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ORIGINAL ARTICLE

**Victor Busov · Richard Meilan · David W. Pearce
Stewart B. Rood · Caiping Ma
Timothy J. Tschaplinski · Steven H. Strauss**

Transgenic modification of *gai* or *rgl1* causes dwarfing and alters gibberellins, root growth, and metabolite profiles in *Populus*

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Abstract In *Arabidopsis* and other plants, gibberellin (GA)-regulated responses are mediated by proteins including GAI, RGA and RGL1-3 that contain a functional DELLA domain. Through transgenic modification, we found that DELLA-less versions of GAI (gai) and RGL1 (rgl1) in a *Populus* tree have profound, dominant effects on phenotype, producing pleiotropic changes in morphology and metabolic profiles. Shoots were dwarfed, likely via constitutive repression of GA-induced elongation, whereas root growth was promoted two- to threefold in vitro. Applied GA_3 inhibited adventitious root production in wild-type poplar, but *gai*/*rgl1* poplars were unaffected by the inhibition. The concentrations of bioactive GA_1 and GA_4 in leaves of *gai*- and *rgl1*-expressing plants increased 12- to 64-fold, while the C_{19} precursors of GA_1 (GA_{53}, GA_{44} and GA_{19}) decreased three- to ninefold, consistent with feedback regulation of GA 20-oxidase in the transgenic plants. The transgenic modifications elicited significant metabolic changes. In roots, metabolic profiling suggested increased respiration as a possible mechanism of the increased root growth. In leaves, we found metabolite changes suggesting reduced carbon flux through the lignin biosynthetic pathway and a shift towards allocation of secondary storage and defense metabolites, including various phenols, phenolic glucosides, and phenolic acid conjugates.

Keywords Metabolic profiling · Adaptation · DELLA proteins · Root formation

V. Busov (✉)
School of Forest Resources and Environmental Science,
Michigan Technological University,
Houghton, MI 49931-1295, USA
E-mail: vbusov@mtu.edu
Tel.: +1-906-4871728

R. Meilan
Department of Forestry and Natural Resources,
Purdue University, West Lafayette, IN 47907-2061, USA

D. W. Pearce · S. B. Rood
Department of Biological Sciences, University of Lethbridge,
T1K 3M4 Lethbridge, AB, Canada

C. Ma · S. H. Strauss
Department of Forest Science, Oregon State University,
Corvallis, OR 97331-5752, USA

T. J. Tschaplinski
Environmental Sciences Division, Oak Ridge National Laboratory,
Oak Ridge, TN 37831-6422, USA

Introduction

Gibberellins (GAs) are a complex family of tetracyclic diterpenoid compounds, some of which are bioactive regulators of diverse developmental processes in plants, including seed germination, flower initiation, fruit development, stem elongation, leaf expansion, and trichome differentiation (Hooley 1994; Davies 1995).

Substantial efforts are underway, predominantly in model annual plants, to understand the molecular mechanisms of these GA-mediated processes (Olszewski et al. 2002). GAs are signaling molecules that exert their effect by triggering signal transduction cascades, activating or repressing downstream genes responsible for carrying out particular developmental and growth processes. Several factors in the signal transduction pathway have been identified through GA response mutants (Thornton et al. 1999; Sun 2000; Richards et al. 2001; Thomas and Sun 2004; Ueguchi-Tanaka et al. 2005). These mutants are either GA-insensitive dwarfs or constitutive GA response mutants (Sun 2000). GA-insensitive mutants show symptoms of GA deficiency, but, unlike GA biosynthesis mutants, cannot be rescued by GA treatment (Olszewski et al. 2002).

GA insensitive (*GAI*), *repressor of GA1* (*RGA*), *RGA-like1* (*RGL1-3*) play a central role in regulating GA responses (Peng et al. 1997; Silverstone et al. 1998). Mutant and transgenic analyses suggest that they are repressors, and belong to the larger GRAS family of transcription factors (Pysh et al. 1999). Although GAI/RGA/RGL1 share high sequence simi-

larity with the other members of the GRAS family, they contain a DELLA domain that is absent in the other family members (Peng et al. 1997; Silverstone et al. 1998). This domain appears to be important in mediating GA responses. Complete deletion or non-synonymous substitutions in this domain are gain-of-function mutations resulting in constitutive inhibition of one or several GA responses (Peng et al. 1997). Although the exact functional significance of this domain is still unclear, recent evidence suggest that it is important during the process of GA-induced phosphorylation followed by ubiquitination, and proteasome-targeted degradation (Sasaki et al. 2003; Fu et al. 2004; Dill et al. 2004). Natural and artificial-induced mutations in DELLA protein genes have been identified in several annual plant species, including *Arabidopsis* (Silverstone et al. 1997; Peng et al. 1997; Lee et al. 2002; Swain et al. 2004; Wen and Chang 2002), rice (Ikeda et al. 2001), wheat (Peng et al. 1999a), maize (Peng et al. 1999a) and barley (Chandler et al. 2002). DELLA mutants are usually significantly dwarfed, display dark green foliage, and have variety of flower and seed developmental defects.

The effects of DELLA proteins on aerial growth are well characterized but their role in the development of roots has remained largely unknown. Recent studies have suggested a putative role for DELLA proteins in the regulation of root elongation (Fu and Harberd 2003). Their role in lateral/adventitious root initiation and growth is completely unknown.

Very little is known about the metabolic changes that occur in DELLA and other GA signaling and biosynthetic mutants. Recent studies have shown that transgenic tobacco plants overexpressing *GA 2-oxidase* has a lower level of lignin (Biemelt et al. 2004). Lignin is produced via the phenylpropanoid pathway, which is extremely branched and under complex regulation. The reported lignin differences suggested possible alterations in other branches of the pathway.

Simultaneous alterations of root and secondary metabolism are of great interest. For example, increases in root biomass and secondary metabolites (which may make plant tissues more refractory to breakdown in the environment) could promote carbon sequestration, drought tolerance, and bioremediation. Here, we report profound effects of *gai*/*rgl1* expression in transgenic poplars on root biomass that are also accompanied by shifts in primary and secondary metabolism.

Materials and methods

Plant material and transformation

We introduced all constructs into hybrid aspen clone INRA 717-IB4 (*Populus tremula* × *P. alba*) via an *Agrobacterium*-mediated protocol (Han et al. 2000). Following regeneration on kanamycin selection media, all lines were verified via PCR for both the presence of *nptII* and either *gai*, *GAI*, or *rgl1* genes. Each line was represented by at least four ramets in all experiments.

Construct preparation

The pD1/SK, pλg/SK, ptG62, and ptg62 plasmids used in the assembly of the *GAI* and *gai* expression constructs were kindly provided by Dr. Nicolas Harberd (John Innes Center, Norwich, UK). pD1/SK and pλg/SK were generated by insertion of 5-kb fragments of *GAI* and *gai* genes, including the promoter and the coding regions into *Sma*I site of pBluescript SK. ptG62 and ptg62 were obtained by inserting the full 1.6 kb coding region of *GAI* and *gai* into pJIT62, downstream of one copy of the 35S promoter and upstream of CaMV terminator. To prepare constructs expressing *GAI* and *gai* under the control of the endogenous *Arabidopsis* promoter, we inserted a 5 kb *Xba*I/*Eco*RI restriction fragment from plasmids pD1/SK and pλg/SK, containing *Arabidopsis GAI* and *gai*, respectively, into the binary vector pG3K. pG3K is a modification of pGREEN II (Hellens et al. 2000), containing an *nptII* gene inserted between nopaline synthase (*nos*) promoter and *nos* terminator. Constructs expressing *GAI* and *gai* under the control of 35S promoter were generated by inserting an *Sst*I/*Xho*I restriction fragments from plasmids ptG62 and ptg62, containing one copy of the 35S promoter, approximately 1.6 kb of the *GAI* and *gai* coding sequences, respectively, and the CaMV terminator into pG3K. The *rgl1* overexpression construct was kindly provided by Dr. Caren Chang (University of Maryland, USA). The *rgl1* cDNA, with complete DELLA truncation, was inserted between the 35S promoter and the *nos* terminator. Details of construct preparation are provided in Wen and Chang 2002. The *PcGA2ox1* overexpression construct *pLARS124* was provided by Dr. Peter Hedden (Rothamsted Research, UK).

All plasmids were transformed into *Agrobacterium tumefaciens* strain C58 via the freeze and thaw method (Holsters et al. 1978). All pGREEN II-derived plasmids were cotransformed into *Agrobacterium* with pSOUP as described (Hellens et al. 2000).

DNA and RNA extraction

DNA and RNA extractions were performed using Qiagen kits with slight modifications (Busov et al. 2003).

PCR and RT-PCR

The following primers were used for PCR verification of transgene integrations: *rgl1*, 5′-CCCGGATTCA AGAAAAGCCTGAC-3′ (forward) and 5′-CAACAA ACAACCTTCATTCTCTTCCAC-3′ (reverse); *gai*/*GAI*, 5′-GCTTGATTCTATGCTCACCGACC-3′(forward) and 5′-CTCTCCTCCACCCGATAACCC-3′ (reverse);

and *nptII*, 5′-ATGCCTGCTTGCCGAATATC-3′ (forward) and 5′-CCAAGCTCTTCAGCAATATCAC-3′ (reverse).

Reverse transcription (RT) PCR was performed on 1.0 μg of total RNA as previously described (Busov et al. 2003) with the following primers: *gai*/*GAI*, 5′-CCGGCGCTTATGCAGGCTCTTGCGCTTCGACC-3′ (forward) and 5′-TGTGCAGCCGCAAACCCAG-CAGACCCGAACC-3′(reverse); and *rgl1*, 5′-TCGGA TCTTGACCCGACCCGGATTCAAGAAAAGCC-3′ (forward) and 5′-GCACCAGCTTGAGAGGACG CG AGTAACCCC-3′ (reverse). The ubiquitin primers have been previously described (Busov et al. 2003).

Metabolic profiling

Analyses were performed on plants grown for 2 months on MS media, free of plant growth regulators. Roots, stems, and leaves were sampled separately. Samples were immediately frozen in liquid nitrogen and kept at −80°C until analyzed. Approximately 200 mg fresh weight (FW) of fine root tissue and 300 mg FW of leaf tissue were ground in liquid nitrogen and extracted overnight with 5 ml 80% ethanol. Sorbitol (200 μl of a 1 mg/ml aqueous solution) was added before extraction as an internal standard to correct for differences in extraction efficiency, subsequent differences in derivatization efficiency, and changes in sample volume during heating. The next day, the extract was transferred to another glass vial before the extraction process was repeated; all extracts from a single sample were combined after 48 h. A 5-ml aliquot of the 10.2-ml root extract and a 4-ml aliquot of the leaf extract were dried under nitrogen stream. For generation of trimethylsilyl (TMS) derivatives, dried extracts were dissolved in 500 μl of silylation-grade acetonitrile followed by the addition of 500 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce Chemical Co. Rockford, IL, USA), and heated for 1 h at 70°C. After 24 h (leaf extracts) to 48 h (fine root extracts), 1-μl aliquots were injected into a Hewlett-Packard HP 5890 Series II gas chromatograph (GC) coupled to an HP 5972 mass spectrometer (MS), fitted with an HP-5MS (cross-linked 5% PH ME Siloxane) 30 m×0.25 mm×0.25 μm (film thickness) capillary column (Hewlett-Packard, Avondale, PA, USA). The standard quadrupole GC-MS was operated in electron impact (70 eV) mode, with 1.5 full-spectrum (50–550 Da) scans per second. Gas (helium) flow was set at 0.6 ml/min with the injection port configured in the splitless mode. The injection port and detector temperatures were set to 250 and 300°C, respectively. The initial oven temperature was held at 100°C for 4 min and was programmed to increase at 8°C per minute to 300°C, where it was held for another 21 min, before cycling to the initial conditions. The stability of the TMS-metabolites was tracked for 72 h after the initial heating to confirm the completion of derivatization, which can be slowed by matrix interactions.

All peaks above a set minimum threshold were integrated, whether their identity was known or not. The low abundance peaks that approached statistically significant thresholds (relative to the control plants) were reintegrated and reanalyzed using a key selected ion, characteristic m/z fragment, rather than the total ion chromatogram, to minimize integrating coeluting metabolites. Overlapping peaks were manually deconvoluted using relatively unique fragments for each metabolite. A large user-created mass spectral database (> 700 metabolites) was used to identify unique fragments for data extraction. Approximately one-half of the peaks were quantified using the total ion chromatogram (TIC), and the remaining metabolites that had obvious peak shoulders (or skewed peak shape), or additional m/z fragments indicative of peak overlap, were quantified using key m/z fragments. To determine TIC peak areas free from coeluting interference, the extracted ion peak areas were multiplied by scaling factors for each fragment that were determined from standards or samples without interfering metabolites. Peaks were quantified by area integration and the relative concentrations were determined based on the quantity of the internal standard (sorbitol) with data then expressed in glucose equivalents. Three replicate samples were analyzed per clone. Student's *t* tests were used to determine statistically significant differences ($P \leq 0.05$) between metabolite concentrations of transgenic and WT clones. Unidentified metabolites were denoted by their retention index (Wagner et al. 2003; Schauer et al. 2005), as well as key m/z fragments or class of compound. Retention indices of unidentified metabolites were determined by interpolation of the retention indices of known metabolites bracketing the unknown, using the average values reported at the Golm Metabolome Database website (http://www.csbdb.mpimp-golm.mpg.de/csbdb/gmd/profile/gmd_smpq.html).

GA analysis

One to two grams of expanding leaves were collected from 3-month-old greenhouse-grown control (WT), and *gai*- and *rgl1*-expressing transgenic plants, each represented by three independent transformation events. The leaves were immediately weighed, frozen in liquid nitrogen, and later lyophilized. Each replicate sample was extracted in 80% MeOH with internal standards of [$^2H_2$]-GA_1, –GA_4, –GA_8, –GA_9, –GA_{19}, –GA_{20}, –GA_{29}, –GA_{34}, –GA_{44}, and –GA_{53} (L. Mander, Australian National University) and reextracted with MeOH (Pearce et al. 2002). The extract was reduced to aqueous, and the GAs extracted into EtOAc at pH 3, then with K-Pi buffer at pH 8.5, and again into EtOAc at pH 3. The sample was further purified on QAE Sephadex A-25 and C_{18} Sep-Pak; methylated with ethereal diazomethane, dried, suspended in water and partitioned with EtOAc. The EtOAc was passed through an NH_2-PrepSep col-

umn (300 mg, Fisher). The eluate was dried, standards of methyl-[^{3}H]-GA_1 and –GA_4 were added, and the methyl-GAs were purified via C_{18} HPLC. Groups of fractions were silylated and analyzed by GC-MS and/or GC-SIM. All GAs were identified from WT samples by GC-SIM (at least five ions), with the exception of GA_4, present in low quantity (M^+ only). In the *rgl1* and *gai* transgenics, GA_1 and GA_4 were identified by full-scan GC-MS; GA_8, GA_{19}, GA_{20}, GA_{29}, and GA_{34} by GC-SIM (at least five ions); and GA_{44} and GA_{53} by M^+ only. GA_9 was not detected in any samples. GAs were quantified by reference to the internal standards using M^+ ratios (or the ratio of the base pair at *m*/*z* 434/436 for GA_{19}/[^{2}H] GA_{19}) in equations for isotope dilution analysis.

Results

DELLA proteins in *Populus*

Using known sequences of DELLA proteins from *Arabidopsis* and other species, we have searched the draft *Populus* genome sequence for putative DELLA proteins. We have identified four sequences with the canonical DELLA domain approximately matching the number of DELLA proteins in *Arabidopsis* (5) (Table 1). The four sequences belong to two distinct lineages, with PtRGL1_1 and PtRGL1_2 having the highest similarity to RGL1 from *Arabidopsis* and PtGAI1 and PtGAI2 having the highest similarity to the *Arabidopsis* GAI protein (Fig. 1). Both pairs of genes show very high sequence similarity, indicative of a recent duplication. The monocot representatives were very well separated in an isolated lineage from the dicot group (Fig. 1). All genes were supported by EST clones originating from shoot tips, terminal vegetative buds, and phloem/cambium tissues (Table 1).

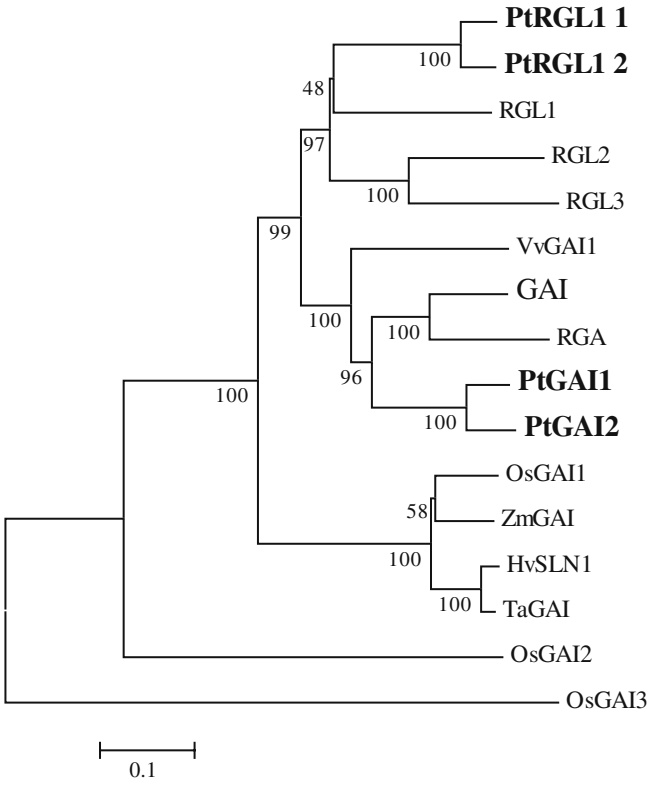

Fig. 1 Unrooted neighbor-joining (Kumar et al. 2001) phylogenetic tree of DELLA domain proteins from seven plant species. Poplar proteins are in *bold* and *larger font*. *Numbers in branch points* indicate bootstrap support from 1,000 iterations. Accession and AGI numbers: *GAI* (*Arabidopsis thaliana*, At1g14920); *RGA* (*Arabidopsis thaliana*, At2g01570); *RGL1* (*Arabidopsis thaliana*, At1g66350); *RGL2* (*Arabidopsis thaliana*, At3g03450); *RGL3* (*Arabidopsis thaliana*, At5g17490); *OsGAI1* (*Oryza sativa*, BAA90749); *OsGAI2* (*Oryza sativa*, AAR31213); *OsGAI3* (*Oryza sativa*, BAD82782); *HvSLN1* (*Hordeum vulgare*, AAL66734); *VvGAI1* (*Vitis vinifera*, AAM19210); *TaGAI* (*Triticum aestivum*, CAB51555); and *ZmGAI* (*Zea mays*, CAB51557)

Transgenic plants overexpressing *Arabidopsis gai*, *GAI* and *rgl1*

We generated transgenic poplars overexpressing *Arabidopsis* GAI proteins with either a WT (*GAI*) or mutated DELLA domain (*gai*), as well as the RGL1 protein without a DELLA domain (*rgl1*) that show the highest similarity to the putative poplar orthologs (Fig. 1, Table 1). We also used versions with the native promoter and WT form of GAI. We recovered more than 20 independent lines (except for the 35S::*gai* construct) (Table 2) and verified the integration of all transgenes via the polymerase chain reaction (PCR) (Table 2).

Table 1 Poplar homologs of DELLA domain proteins

Name	Gene model	EST support source
PtRGL1_1	eugene3.00040654	Shoot tips
PtRGL1_2	eugene3.00002319	Phloem and cambium
PtGAI1	eugene3.00081230	Terminal vegetative buds
PtGAI2	eugene3.00101036	Terminal vegetative buds

The EST source was that of the EST sequence producing the greatest similarity to the gene model sequence

Examination of the plants in vitro and under greenhouse conditions indicated that overexpression of *gai* under its native *Arabidopsis* promoter and *rgl1* under the 35S promoter caused reduced stature in multiple independent transgenic lines (Table 2, Fig. 2). Approximately 90% of the lines containing the *rgl1* transgene were severely dwarfed and 30% of the lines transformed with *gai* under the control of its native *Arabidopsis* promoter showed visible dwarfing (Table 2). We did not observe any effect from WT *GAI* expression driven by either the native *Arabidopsis* or 35S promoter. More subtle phenotypic effects may be detected later under field conditions.

It was very difficult to recover transgenic plants with the 35S::*gai* construct. After screening more than

Table 2 Proportion of mutant poplars in tissue culture after transformation with *gai*, *GAI*, and *rgl1*

Transgene	Promoter	Lines with Transgene[a]	Lines showing strong dwarf phenotype[b]	Mutant proportion
Gai	*PGAI*[c]	30	9	0.35
Gai	*CamV35S*	11	1	0.10
GAI	*pGAI*	38	0	0.00
GAI	*CamV35S*	34	0	0.00
Rgl11	*CamV35S*	26	26	1.00

[a]Lines that were verified by PCR for the integration either *gai*, *GAI* or *rgl1* transgenes
[b]Lines that were decisively smaller than WT
[c]Native *Arabidopsis GAI* promoter

threefold explants (total of ~700) than had been done for the other transgenes, we were able to recover only 11 lines containing 35S::*gai*, and only one of them showed an obvious dwarfing phenotype (Table 2). We recovered many plants with a truncated T-DNA insert in which *nptII* but not the *gai* gene was integrated (data not shown), an effect that was not observed with any of the other constructs. We believe that deleterious effects of

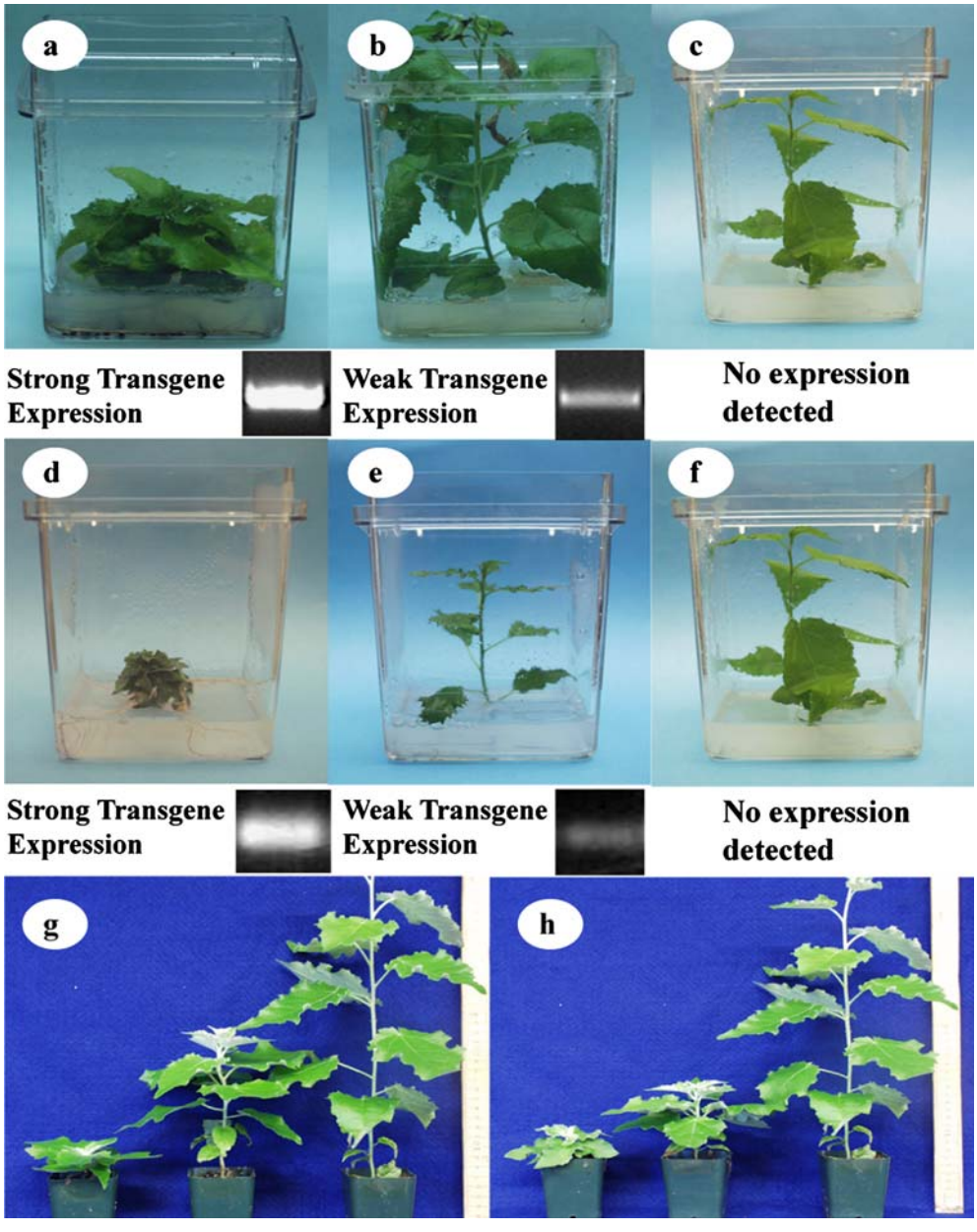

Fig. 2 Effect of *gai* and *rgl1* expression in transgenic poplars. **a** Severely dwarfed line with high levels of *gai* expression. **b** WT-like transgenic line with low levels of *gai* transgene expression. **d** Severely dwarfed line with high levels of *rgl1* expression. **e** WT-like transgenic line with low levels of *rgl1* expression. **c**, **f** Untransformed WT (control) plants. **g** Same plants from left to right as shown in panels **a–c** after 4 months' growth in a greenhouse. **h** Same plants from left to right as shown in panels **d–f** after 4 months' growth in greenhouse. Plants shown in photos are representative of at least five ramets of the same line. Expression was evaluated in three independent PCR reactions in three independent ramets of each line

high *gai* expression may have affected regeneration and contributed to observed recovery of events with either incomplete integration or low transgene expression.

To confirm transgene expression, we extracted RNA and performed RT-PCR on six independent *gai*- and *rgl1*-expressing lines that showed varying degrees of dwarfing. We were able to detect the presence of transgene transcripts in all lines that were examined. The level of transgene expression was measurably higher in most dwarfed plants compared to less dwarfed plants for both *rgl1* and *gai* transgenics (Fig. 2). The ubiquitin gene that was used as a loading control showed equal expression across all the transgenic and WT lines examined (data not shown).

GA metabolic profile changes in *gai* and *rgl1* plants

GA insensitive and RGA exert feedback effects on GA metabolism in herbaceous plants (Fujioka et al. 1988; Talon et al. 1990; Tonkinson et al. 1997; Chandler et al. 2002) but there has been no report on the effect of any of the RGL proteins. No information is available on the effect of any of the DELLA proteins on GA metabolism in woody plants. Consequently, we measured key GAs in leaves of the *rgl1* and *gai* transgenics. We found similar perturbations in GA metabolism for both *gai*- and *rgl1*-expressing transgenics. Concentrations of the bioactive GA_1 and GA_4 increased 12- to 64-fold (Table 3). Their C_2-hydroxylated catabolites, GA_8 and GA_{34} also increased, but to a much lesser extent (two- to ninefold). In contrast, the C_{20} GA precursors of GA_1 (GA_{53}, GA_{44}, and GA_{19}) decreased three- to fivefold. The concentration of GA_{20} did not change significantly, while its C_2-hydroxylated metabolite GA_{29} decreased several-fold.

rgl1/gai effect on root formation

We observed greatly enhanced root system development for both *rgl1* and *gai* transgenics grown in vitro. Roots of transgenics had two to six times higher fresh mass than those of the WT plants (Fig. 3). The roots of *rgl1* and *gai* poplars were thick, ruddy, and displayed a proliferation of lateral roots (Fig. 4).

By providing an exogenous supply of bioactive GA_3 in the tissue culture medium, we tested the GA response of six independent poplars lines that expressed each of the three transgenes: *gai*-, *GAI*- and *rgl1*. For controls, we used WT and transgenic plants that overexpressed bean (*Phaseolus coccineus*) *PcGA2ox1*. PcGA2ox is a GA biosynthesis enzyme that deactivates both bioactive $GA_{1/4}$ and their immediate precursors ($GA_{20/9}$) and has strong dwarfing effect in poplar (Busov et al. 2003). PcGA2ox cannot metabolize GA_3.

The presence of GA_3 in the medium induced rapid elongation in WT, *PcGA2ox*, and *GAI* transgenics, but had little to no effect on the dwarfed *gai*- and *rgl1*-expressing poplars (Fig. 5).

In response to GA_3 treatment, we observed a dramatic reduction in the formation of adventitious roots in WT, *GA2ox*, and *GAI* overexpressing plants. On average, there was a two- to fourfold reduction in both the number of adventitious roots (Fig. 6) and the percentage of rooted plants (data not shown). In contrast, *rgl1*- and

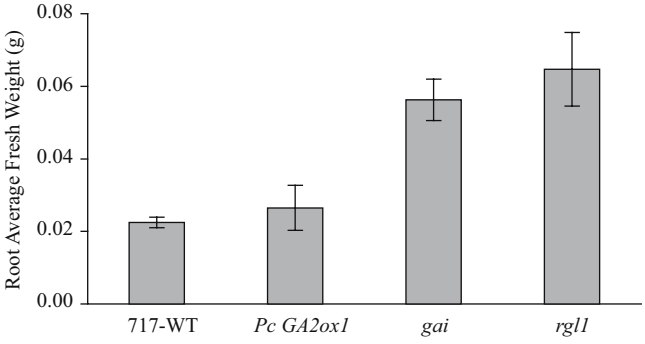

Fig. 3 Root systems of dwarf transgenic poplars produce high biomass. Ten lines and five ramets per line were grown for 1 month under the same conditions before roots were washed and weighed. *Bars* show one standard error over line means

Table 3 Gibberellin content in expanding leaves of *rgl1*, *gai*, and WT plants

GA	Wild-type (ng/g dry wt^{-1})	*rgl1*	*gai*	Ratio	
				rgl1:WT	*gai*:WT
GA_{53}	3.6 (0.7)	1.0 (0.1)[b]	0.4 (0.2)[b]	0.3	0.1
GA_{19}	20 (1.6)	4.6 (1.1)[a]	2.5 (0.3)[a]	0.2	0.1
GA_{44}	1.6 (0.2)	0.6 (0.1)[a]	0.4 (0.1)[a]	0.4	0.2
GA_{20}	3.1 (1.1)	2.0 (0.6)	2.6 (0.2)	0.6	0.7
GA_1	2.6 (0.6)	117 (21)[a]	31 (4.5)[a]	44.3	11.5
GA_4	0.9 (0.2)	55 (12)[a]	19 (4.8)[b]	63.7	22.1
GA_8	37 (6.5)	106 (21)[a]	77 (5.6)[a]	2.9	2.1
GA_{29}	11 (2.9)	0.8 (0.1)[b]	2.2 (0.3)[b]	0.1	0.2
GA_{34}	1.2 (0.3)	11 (1.0)[a]	4.1 (0.6)[a]	8.6	3.3

[a, b]Means with SE for three replicates of each genotype, one from each of three ramets
Difference from WT significant at [a]$P < 0.05$ or approaching significance [b]$P < 0.11$, in Student's *t* test with equal or unequal variances as appropriate

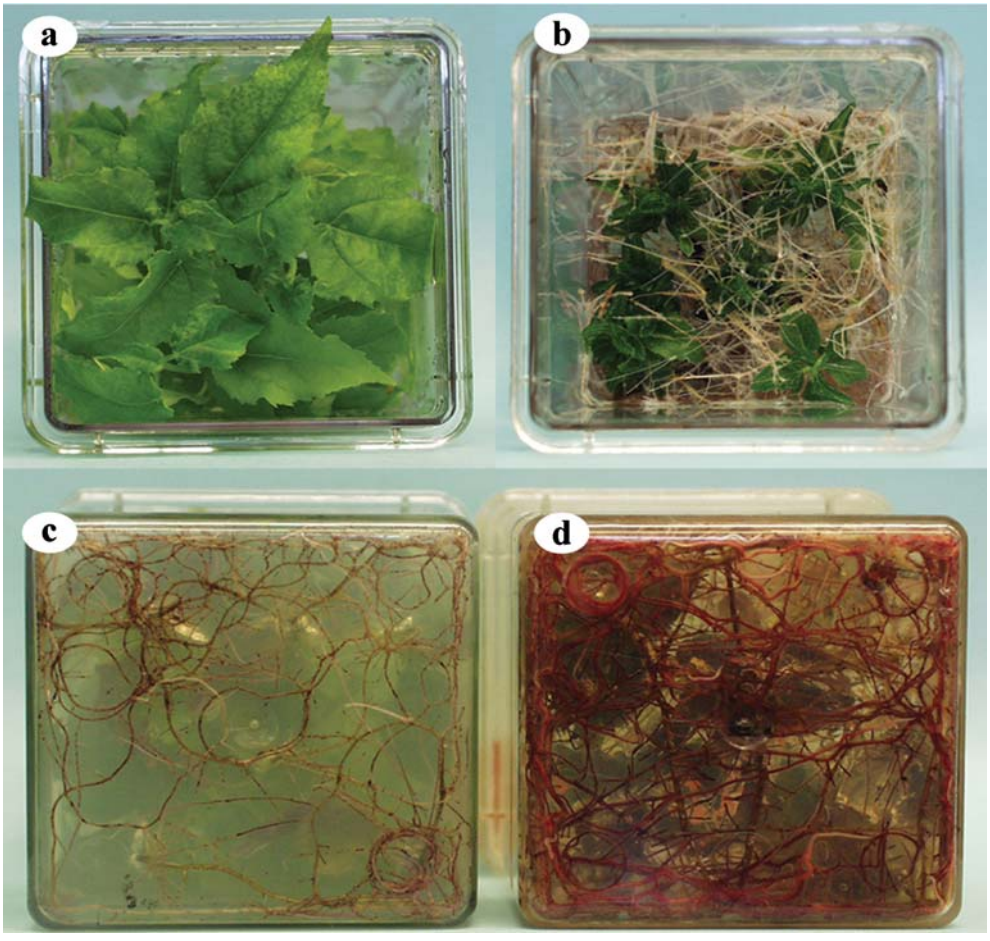

Fig. 4 Dwarf transgenic poplars produce large and morphologically distinct root systems. One-month-ld *rgl1* transgenic plants are pictured. **a**, **c** WT plants. **b**, **d** Transgenic plants expressing *rgl1*. The bottom panel shows the root systems of the same plants shown in the top panel. Plants are representative of all ramets (at least five) of a given line

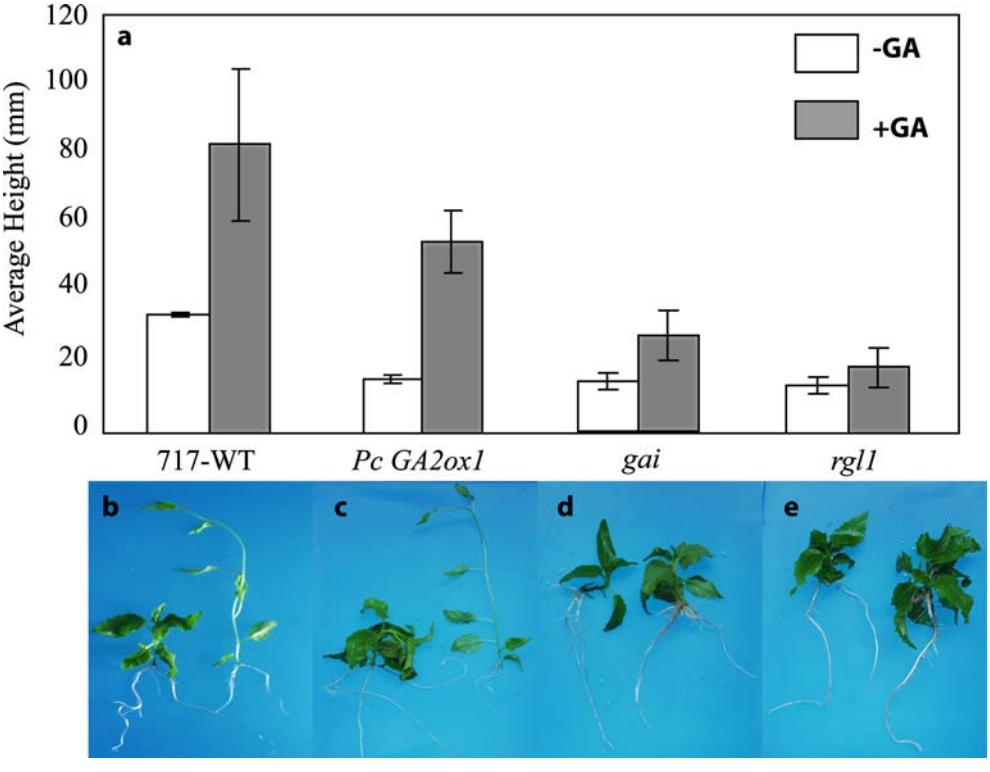

Fig. 5 *gai* or *rgl1*-expressing poplars do not respond to GA-induced stem elongation. **a.** Average height of WT and transgenic plants grown in vitro on media with or without 100 μM GA_3 (+GA and −GA, respectively) for 4 weeks. *Bars* show one standard error over five line means. **b–e** Photos below each *bar pair* represent typical plants that were used in the measurements. Plants to the *left* and *right* in each panel from control (−GA) and GA treatment (+GA), respectively

gai-transformed plants appeared to be completely unaffected by the treatment, producing roots despite the GA_3 incorporated in the medium.

Metabolic profiles in roots of *gai* and *rgl1* dwarfs

To gain insight into the biochemical changes that might be associated with the dramatic architectural changes of the dwarfed transgenic plants, we performed metabolic profiling in different organs from plants grown under the same conditions in the greenhouse. Roots of both types of mutants had similar metabolic profiles, including monosaccharide (glucose, fructose, and galactose) concentrations that were $\leq 50\%$ that of the WT plants, coupled with two- to threefold accumulations of citric acid and several amino acids, including Glu, Arg, GABA, and Asp (Table 4). Both *gai* and *rgl1* transgenic plants accumulated two unidentified glucosides (retention index: 3238.0 and 3002.5) that were either not present in the WT or were below the detection threshold.

We also observed specific metabolic differences associated with either *gai* or *rgl1* presence. For example, roots of *rgl1* transgenics accumulated Asn (4.39-fold that of WT) and carbamoyl aspartate (3.42-fold), while the levels of these metabolites were not significantly higher in the *gai* transgenics. Transgenics expressing *gai* accumulated salicin (5.27-fold), and threonic acid (1.95-fold) while these metabolite changes were not found in *rgl1* expressing plants.

Metabolic profiles in leaves of *gai* and *rgl1* dwarfs

Many more metabolites were affected in leaves than in roots of both *gai* and *rgl1* transgenic types. Most of the detected changes were associated with various products and intermediates of the phenylpropanoid biosynthetic pathway. Both *gai* and *rgl1* transgenics accumulated three major phenoloic glucosides—salicin, tremulacin, and syringin (Table 5). Catechol was also significantly increased in *gai* transgenics, and the same trend was evident in *rgl1* plants; both transgenics showed a 30–33% decline in the precursor metabolite, salicylic acid. Plants containing *rg1l* accumulated chlorogenic acid (3-*O*-caffeoylquinic acid), which was coupled with a decline in quinic acid, one of its monomers and a decline in the related, minor phenolic acid–quinic acid conjugates. The latter included 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 1-*O*-feruloylquinic acid, and 5-*O*-feruloylquinic acid. These declines were accompanied by reductions in other phenolic acid conjugates, such as galloylglucoside and caffeoylglucoside (Table 5), but there were no effects on the phenolic acid monomers themselves (data not shown). Levels of phenylalanine, a key upstream precursor of the phenolic acid biosynthetic pathway, were reduced by 31–37%.

Plants expressing either *gai* or *rgl1* also accumulated the same unidentified glucoside (retention index 3002.5), and another unidentified glucoside (retention index 3200.2) containing a relatively unique fragment (315 m/z, Table 5) which was not detectable in WT plants.

In contrast to roots, the levels of many *N*-containing metabolites were lower in the leaves of *rgl1*, including ethylamine, ethanolamine, glutamine, and Gly, Ala, Val, GABA were also present at lower levels in leaves of *gai* transgenics, but not in leaves of the *rgl1* plants.

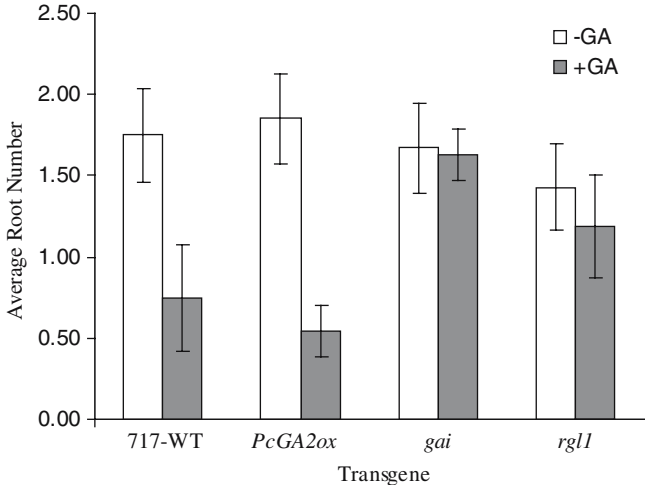

Fig. 6 Inhibition of GA-induced adventitious rooting. Average root number of WT and transgenic plants grown on tissue culture media with and without 10 μM GA_3 (+GA and −GA, respectively) for 4 weeks. *Bars* show one standard error over five line means

Discussion

The role of GA in shoot development has been well characterized (Hooley 1994). However, the way in which GA regulates root development is still poorly understood (Fu and Harberd 2003). We have demonstrated that exogenously applied GA has an inhibitory effect on adventitious root formation. Similar effects of GA on root development were also reported for transgenic poplars overexpressing an *Arabidopsis GA 20-oxidase* gene (Eriksson et al. 2000), resulting in the overproduction of GA. Impaired root growth was also found in rice with a null mutation at the *Slender-1* locus, encoding a GAI-like protein (Ikeda et al. 2001). We have shown that *rgl1* and *gai* presence can overcome this repression. In addition to negating the repressive effect of GA on adventitious rooting, *rgl1* and *gai* expression also alters root morphology, causing development of a more extensive root system.

The mechanism of the increased root development is still unknown. Fu and Harberd (2003) recently suggested a role for DELLA proteins in root development. In *Arabidopsis* RGA, (GAI-like) protein, although present in the root elongation zone was completely excluded when roots were rapidly elongating in response to

Table 4 Root metabolite responses of *gai* and *rgl1* transgenic plants relative to wild-type (WT) plants, and relative metabolite concentrations (μg glucose equiv./g FW) of transgenics, as determined by gas chromatography-mass spectrometry with electron impact ionization

Metabolite	*gai*/WT	*P*-value	*gai*	Metabolite	*rgl1*/WT	*P*-value	*rgl1*
3002.5–286,242	Novel	0.000	42 (9)	3002.5–286,242	Novel	0.000	30 (4)
3238.0–363,273	Trace in WT	0.000	106 (36)	3238.0–363,273	Trace in WT	0.000	92 (24)
Salicin	5.27	0.017	302 (57)	Asparagine	4.39	0.025	1159 (254)
GABA	3.18	0.035	12 (3)	*N*-Carbamoylaspartic acid	3.42	0.026	330 (65)
Citric acid	3.07	0.042	155 (34)	Uracil	3.30	0.065	86 (24)
Arginine	2.92	0.002	1366 (11)	GABA	3.27	0.007	13 (2)
Glutamic acid	2.62	0.003	41 (3)	Arginine	2.81	0.007	1314 (150)
Uracil	2.60	0.001	68 (5)	Glutamic acid	2.64	0.010	41 (5)
Asparagine	2.39	NS	631 (187)	Citric acid	2.49		126 (14)
N-Carbamoylaspartic acid	2.38	0.069	230 (51)	Aspartic acid	2.07	0.017	126 (16)
Aspartic acid	2.18	0.005	133 (12)	Glutamine	1.76	NS	1775 (547)
Glutamine	2.00	0.026	2022 (207)	Salicin	1.49	NS	86 (23)
Threonic acid	1.95	0.035	4 (1)	Threonic acid	1.00	NS	4 (1)
Myoinositol	1.41	NS	1773 (560)	Myoinositol	0.70	0.002	885 (43)
Salicylic acid glucoside	1.37	0.035	13 (6)	Salicylic acid glucoside	0.57	NS	5 (1)
Glucose	0.53	0.009	2907 (397)	Glucose	0.54	0.013	2978 (464)
Fructose	0.38	0.029	1366 (111)	Fructose	0.48	0.037	1314 (150)
Galactose	0.33	0.013	54 (15)	Galactose	0.40	0.046	65 (28)

Only those metabolites (analyzed as trimethylsilyl derivatives) of transgenic plants whose concentrations were statistically different ($P \leq 0.05$) from WT plants are shown. Data are the mean (SEM) of three replicates. Unknowns are denoted by retention index and key m/z fragments

GA treatment (Fu and Harberd 2003). The destabilization of RGA likely released the growth-restraining effects of DELLA proteins in the root elongation zone. It could be speculated that because DELLA-less forms of these proteins are more resistant to degradation, they will have strong inhibitory effect on root elongation growth, and thus promote faster transition to differentiation zone where most of the lateral roots initiate. Our observation that *rgl1*/*gai* overexpression promotes lateral root formation and overcomes the inhibitory effect of GA on adventitious root formation, a process very similar to lateral root formation (Smith and Federoff 1995), supports this hypothesis.

Another explanation of the increased root growth is suggested by the changes in root metabolic profiles, which indicate an increased rate of respiration in roots of *gai* and *rgl1* expressing transgenic poplars. Depletion of monosaccharides in roots, coupled with the accumulation of citric acid, amine-rich amino acids, such as Asn and Arg, could be due to increased respiratory consumption of monosaccharides to generate Krebs cycle organic acids that are required for amino acid synthesis and root growth. The concurrent decline in Gln and other N-containing metabolites, including phenylalanine, in *rgl1* leaves is consistent with increased N allocation to roots via perturbations in the secondary carbon pathways. Increased respiration in roots coupled with higher root biomass allocation has been reported for GA-deficient tomato (Nagel and Lambers 2002). Interestingly, the increased biomass allocation to roots was found to be a direct consequence of GA deficiency and not an indirect effect of the reduced growth. Thus, it appears that GA may have control over plant architecture and biomass allocation via regulating a number of primary and secondary biosynthetic pathways coordinately.

Changes in the metabolic profiles in leaves of dwarf plants indicate reduced carbon flux through the lignin biosynthetic pathway and a shift to storage and defense compounds associated mainly with phenylpropanoid pathway. For example, the accumulation of syringin (sinapyl alcohol glucoside), likely reflected the reduced shoot growth of the transgenics, leading to a build up of a storage form of the monolignol precursor. Phenolic glucosides like salicilin and tremulacin that may divert carbon flux through the lignin pathway, and play a role in defense were similarly found at much higher levels in both *gai* and *rgl1* expressing plants. The accumulation of 3-*O*-caffeoylquinic acid, coupled with declines in quinic acid and other phenolic acid conjugates, may also indicate reduced carbon flux through the lignin biosynthetic pathway, given the conjugation of a key phenolic acid precursor of monolignol biosynthesis with an upstream organic acid intermediate of the shikimic acid pathway. A reduced level of lignification and changes to the expression patterns of a number of genes in the phenylpropanoid pathway was recently reported for GA-deficient transgenic tobacco (Biemelt et al. 2004). The phenylpropanoid pathway in trees plays a major role in productivity, herbivore defense, and adaptation (Hu et al. 1999; Peters and Constabel 2002), and has been intensively studied with the goal of modification of wood and pulping properties (Chiang 2002; Baucher et al. 2003). Regulation of the pathway via GA may provide new insights on the adaptive mechanisms of trees, as well as provide new avenues for wood modification.

Table 5 Leaf metabolite responses of *gai*, and *rgl1* transgenics relative to wild-type (WT) plants, and relative metabolite concentrations (μg glucose equiv./g FW) of transgenic clones, as determined by gas chromatography-mass spectrometry with electron impact ionization

Metabolite	*gai*/WT	*P*-value	*gai*	Metabolite	*rgl1*/WT	*P*-value	*rgl1*
3002.5–286,242 glucoside	Novel	0.001	71 (2)	3002.5–86,242 glucoside	Novel	0.001	19 (5)
3200.2–30,315 glucoside	Novel	0.001	2 (0)	3200.2–30,315 glucoside	Novel	0.001	5 (1)
Tremulacin	6.49	0.003	26 (3)	Tremulacin	9.29	0.013	37 (7)
Catechol	4.00	0.049	37 (10)	Syringin	3.32	0.036	29 (6)
Syringin	2.72	0.037	24 (5)	Catechol	2.81	0.057	26 (6)
Salicin	1.83	0.029	7168 (931)	3-O-Caffeoylquinic acid	2.02	0.050	48 (25)
Raffinose	1.56	0.010	10 (0)	Salicin	1.64	0.031	6,408 (699)
3-*O*-Caffeoylquinic acid	1.24	NS	89 (11)	Valine	0.96	NS	11 (3)
Glutamine	0.92	NS	1528 (340)	Raffinose	0.83	NS	5 (1)
Threonic acid	0.90	NS	376 (40)	GABA	0.83	0.049	27 (1)
Galloylglucoside	0.88	NS	191 (18)	Threitol	0.81	0.002	77 (4)
Myoinositol	0.79	NS	11223 (560)	Salicylic acid	0.70	0.041	7 (1)
Quinic acid	0.72	0.033	2549 (113)	Phenylalanine	0.69	0.013	6 (0)
Salicylic acid	0.67	0.002	6 (0)	Glutamine	0.67	0.006	1,109 (82)
Valine	0.67	0.047	8 (0)	Ethylamine	0.65	0.019	29 (2)
(D+)-Arabitol	0.63	NS	40 (13)	Glycerol	0.62	0.035	25 (2)
Phenylalanine	0.61	0.007	5 (0)	Threonic acid	0.61	0.032	252 (37)
Galactaric acid	0.61	NS	6 (1)	Myoinositol	0.59	0.043	8,401 (642)
2294.8—Inositol-phosphate	0.61	NS	63 (27)	Alanine	0.57	0.057	25 (2)
GABA	0.60	0.003	17 (1)	Ethanolamine	0.53	0.039	54 (7)
Salirepin	0.60	NS	511 (52)	(D+)-Arabitol	0.45	0.024	28 (4)
Glycerol	0.58	0.036	24 (3)	*β*-sitosterol	0.41	0.033	63 (12)
4-*O*-Caffeoylquinic acid	0.56	0.042	7 (1)	Quinic acid	0.41	0.006	1,443 (265)
Glucaric acid	0.55	0.048	38 (9)	4-O-Caffeoylquinic acid	0.38	0.012	5 (0)
Alanine	0.54	0.045	24 (2)	Glycine	0.37	0.006	9 (3)
Ethylamine	0.51	0.014	23 (4)	Caffeoylglucoside	0.27	0.016	42 (9)
Threitol	0.44	0.001	43 (3)	2294.8—Inositol-phosphate	0.26	0.036	27 (2)
1-*O*-Feruloylquinic acid	0.42	0.001	14 (1)	Galloylglucoside	0.26	0.014	56 (13)
Ethanolamine	0.42	0.011	43 (1)	Galactaric acid	0.25	0.019	2 (1)
Ethyl phosphate	0.39	0.081	433 (149)	Ethyl phosphate	0.23	0.031	262 (59)
β-sitosterol	0.37	0.021	58 (2)	Phosphate	0.22	0.007	2,799 (814)
5-*O*-Feruloylquinic acid	0.36	0.005	10 (0)	Glucaric acid	0.16	0.001	11 (3)
Phosphate	0.36	0.012	4565 (669)	1-*O*-Feruloylquinic acid	0.15	0.008	5 (1)
Caffeoylglucoside	0.35	0.023	55 (8)	Salirepin	0.15	0.020	129 (19)
Glycine	0.28	0.001	7 (1)	5-*O*-Feruloylquinic acid	0.13	0.002	3 (1)
5-*O*-Caffeoylquinic acid	0.24	0.021	11 (1)	5-*O*-Caffeoylquinic acid	0.05	0.010	2 (1)

Only metabolites (analyzed as trimethylsilyl derivatives) that were statistically different ($P < 0.05$) between transgenic and WT plants are shown. Data are the mean (SEM) of three replicates. Unknowns are denoted by retention index and key m/z fragments or class of compound

This is the first description of the effects of expression of *rgl1* on GA content in any species, and the first report of the effects of *gai* in a woody plant. The effects of both genes on GA content in poplar were similar to those found in studies of dominant dwarf DELLA mutants in a variety of herbaceous plants (Fujioka et al. 1988; Talon et al. 1990; Tonkinson et al. 1997; Peng et al. 1999b; Chandler et al. 2002). In all cases, the content of the biologically active GAs (GA_1, GA_3, or GA_4) were manyfold higher than in the WT controls and the content of C_{20} GAs (GA_{53}, GA_{19}, and GA_{44}) was reduced. Such effects have been linked to feedback regulation of *GA 20-oxidase* or *GA 3-oxidase* (Xu et al. 1995; Peng et al. 1997; Cowling et al. 1998; Fu et al. 2001). It has also been predicted, without direct evidence at the transcript or protein levels, that activity of *GA 2-oxidase* genes might be reduced by feed-forward regulation, to further increase the content of GA_1 and GA_4 (Olszewski et al. 2002). Consistent with lowered activity of GA 2-oxidase(s), we found the ratio of the C_2-hydroxylated GA_8 to its precursor, GA_1, decreased more than tenfold in both *rgl1* and *gai*, that the ratio of C_2-hydroxylated GA_{34} to GA_4 decreased about sevenfold, and the ratio of C_2-hydroxylated GA_{29} to GA_{20} also decreased severalfold.

The pleiotropic responses described in this paper provide new insights into the integration of ecophysiological adaptation of poplars and suggest that tree breeding that is directed at GA regulatory genes could have important benefits for drought tolerance, bioremediation, pest tolerance, and carbon sequestration.

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