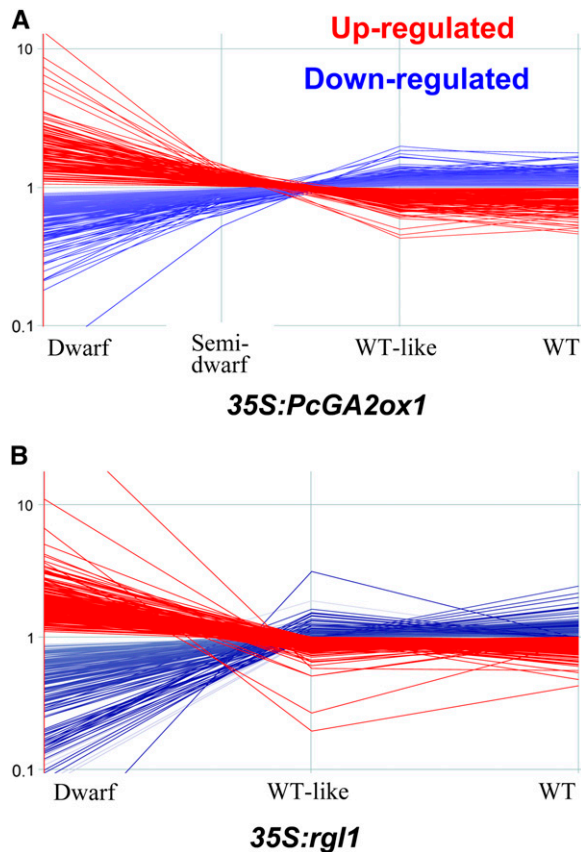
response factors that play repressive role in auxin signaling to be downregulated (Ellis et al., 2005). Three auxin-inducible Aux/indole-3-acetic acid (IAA) genes (Abel, 2007) were upregulated, but another AUX/IAA and two auxin-responsive SAUR (small-auxin-up-RNA) genes (Jain et al., 2006) were downregulated. Two efflux (Pt *PIN3* and 9, similar to At *PIN1* and 2, respectively) and one influx (similar to *Arabidopsis* Like AUX3-At *LAX3*) carriers involved in auxin transport were upregulated. However, another efflux carrier (Pt *PIN6*, similar to At *PIN3*) was downregulated. Although complex, some of the observed patterns are consistent with published effects of auxin-associated genes in LR formation. For example, homologs of positive regulators of LR initiation and emergence like *IAA7* (Muto et al., 2007), *TIR1* (Perez-Torres et al., 2008), and *LAX3* (Swarup et al., 2008) were all upregulated in transgenic roots.

### Transcription Factors

About 10% of the differentially regulated genes encode putative transcription factors (TFs) (see Supplemental Data Set 1I online), including 57 downregulated and nine upregulated genes. Therefore, the majority of the TFs showed a downregulation trend. Nearly half of all differentially regulated TFs (28) were from the MYB, bHLH, or bZIP families. The remaining TFs belong to various families, including some with a role in initiation and subsequent stages of LR formation (e.g., SCARECROW and KNAT6) (Malamy and Benfey, 1997; Dean et al., 2004).

### Transgenic Plants Exhibited Tissue-Specific Changes in GA, ABA, and IAA Concentrations

Significant changes in expression of genes associated with GA, ABA, and IAA metabolism and signaling prompted us to measure



**Figure 5.** Genes That Were Differentially Expressed in Both 35S: *PcGA2ox1* and 35S: *rgl1* Transgenics in Relationship to Their Phenotype.

35S: *PcGA2ox1* (A) and 35S: *rgl1* (B) transgenics. Blue indicates down-regulated and red indicates up-regulated genes. Details about the displayed genes are provided in Supplemental Data Set 1B online.

the concentrations of these hormones in transgenic plants. Consistent with our earlier findings (Busov et al., 2003, 2006), levels of the two bioactive GAs ( $GA_1$  and  $GA_4$ ) were significantly decreased in both roots and leaves of GA-deficient transgenics but increased in GA-insensitive transgenic plants (Table 2). The latter is likely the result of a feedback regulation of GA insensitivity (Chandler et al., 2002). ABA concentrations significantly decreased in the roots of both GA-deficient and GA-insensitive

transgenic plants but increased in the leaves (Table 2). This result, along with the microarray data, point to synergistic interactions between GA and ABA in suppression of LR development in *Populus*. IAA concentration, on the other hand, was unchanged in the leaves but slightly increased in roots of both transgenic types (Table 2).

#### Differentially Expressed Pt *PIN9* Modifies LR Formation in Transgenic Plants

A putative auxin efflux carrier (Pt *PIN9*) gene was found to be highly upregulated in both transgenic types (see Supplemental Data Set 1H online; gene model fgenes4\_pm.C\_LG\_XVIII000434). RT-PCR analysis across a variety of poplar tissues revealed that Pt *PIN9* was predominantly expressed in roots (Figure 6A), suggesting a specific role in root development. Transgenic *Populus* plants harboring either sense or antisense Pt *PIN9* under the cauliflower mosaic virus 35S promoter were regenerated, and events with confirmed Pt *PIN9* upregulation (fourfold) or downregulation (fivefold) were selected for further analysis. Transgenic *PIN9* plants were morphologically similar to the wild type in their aerial organ development (Figure 6D). However, as suggested by the microarray, upregulation of Pt *PIN9* in 35S:Pt*PIN9* transgenic plants led to a significant increase in LR formation and LPR initiation (Figures 6B to 6D). No significant changes were observed in antisense plants relative to the wild type, likely due to gene redundancy (the PIN family in *Populus* comprises 15 members). The results showed that differentially expressed genes identified by the microarray indeed affected LR formation at the primordium initiation stage.

To further understand the role of Pt *PIN9* in relation to the GA response, we tested if the gene is GA responsive. Indeed, exogenous treatment of GA had a strong repressive effect on Pt *PIN9* abundance as early as 1 d after treatment and persisted as long as 12 d after treatment (Figure 6E). We also tested if the positive effect on LR formation from Pt *PIN9* overexpression can be reversed by GA. Exogenous application of GA on 35S:Pt*PIN9* plants did inhibit the LR formation but to much less effect compared with the wild type (Figures 6F and 7B).

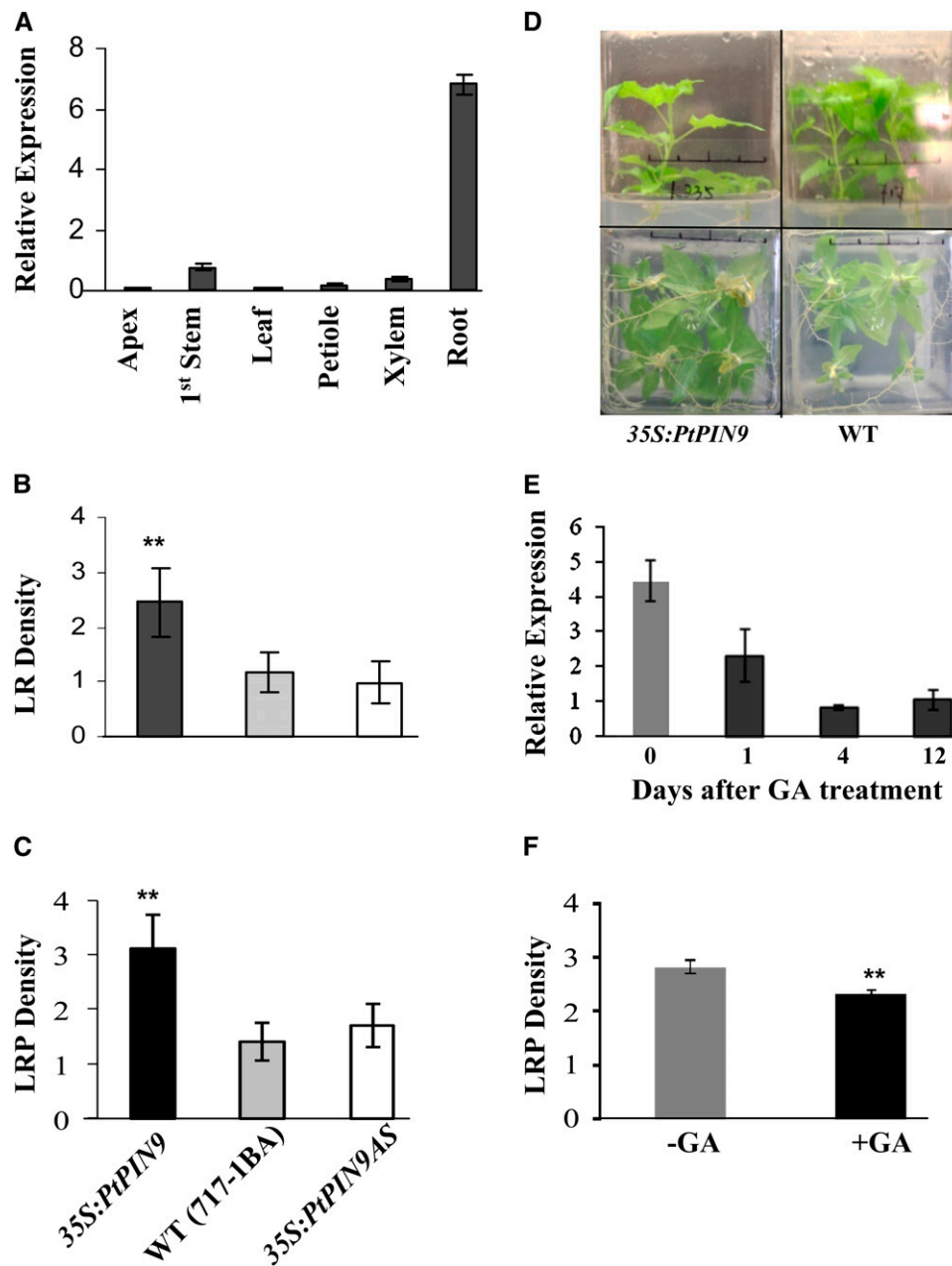
#### GA Inhibited LR Formation via Repressing Primordium Initiation

To establish a causative relationship between GAs and the observed root phenotypes, we tested whether exogenously

**Table 2.** Phytohormone Concentrations in Leaves and Roots of the Wild Type and the Two Transgenic Types

Genotype	Organ	$GA_1$	$GA_4$	IAA	ABA
Wild-type control	Leaf	58.1 ± 15.4	6.64 ± 3.18	22.5 ± 3.1	185.2 ± 28.9
	Root	77.1 ± 29.3	2.24 ± 0.74	61.4 ± 4.1	72.2 ± 13.5
35S: <i>PcGA2ox1</i>	Leaf	19.9 ± 9.4**	5.53 ± 2.33*	21.1 ± 5.9	232.3 ± 32.1**
	Root	48.8 ± 9.6**	1.15 ± 0.62**	72.9 ± 5.2*	49.5 ± 12.1**
35S: <i>rgl1</i>	Leaf	139.6 ± 21.9**	12.2 ± 3.6**	19.6 ± 3.7	207.1 ± 16.7**
	Root	97.7 ± 31.5**	3.93 ± 0.68**	69.1 ± 9.7*	54.7 ± 17**

Hormone concentration in  $ng\ g^{-1}$  dry weight; \*\* and \* indicate significant differences compared to wild-type plants as determined by Student's *t* test at  $P < 0.01$  and  $P < 0.05$ , respectively



**Figure 6.** Pt *PIN9* Expression and Functional Characterization.

(A) Pt *PIN9* is predominantly expressed in roots.

(B) LR formation significantly increased in *35S:PtPIN9* transgenics but not in *35S:PtPIN9AS* transgenics.

(C) LRP density significantly increased in *35S:PtPIN9* transgenics but not in *35S:PtPIN9AS* transgenics.

(D) Photos show in vitro aerial (top panels) and root (bottom panels) phenotypes of representative *35S:PtPIN9* transgenics and wild-type control plants. Top and bottom panels show photos of the same plants (1 month old) taken from the side and bottom of the Magenta box, respectively.

(E) Pt *PIN9* expression is repressed after GA treatment.

(F) LR formation is repressed in *35S:PtPIN9* transgenics by GA application but to much less extent. Black and gray bars in (E) and (F) correspond to GA-treated and untreated samples; \*\* indicates significance at  $P < 0.01$  as determined by Student's *t* test.

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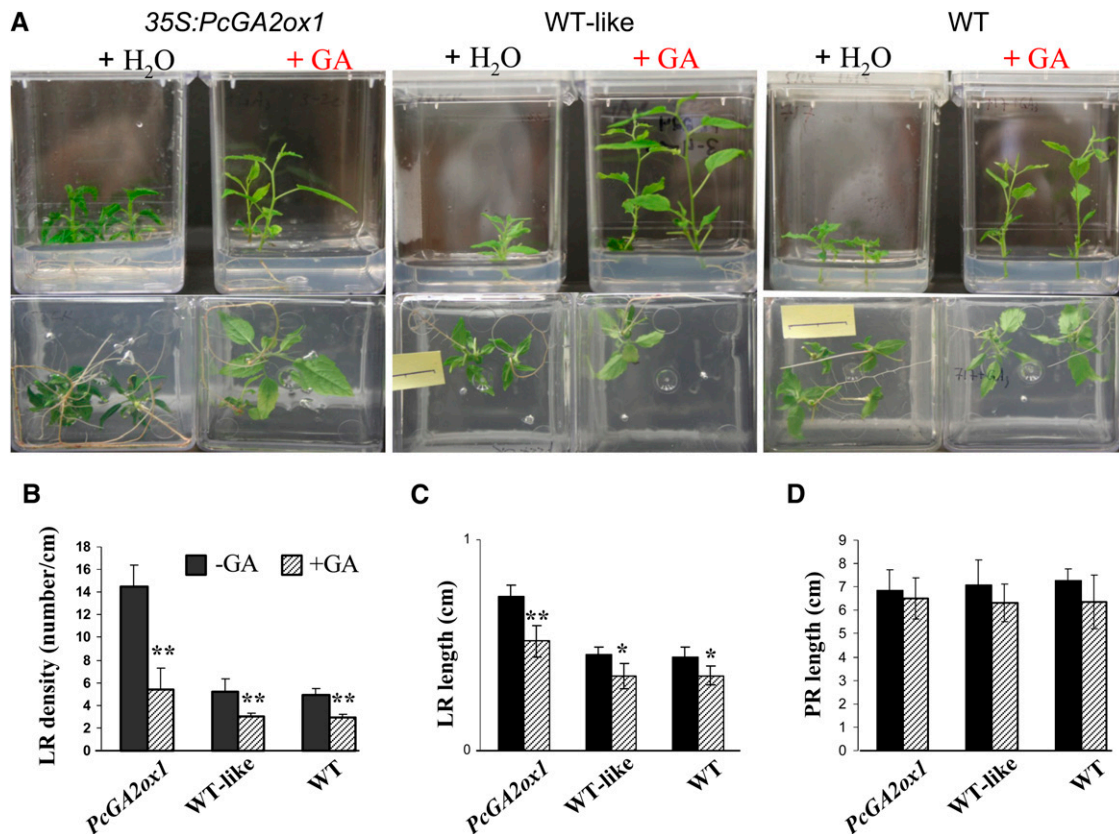
applied GA could reverse them. We found a typical and rapid stem elongation response to the hormone application, with a concomitant suppression in LR development in both GA-deficient transgenics and wild-type control (Figure 7A). As expected, GA-insensitive *35S:rg1* transgenics did not show any change in either aerial or root development in response to exogenous GA application, due to the constitutive block in GA response (see Supplemental Figure 1 online). Root measurements showed that GA application significantly decreased the number, and to a lesser extent, the length of LRs (Figures 7B and 7C). The effect was most pronounced in GA-deficient plants, as both the LR density and length became similar to those of the wild-type control following exogenous GA treatment. No change in primary root length was observed (Figure 7D).

The decreased number of LRs may be a result of the suppressed initiation of lateral root primordia (LRP) or arrest of already initiated LRP (Zhang et al., 1999; Casimiro et al., 2003). To distinguish between the two, we examined the number of LRP in transgenic and wild-type plants before and after GA application, following the five stages of LRP development (from stage I of

the first periclinal division to stage V of emerged LR with mature tissue pattern; see Supplemental Figure 2A online) as previously defined (Laskowski et al., 1995; Malamy and Benfey, 1997). GA treatment significantly decreased the number LRP across all developmental stages in both transgenic and wild-type plants (Figure 8A; see Supplemental Figure 2B online). Collectively, GA-deficient plants had nearly twice as many LRP as the wild type prior to GA application, and the suppression effect was much greater in GA-deficient plants after GA application (Figure 8B). It therefore appears that GA primarily suppresses LR formation at the early stages of LRP initiation.

#### GA Application Reversed Gene Expression Changes in the Transgenic Plants

Because exogenous GA application rapidly reversed the root phenotypes of the GA-deficient dwarf plants, we sought to investigate if altered gene expression in the transgenics, as revealed by microarray analysis, is also reversible by GA treatment. We selected eight representative genes from different

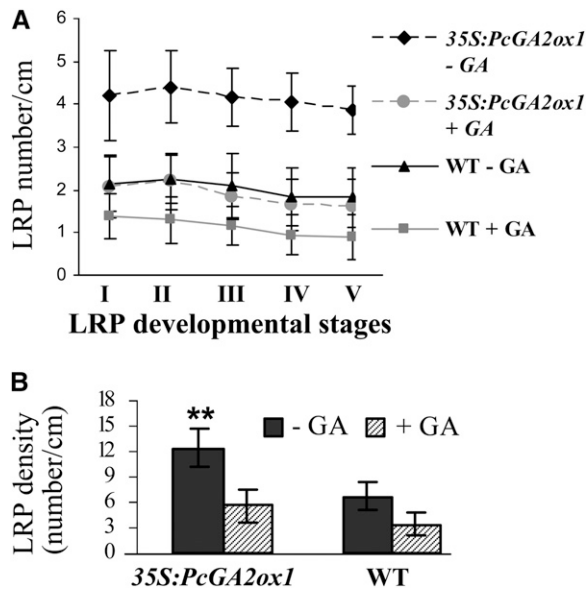


**Figure 7.** Effect of Exogenous GA<sub>3</sub> Application on LR Development of *35S:PcGA2ox1* Transgenic and Wild-Type Plants.

(A) Effect of GA<sub>3</sub> application on aerial and root development in *35S:PcGA2ox1* transgenic and wild-type plants. Photographs were taken 6 d after GA<sub>3</sub> application. Top and bottom panels show aerial and root development, respectively, of plants in the same Magenta box.

(B) to (D) Quantitative analysis of LR density (B), LR length (C), and primary root (PR) length (D) at 12 d after GA<sub>3</sub> treatment. Bars represent means and SE of two independent experiments where each experiment included at least six ramets per event. \*\* and \* indicate significance at  $P < 0.01$  and  $P < 0.05$ , respectively.

[See online article for color version of this figure.]



**Figure 8.** LRP Density Dramatically Decreased in Wild-Type and 35S: *PcGA2ox1* Expressing Plants after GA<sub>3</sub> Application.

(A) Distribution of LRP among the five developmental stages in the 35S: *PcGA2ox1* and wild-type plants before and after GA<sub>3</sub> treatment. Bars show means and SE over eight 1-month-old roots per event. All differences between genotypes as well as within genotype before and after GA<sub>3</sub> treatment were significant ( $P < 0.01$ ) as determined by Student's *t* test.

(B) Total number of LRP in all five stages before and after GA<sub>3</sub> treatment in the 35S: *PcGA2ox1* and wild-type plants. Bars show means and SE of the averaged LRP number of all five stages as illustrated in Supplemental Figure 2 online. Data were collected at 4 d after GA treatment. \*\*, Statistically significant differences compared with wild-type control, as determined by Student's *t* test at  $P < 0.01$ .

functional categories that showed some of the most pronounced transcriptional changes in the transgenic roots. RT-PCR analyses revealed that seven of the eight genes were indeed regulated by GA (Figure 9). The *Arabidopsis* putative orthologs of *CYCD3;1* and *KipRP* are involved in initiation of LRP (Himanen et al., 2002). In accordance with our finding that GA acts very early during LRP initiation (see Supplemental Figure 2 online), GA application caused a decline in *CYCD3;1* and an increase in *KipRP* abundance as early as 1 d after treatment. Similarly, two glucosyl hydrolase genes (*GHF17* and *GHF19*) involved in cell wall loosening and the TF *SCARECROW*, which is involved early stages of LR formation (Heidstra et al., 2004), were also rapidly and significantly ( $P < 0.01$ ) downregulated by GA application. GA had no effect on the expression *ACCOx*, which is involved in ethylene biosynthesis (Bleecker and Kende, 2000), but upregulated *NCED1* associated with ABA biosynthesis (Figure 9). Consistent with this, a poplar putative ortholog for *ATHB12*, a downstream target of ABA signaling in *Arabidopsis* (Olsson et al., 2004), was also upregulated, but at a slower pace. This suggests that GA likely increases ABA levels, which in turn activates the ABA signaling and response pathway.

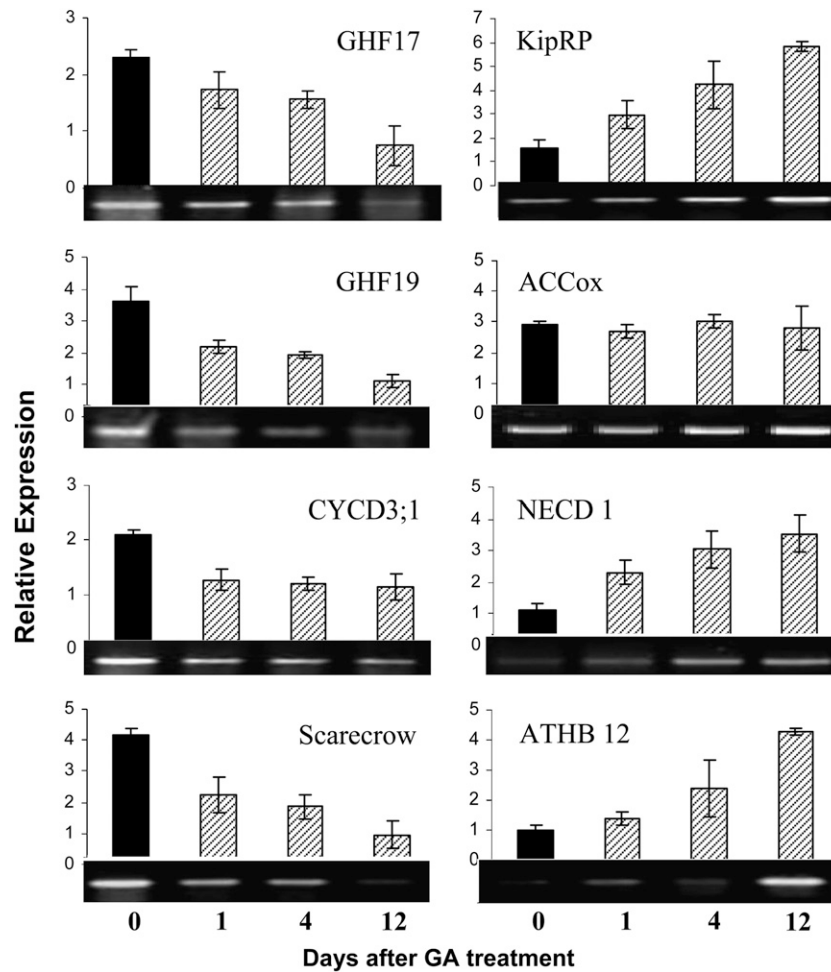
We searched the upstream putative promoter regions of all eight genes for GA response elements (GAREs) (Skriver et al., 1991; Ogawa et al., 2003; Sutoh and Yamauchi, 2003). Consistent with the expression data (Figure 9), GARE *cis*-elements are present in six of the eight gene promoters. GARE is not found in the promoter of *ACCOx* that did not respond to GA application nor in the promoter of *ATHB12* that was upregulated at a much later time (4 d), likely as a result of increased ABA biosynthesis. Furthermore, for four (*NCED1*, *KipRP*, *GHF17*, and *GHF19*) of the six genes that showed a rapid response to GA treatment, we found two GARE elements in the putative promoter regions (Table 3). The data suggest that these GA-responsive genes are likely direct targets of GA signaling.

### Root-Specific Poplar GA 2-Oxidases Regulate GA Levels and LR Formation

Because of the strong indication that GA catabolism and response may regulate LR development, we identified seven *GA2ox* in the poplar genome sequence (see Supplemental Data Set 1J online), two of which (Pt *GA2ox2* and 7) were predominantly expressed in roots (Figure 10A). Their high sequence similarity (see Supplemental Figure 3 online) and similar expression patterns suggest paralogous relationships (poplar has experienced a recent genome-wide duplication, and many gene family members contain closely related paralogs originating from the salicoid-specific duplication) (Tuskan et al., 2006). An RNA interference (RNAi) construct was therefore designed to suppress both genes via targeting of a highly similar gene fragment (see Supplemental Figure 3 online). More than 20 transgenic events were recovered and PCR verified for the presence of the transgene and isolated events that showed suppression of the two targeted genes (Figure 10B). We selected two events with moderate and severe reduction of the two genes for further study. Consistent with their root preferential expression, no effect on aerial development was found (Figure 10C). Quantitative analysis revealed that LR number was significantly decreased in both RNAi transgenics (Figure 10D). As expected based on their putative function in bioactive GA catabolism, levels of GA<sub>1</sub> and GA<sub>4</sub> in the RNAi transgenic roots were increased (Table 4).

### Role of GA in LR Initiation

Quantitative analyses of LR development in GA-deficient and GA-insensitive *Populus* showed an increase in LR density and elongation. LR development was inhibited by exogenous application of GA *in vitro* or by elevated GAs *in vivo* due to RNAi silencing of two root-specific *GA2ox* genes. This GA-mediated repressive effect appears to result from a dramatic reduction of LRP initiation. Consistent with this, GA-deficient transgenics have many more LRP. Therefore, GA appears to negatively regulate the early initiation step of LR formation. One of the first events for LR initiation is dedifferentiation and cell cycle reentry (G1 → S transition), followed by a series of divisions and subsequent organization of a functional root meristem. The role of GA in this sequence of events is unknown. However, studies from the much better understood shoot apical meristem indicate that GA



**Figure 9.** Response of Eight Differentially Expressed Genes after GA<sub>3</sub> Treatment of 35S:PcGA2ox1 Transgenic Plants.

0, Roots sampled before treatment; 1, 4, and 12 indicate days after GA application, respectively. Abbreviations used in the figure correspond to the names used in Supplemental Data Set 1K online. Bars are means and SE of three biological replications.

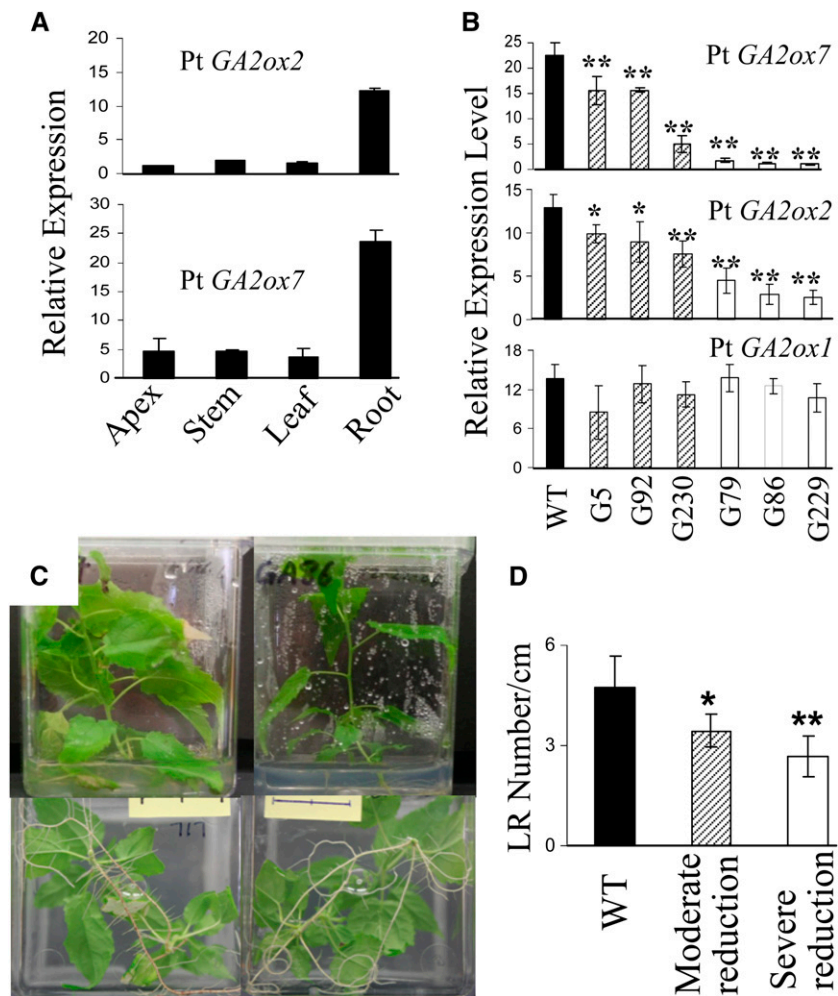
is selectively excluded from the shoot apical meristem via downregulation of *GA2ox* in the meristem dome and upregulation of *GA2ox* at the boundary with emerging leaf primordia (Sakamoto et al., 2001a, 2001b). It is believed that reduced GA levels are needed for the undetermined meristem cell fate

maintenance (Sakamoto et al., 2001a), whereas high GA concentrations promote cell differentiation and expansion (Jasinski et al., 2005; Shani et al., 2006). Because of the requirement for dedifferentiation and de novo organization of LR meristems, GA deficiency and insensitivity in roots would facilitate the process

**Table 3.** GARE *cis*-Element Motifs in the Promoter of Differentially Regulated Genes

Gene Model	Closest At Hit	Gene Name/Description	GARE Site	GA Response
eugene3.19440001	AT3G58100	GHF (glycosyl hydrolase family) 17	-566, -829	Yes
estExt_fgenesh4_pg.C_LG_XIX0853	AT3G54420	GHF (glycosyl hydrolase family) 19	-707, -1397	Yes
grail3.0040026601	AT4G34160	CYCD3;1	-573	Yes
gw1.III.2060.1	AT4G00150	SCARECROW	-1081	Yes
estExt_fgenesh4_pg.C_LG_IX0597	AT1G49620	KipRP	-1257, -1479	Yes
eugene3.00110845	AT3G14440	NCED1	-763, -1450	Yes
eugene3.00140486	AT3G61890	ATHB-12	No	No
fgenesh4_pg.C_LG_VI000988	AT2G19590	ACCOx (ACC oxidase)	No	No

GA response is based on Figure 9.



**Figure 10.** Identification and Functional Characterization of Root-Specific Poplar GA 2-oxidases.

**(A)** Expression of Pt *GA2ox2* and 7 in different tissues. Bars represent means and SE of at least three biological replications. All expression analyses were performed on greenhouse-grown plants of the same WT-717 genotype used in the transgenic analysis.

**(B)** Significant downregulation of Pt *GA2ox2* and *GA2ox7* in RNAi transgenic *Populus*. Three representative events of moderate reduction (diagonally striped) and severe reduction (white) of the targeted genes compared with the wild-type control (black) are shown. A nontargeted Pt *GA2ox1* was not affected in the same transgenic events. The RNAi fragment used in the RNAi construct had 100% homology to Pt *GA2ox7* and 86% homology to Pt *GA2ox2*. Note the higher gene silencing efficiency against Pt *GA2ox7*. Bars show means and SE over three independent experiments where each experiment was performed with at least five ramets per event.

**(C)** Aerial and root appearance of wild-type control and *GA2ox\_RNAi* transgenics in vitro; top and bottom panels represent photos of the same plants (1 month old) taken from the side and bottom of the Magenta boxes.

**(D)** LR density (averaged LR number per centimeter of primary root) in two representative RNAi events, with a moderate reduction (diagonally striped) and a severe reduction (white) of the two targeted genes, respectively; \*\* and \* indicate significance at  $P < 0.01$  and  $P < 0.05$ , respectively.

[See online article for color version of this figure.]

of new LRP formation. In accordance with this hypothesis, expression of genes like *CYCD3;1* and *KipRP* appeared to be spatiotemporally regulated in a fashion consistent with their distinct roles in G1 → S transition of *Arabidopsis* LR formation (Himanen et al., 2002; De Smet et al., 2003). *CYCD3;1* was upregulated and *KipRP* downregulated in both GA-deficient and GA-insensitive transgenic plants. Rapid reversal of these trends following GA treatment, coupled with the presence of GARE cis-elements in the promoter regions of these two genes, suggest

that they may be direct targets of GA signaling. Putative orthologs of *Arabidopsis* genes associated with early stages of LRP formation, like *SCARECROW* (Malamy and Benfey, 1997), *AIR12* (Neuteboom et al., 1999), and *IAA19* (Muto et al., 2007), were also abundantly expressed in the roots of GA-modified transgenic plants. Precise localization of GA metabolic and signaling genes and proteins during LRP formation would help to pinpoint the exact tissues, stages, and roles GA plays in LR development.

**Table 4.** GA Levels in GA2ox\_RNAi Transgenics and Wild-Type Control

Genotype	Organ	GA <sub>1</sub>	GA <sub>4</sub>
Wild-type control	Leaf	58.1 ± 15.4	6.64 ± 3.18
	Root	77.1 ± 29.3	2.24 ± 0.74
Transgenics	Leaf	65.4 ± 17.2**	8.87 ± 4.77*
	Root	95.8 ± 29.5**	3.93 ± 1.61**

GA concentration in ng g<sup>-1</sup> dry weight; \*\* and \* indicate significant differences compared with the wild type as determined by Student's *t* test at *P* < 0.01 and *P* < 0.05, respectively.

### Mechanistic Insights from Transcriptomic Analysis

Our data suggest possible mechanisms of GA action in LR development. Several lines of evidence suggest crosstalk with auxin. First, the auxin levels were increased in GA-deficient and insensitive transgenic roots. Second, genes encoding key regulators of auxin responses and transport system showed changes in their expression patterns consistent with their involvement in regulation of LR development. Finally, transgenic upregulation of one of these genes, which encodes a putative auxin efflux carrier (Pt *PIN9*), led to increased LR formation similar to that found in the GA-deficient and insensitive transgenics. Expression of this same gene was repressed by exogenous application of GA as early as 1 d after application of the hormone, further suggesting that GA interacts with the poplar auxin transport to modulate LR development. Among the other poplar PIN-encoding genes, Pt *PIN9* shows the most root-specific expression pattern, further accentuating its central role in root development. Although overexpression of Pt *PIN9* alleviated the GA inhibitory effect on LR formation, it did not completely block it. This suggests that GA likely affects LR development through convergence of multiple signaling pathways, which may in turn interact with each other as discussed below.

Although auxin can directly stimulate LR formation, it also affects the biosynthesis and response of ABA, a hormone that generally represses LR formation (Suzuki et al., 2001; De Smet et al., 2003, 2006; Razem et al., 2006). LR proliferation in GA-deficient and GA-insensitive transgenics was associated with reduced ABA concentrations and the downregulation of a suite of ABA biosynthetic and signaling genes. The two major steps of ABA biosynthesis, performed by *ABA1* and *NCED1* (Nambara and Marion-Poll, 2005), were significantly repressed, and consequently many ABA-responsive genes were significantly downregulated. Poplar *NCED1*, in particular, displayed very low abundance in GA-modified transgenic roots, but its expression increased sharply upon GA treatment within 1 d. In addition, putative orthologs of *AIR12* and *IAA19*, which are auxin inducible and known to be repressed by ABA (De Smet et al., 2003, 2006) and highly expressed during LR initiation in *Arabidopsis* (Nibau et al., 2008), were upregulated in GA-modified transgenic poplar roots. Taken together, our data suggested that one mechanism of the GA deficiency-induced root proliferation is through auxin-mediated or direct downregulation of ABA biosynthesis.

### Conclusions

The observed responses of root development to GA signal are consistent with GA having an important role in regulation of plant adaptation to stress. GA signaling may enable integration of aerial and root development, where the attenuation of aerial growth and concomitant stimulation of root development caused by reductions in GA produces a smaller plant with lower demands on environmental resources and a root system that is more actively exploring the soil environment (Chaves et al., 2002; Sharp and LeNoble, 2002). This hypothesis is supported by recent studies in *Populus*, where induction of an *RG*A-like gene (repressor of the GA response) was observed in leaves under drought conditions (Street et al., 2006). Similarly, in *Arabidopsis*, DELLA proteins are known to mediate the response to salt and other stresses (Achard et al., 2006). Treatment with paclobutrazol, a GA biosynthesis inhibitor, has been long known among horticulturists to reinvigorate stressed ornamental trees via promotion of root system development (Chaney, 2003; Watson, 2004). Overall, our data indicate that modulation of GA metabolism and response provides an important signaling mechanism that helps plants respond to stress via the coordinated suppression of aerial growth and stimulation of LR growth.

### METHODS

#### Plant Materials and Treatments

The generation and initial characterization of transgenic plants used in this study were previously reported (Busov et al., 2003, 2006). For microarray experiments, roots from 7-week-old in vitro-grown plants were harvested. All plants were grown in hormone-free half-strength Murashige and Skoog (Sigma-Aldrich) media containing 0.7% Phytagar (Gibco-BRL) and 1% sucrose and maintained at 26°C under a 16/8-h photoperiod. LRs were carefully removed from media, washed, snap-frozen in liquid nitrogen, and stored at -80°C until use. Ten microliters of aqueous 3 mM GA<sub>3</sub> solution were directly applied to the shoot apex of 2-week-old in vitro-grown plants at 4-d intervals for 2 weeks.

#### LR Quantification

To measure LR development, 5-cm cuttings were grown in vitro as described above. Digital images of roots were obtained in the presence of a scale bar and quantified using ImageJ 1.63 software (<http://rsbweb.nih.gov>). We counted the number of emerged roots and measured LR length in a 2-cm section starting at the root tip. Each experiment was repeated three times with at least five plants per replication. Height and biomass measurements were performed on 3-month-old plants maintained in a greenhouse. Stems were separated from roots, and the latter were carefully cleaned to remove soil. Dry weights of stem and root fractions were determined after incubation in an oven at 70°C for 3 d. We used two independent experiments and five plants per event.

#### Microarray Hybridization and Data Analysis

We used a total of six individual genotypes as follows: the wild type, 35S: *PcGA2ox* (dwarf event), 35S: *PcGA2ox* (semidwarf event), 35S: *PcGA2ox1* (wild-type-like event), 35S: *rgl1* (dwarf event), and 35S: *rgl1* (wild-type-like event). Two independent biological replicates per genotype were used, each pooled from 20 clonally propagated plants, for a total of 12 hybridizations (six genotypes × two biological replicates). RNA was

isolated as previously described using the Qiagen RNeasy plant kit (Busov et al., 2003). Prior to labeling, RNA quality was assessed by Agilent Bioanalyzer (Agilent Technologies), and 3  $\mu$ g of total RNA was used to prepare biotinylated complementary RNA. The labeling, hybridization, and imaging procedures were performed according to Affymetrix protocols at the Center for Genomics Research and Biocomputing, Oregon State University (<http://corelabs.cgrb.oregonstate.edu/affymetrix>) using the Affymetrix Poplar GeneChip. Data were analyzed using GeneSpring GX10 (Agilent Technologies). Raw data were preprocessed by the GC-RMA algorithm (Wu et al., 2004) and per-gene normalized to control (wild-type) samples. Probes were filtered using raw expression level at  $\geq 100$ . Statistical significance between mean values across the six genotypes was determined using one-way analysis of variance with Benjamin Hochberg multiple testing correction at a false discovery rate of  $\leq 0.05$ . The Tukey's post-hoc test was used to identify significant differences between genotypes when the analysis of variance was significant. GO enrichment analysis was performed with the set of genes (2069) that showed significant differences between wild-type and the dwarf plants from both transgenic types using a hypergeometric test with Benjamin Hochberg multiple testing correction at a false discovery rate of  $\leq 0.05$ . Hierarchical clustering ( $R^2 > 0.9$ ) (Bar-Joseph et al., 2003) was used to identify genes whose expression correlates with phenotypic severity of root traits.

#### RT-PCR Analysis

Reverse transcription was performed using 3  $\mu$ g of DNaseI-treated total RNA with a SuperScript III reverse transcription kit (Invitrogen) and oligo (dT) primers. Gene-specific primers were used for PCR amplification of each gene (see Supplemental Data Set 1K online). Poplar elongation factor 1 $\alpha$  (gw1.X.2539.1) and ubiquitin4 (eugene3.00011726) expression levels were used to correct for variations in loadings. Relative expression was estimated by measuring band intensity on ethidium bromide-stained gels using the GelDoc-It Imaging System and analyzed via Launch-VisionWorksLS software (Ultra-Violet Products). At least two biological and two technical replications were used in measuring each expression value, and the data were analyzed using KaleidaGraph 3.0 (Synergy Software).

#### Quantification of LRP

To study LRP, sections were taken spanning the region of 0.5 to 2.5 cm from the root tip. Primordia were stained by the Feulgen method as previously described (Dubrovsky et al., 2000) but with a hydrolysis step using 1 N HCl at 60°C for 10 min after tissue dehydration. Digital photographs were taken with the aid of a Leica MZ10F fluorescence microscope with a Retiga 2000 fast monochrome cooled camera and Q-imaging system at 560 to 570 nm.

#### Promoter Analysis

Approximately 2000 bp upstream of the translation start site were downloaded using the JGI Poplar Genome Browser ([http://genome.jgi-psf.org/Poptr1\\_1/Poptr1\\_1.home.html](http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html)). The region was examined for the presence of putative GARE *cis*-elements (TAACAAA/G) (Skriver et al., 1991; Ogawa et al., 2003; Sutoh and Yamauchi, 2003) using the PLACE database (<http://www.dna.affrc.go.jp/PLACE/>).

#### Generation of Binary Vectors and Transformation

A fragment of high sequence homology between Pt *GA2ox 2* and *7* was selected for downregulation of both genes (see Supplemental Figure 3 online). The fragment was PCR amplified using primers attB1\_GA2ox7-F (5'-GGGGACAAGTTTGTACAAAAAGCAGGCTTGTTAGTTCCTTCTCC-

AACA-3') and attB2\_GA2ox7-R (5'-GGGGACCACTTTGTACAAGAAA-GCTGGGTGCTGGTTTCCGAAAAACG-3') and inserted into binary RNAi vector *pHELLSGATE* using BP clonase (Wesley et al., 2001). Pt *PIN9* sense and antisense plants were generated using the pART7/pART27 system as previously described (Gleave, 1992). The open reading frame of Pt *PIN9* was PCR amplified using the following primers: F1, 5'-GAATTCATGATCACTGGCAAGGACA-3', and R1, 5'-TCTAGATCAAACGCCAAGAAGCAC-3'. The amplified fragment was inserted into the pCR 4-TOPO vector using the TOPO-TA cloning kit (Invitrogen). The *EcoRI* fragment was subcloned into pART7 and sequence-verified. Expression cassettes carrying Pt *PIN9* in sense and antisense orientations were removed from pART7 using *NotI* and inserted into the respective site of pART27. All binary vectors were transformed into *Agrobacterium tumefaciens* strain C58 via the freeze and thaw method (Holsters et al., 1978). The construct was transformed via *Agrobacterium*-mediated transformation as previously described (Han et al., 2000) into the same genetic background as used for generation of GA transgenics (e.g., clone INRA 717-IB4-*Populus tremula*  $\times$  *Populus alba*). Validation of Pt *PIN9* overexpression/suppression was performed using the following primers: PIN9-F, 5'-GAATTCATGATCACTGGCAAGGACA-3'; PIN9-R, 5'-TCTAGATCAAACGCCAAGAAGCAC-3'. RNA extraction and RT-PCR were performed as described above.

#### Phytohormone Analysis

Three grams of roots and expanding leaves were respectively harvested from 3-month-old greenhouse-grown transgenic plants, each line represented by three independent plants. The samples were immediately frozen and powdered in liquid nitrogen and then lyophilized. Each replicate sample was extracted in 80% methanol with internal standards of [<sup>2</sup>H<sub>2</sub>]-GA<sub>1</sub>, -GA<sub>4</sub>, -ABA, and [<sup>13</sup>C<sub>6</sub>]-IAA and reduced to aqueous phase. The aqueous phase was extracted with EtOAc at pH 3, then with K-Pi buffer at pH 8.5, again into EtOAc at pH 3, and further purified on C<sub>18</sub> Sep-Pak and MCX SPE columns (Qasis; Waters). The eluant was dried and redissolved with HPLC initial solution, filtered through a 0.22- $\mu$ m filter, and analyzed with a liquid chromatography-mass spectrometry system (LCQ Deca AMX, HPLC-ESI-MS; Thermo-Finnigan). Tandem mass spectrometry data were then analyzed using software Xcalibur 2.1 (Thermo-Finnigan) and quantified by reference to the internal standards using M<sup>+</sup> ratios in equations for isotope dilution analysis.

#### Accession Number

All microarray data have been deposited in the Gene Expression Omnibus database under accession number GSE16888.

#### Supplemental Data

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

**Supplemental Figure 1.** Effect of GA<sub>3</sub> Application on Aerial and Root Development in *35S:rg11* Transgenic and Wild-Type Plants.

**Supplemental Figure 2.** Lateral Root Primordium Development.

**Supplemental Figure 3.** Alignment of Pt *GA2ox2* and Pt *GA2ox7* Nucleotide Sequences.

**Supplemental Data Set 1A.** Differentially Expressed Genes as Determined by ANOVA (FDR < 0.01).

**Supplemental Data Set 1B.** Genes Whose Expression Correlates with the Strength of the Root Phenotype.

**Supplemental Data Set 1C.** Cell Wall Loosening.

**Supplemental Data Set 1D.** Cell Proliferation and Growth.

**Supplemental Data Set 1E.** GA Biosynthesis, Metabolism, and Signaling.

**Supplemental Data Set 1F.** ABA Biosynthesis, Signaling, and Response.

**Supplemental Data Set 1G.** Ethylene Biosynthesis, Signaling, and Response.

**Supplemental Data Set 1H.** Auxin Transport, Signaling, and Response.

**Supplemental Data Set 1I.** Transcription Factors.

**Supplemental Data Set 1J.** Seven GA 2-Oxidase Genes Identified in *Populus*.

**Supplemental Data Set 1K.** Primers for RT-PCR Verification.

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# Gibberellins Regulate Lateral Root Formation in *Populus* through Interactions with Auxin and Other Hormones

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