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ORIGINAL ARTICLE

Repression of gibberellin biosynthesis or signaling produces striking alterations in poplar growth, morphology, and flowering

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Abstract We modified gibberellin (GA) metabolism and signaling in transgenic poplars using dominant transgenes and studied their effects for 3 years under field conditions. The transgenes that we employed either reduced the bioactive GAs, or attenuated their signaling. The majority of transgenic trees had significant and in many cases dramatic changes in height, crown architecture, foliage morphology, flowering onset, floral structure, and vegetative phenology. Most transgenes elicited various levels of height reduction consistent with the roles of GA in elongation growth. Several other growth traits were proportionally reduced, including branch length, internode distance, and leaf length. In contrast to elongation growth, stem diameter growth was much less affected, suggesting that semi-dwarf trees in dense stands might provide high levels of biomass production and carbon sequestration. The severity of phenotypic effects was strongly correlated with transgene expression among independent transgenic events, but often in a non-linear manner, the form of which varied widely among constructs. The majority of semi-dwarfed, transgenic plants showed delayed bud flush and early bud set, and expression of a native GAI transgene accelerated first time flowering in the field. All of the phenotypic changes observed in multiple years were stable over the 3 years of field study. Our results suggest that transgenic modification of GA action may be useful for producing semi-dwarf trees with modified growth and morphology for horticulture and other uses.

Keywords Hormones · Populus · Growth · Gibberellin

Abbreviations

GA	Gibberellins
GA2ox	Gibberellin 2 oxidase
GAI	GA insensitive
RGL	Repressor of GA1 like

Introduction

Semi-dwarfism is an important production trait in many crop species. The 'green revolution' varieties of rice and wheat are semi-dwarfs which have brought about major increases in grain yields world-wide (David and Otsuka 1994; Nagano et al. 2005). In fruit trees, semi-dwarf varieties allow dense field cultivation; facilitate mechanized maintenance; increase efficiency of pollination and fruit collection; allow more precise pesticide application (Webster 2002); and when used as a rootstock elicits precious and profuse flowering (Atkinson and Else 2001). Semi-dwarf trees are also desirable for ornamental purposes, such as in street and backyard trees, and for planting under power lines. Trees with much reduced growth in height relative to girth have been proposed as desired

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ideotypes for bioenergy plantings (Ragauskas et al. 2006). Semi-dwarfism has also been considered as a biosafety trait due to the decreased fitness of such plants in competition for light and other resources (Gressel 1999), potentially limiting the spread of linked transgenes. However, classical tree breeding to select for traits like reduced stature, while retaining tree health, may be difficult due to the scarcity or complete absence of such alleles in wild populations (Bradshaw and Strauss 2001).

Dwarfism is associated with deficiencies in gibberellin (GA) levels or perturbations in GA signaling pathways (Peng et al. 1999; Spielmeyer et al. 2002). Higher levels of several bioactive GA forms induce rapid shoot elongation. Conversely, low levels of these bioactive forms, or blocks in the response to GA, causes reduced elongation and thus dwarfism (Davies 1995). GA metabolism and signal transduction pathway are well studied in *Arabidopsis* and several other annual plants (Hedden et al. 2001; Olszewski et al. 2002; Itoh et al. 2008). Many of these genes are also found to show similar effects when heterologously expressed in different plant species (Fu et al. 2001; Biemelt et al. 2004; Dijkstra et al. 2008).

The *GA 2-oxidases* and *DELLA* genes are key regulatory genes that decrease bioactive GA and its signaling. *GA 2-oxidases* (*GA2ox*) are small gene families that encode major enzymes involved in GA catabolism (Rieu et al. 2008; Lo et al. 2008). Increased expression of *GA2ox* in transgenic plants decreases bioactive GA and results in dwarfed phenotypes (Schomburg et al. 2002; Curtis et al. 2005; Lee and Zeevaart 2005; Dijkstra et al. 2008). DELLA domain proteins belong to a subfamily within the larger GRAS transcription factor family (Pysh et al. 1999). The conserved DELLA domain in these proteins is important for their destabilization and degradation, and when mutated constitutively blocks one or more GA responses (Itoh et al. 2008; Schwechheimer and Willige 2009; Harberd et al. 2009). Transgenic overexpression of DELLA domain proteins and particularly versions of the protein with deletion of the N-terminal DELLA region, confers a GA-unresponsive and constitutive dwarf phenotype (Fu et al. 2001; Hynes et al. 2003).

Although the regulatory role of GA has been well studied in herbaceous annuals under laboratory conditions, their role in woody perennial species, particularly in natural environments is poorly understood. For example it has been long speculated that GA plays a role in short day (SD)-induced cessation of shoot growth, which precedes entry into dormancy—an adaptive mechanism of temperate trees to survive harsh winter weather. Correlative measurements suggest role of GA metabolism but direct evidence from gene/transgene manipulations are absent (Olsen 2010). Furthermore, flowering in trees occurs after a period of juvenile vegetative growth which can last from several years to decades (Brunner and Nilsson 2004). Although very little is known about the role of GA in this phenomenon, lesions in the DELLA domain protein in *Vitis* causes precocious first flowering (Boss and Thomas 2002).

Here we expressed several forms of well-characterized GA2ox and DELLA domain proteins in poplar trees and studied their effects on growth, form and phenological characteristics over a 3-year period in the field. We report that these genes elicit a wide variety of phenotypic effects on all of these traits, and show a strong but non-linear association with the intensity of transgene expression.

Materials and methods

Transgenics, field trial design, and phenotypic measurements

All transgenic manipulations were performed in clone INRA717-1B4 (*P. tremula* × *P. alba*) using standard *Agrobacterium*-mediated transformation procedures (Filichkin et al. 2006). The constructs used are described in Table 1. All transgenic plants were PCR-verified for the presence of the transgene as previously described (Busov et al. 2003, 2006). RT-PCR as described below was used to validate the expression of all transgenes. Approximately, 6-month-old containerized seedlings were planted in western Oregon (Corvallis) on flat, former agricultural land at two different dates (summer or fall of 2003) (Table 1). Ramets of approximately 20 independent events per construct were planted in pairs in two-tree plots at spacing of approximately 2 × 3 m between trees, and each plot was replicated twice in a randomized design. Weeds were controlled as needed by herbicide application and manual cultivation, and irrigation was applied during the first two growing seasons. Regular phenotypic measurements and observations were made for three growing seasons. Details about measurements of different traits are described in Table 2. Bud set was recorded on 10 October 2004. Only the apical bud of the stem leader of each tree was scored for presence of a fully developed vegetative bud. The presence of flowers was recorded once a month during the growing season (March–August).

Sampling, RNA extraction and reverse transcription (RT) reaction

Fresh samples, including leaves, petioles and stems from the apical part of branches, were collected from field-grown plants, immediately frozen in liquid nitrogen, and stored at −80°C. Total RNA was isolated from collected samples using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). To remove traces of genomic DNA, total RNA

Table 1 Constructs used in generating transgenic poplars

Construct name	Events	Gene	Source	Promoter	Accession	Type
35S::PcGA2ox	27	*PcGA 2-oxidase*	*Phaseolus*	CaMV 35S	AJ132438	Wild type
35S::PtGA2ox	19	*PtaGA 2-oxidase*	*Poplar*	CaMV 35S	AY392094	Wild type
35S::rgl	18	*rgl-1*	*Arabidopsis*	CaMV 35S	AY048749	Mutant (51 bp in-frame deletion of the DELLA domain)
35S::gai	12	*gai*	*Arabidopsis*	CaMV 35S	Y15193	cDNA, mutant (51 bp in-frame deletion of the DELLA domain)
35S::GAI	19	*GAI*	*Arabidopsis*	CaMV 35S	Y15193	cDNA, wild type
pGAI::gai	30	*gai*	*Arabidopsis*	Native GAI	Y15193	Genomic, mutant (51 bp in-frame deletion)
pGAI::GAI	29	*GAI*	*Arabidopsis*	Native GAI	Y15193	Genomic, wild type

Table 2 Description of measured traits in the transgenic field trail

Variables	Description
2004	
Height	Total height (m)
Diameter	Measured at 10 cm from the base of plant (cm)
Volume	Basal area × height (cm^3)
Branch height	Height from the base of the plant to the two longest branches (cm)
Internodal distance	Distance between 1st and 20th internode (cm)/20
Leaf length	Leaf sampled at leaf plastochron index 8 (LPI) (cm)
Leaf width	Leaf sampled at leaf plastochron index (LPI) (cm)
Leaf length/width (L/W)	The ratio of leaf length to width
Crown width	Mean of two widths taken at 90° from one another (cm)
Diameter/height (Dia/Ht)	Ratio of diameter to height
Bud set (BS)	Bud set (BS)
2005	
Branch angle (°)	Angle (°) formed between the main stem and two representative branches
Days to bud flush (DBF)	Days to bud flush measured from 1 January
Relative height growth (RelHtG)	(Height 2005 − height 2004)\(height 2004)
Relative diameter growth (RelDiaG)	(Diameter 2005 − diameter 2005)\(diameter 2004)
Flowering	Number of events that flowered

(18.5 μg) was treated with DNaseI using the DNA-free kit (Ambion, Austin, TX, USA). Two micrograms of DNaseI-treated total RNA was used to synthesize first-strand cDNA employing oligo-dT primer and Superscript II (Invitrogen, Carlsbad, CA, USA). Following cDNA synthesis all samples were treated with RNase H (New England Biolabs, Ipswich, MA, USA).

Real-time RT-PCR

The real-time PCR reactions were performed with a MX3000P Real-time PCR system (Stratagene, La Jolla, CA, USA) using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). Primers were designed using Genetics Computer Groups (GCG) (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA). The sequences of the primers used for PCR amplification of different transgenes are as follows: GAI-FWD: 5′-CTT GATTCTATGCTCACCGAC-3′; GAI-REV: 5′-TCAAC CAGGACAACATGCC-3′; GA2 oxidase-FWD: 5′-TGT CTCAGCCAGCATTGAACCAGT-3′; GA2 oxidase-REV: 5′-ATCACCGTTTGGGCCAATCCTCTT-3′; UBQ-FWD: 5′-AGAGTGTGAGAGAGAGAAGAG-3′; UBQ-REV: 5′-CGACGACCATCAAACAAGAAG-3′. Optimization of primer and cDNA concentrations were performed according to manufacturer's instructions (Stratagene, La Jolla, CA, USA). All PCR reactions were performed at the following conditions: 95°C for 15 s, 60°C for 30 s and 72°C for 90 s for 40 cycles and then followed by a denaturation step at 95°C, annealing at 55°C and a gradual increase in temperature up to 95°C to generate a dissociation curve.

Each assay was performed on 66.6 ng of sample cDNA, in triplicates and also included standard curve of six cDNA dilutions and no template negative control (sterile RNAse/DNAse-free distilled water). Expression of ubiquitin-like (*UBQ*) gene (*POPTR_0002s21690*) was used to correct for small loading differences. Relative transcript abundance was calculated using the standard curve method (http://www.appliedbiosystems.com).

Statistical analysis

All statistical analysis was performed as described below using SAS 9.1 (Cary, NC) unless otherwise noted. Graphs were made in SigmaPlot (Systat Software Inc., CA), Excel 2003 (Microsoft Corporation), and PRISM (PRISM version 5; Graphpad Software, CA).

Analysis of variance (ANOVA) was used to determine the effect of construct and event on measured traits (Table 2). This experiment was considered a fixed effects design with two nested factors, constructs and events within constructs (Milliken and Johnson 1984). Because of the different planting dates, we weighted trait values relative to their corresponding controls planted at equivalent times. The effects model was used to identify significant differences between constructs. Tukey's honestly significant difference (HSD) was used after significant differences between the constructs were identified to determine which constructs produced different responses. The means model (Milliken and Johnson 1984) was employed to evaluate the differences between events both within and between constructs. The least significant difference (LSD) multiple range test was used after significant differences were identified to determine which events within constructs produced different responses.

Principle component analysis (PCA) was performed on the correlation matrix of variables. Significant components were determined using the broken stick method (Jackson 1993). Principle component (PC) loadings (eigenvectors) were used to assess patterns of association among traits.

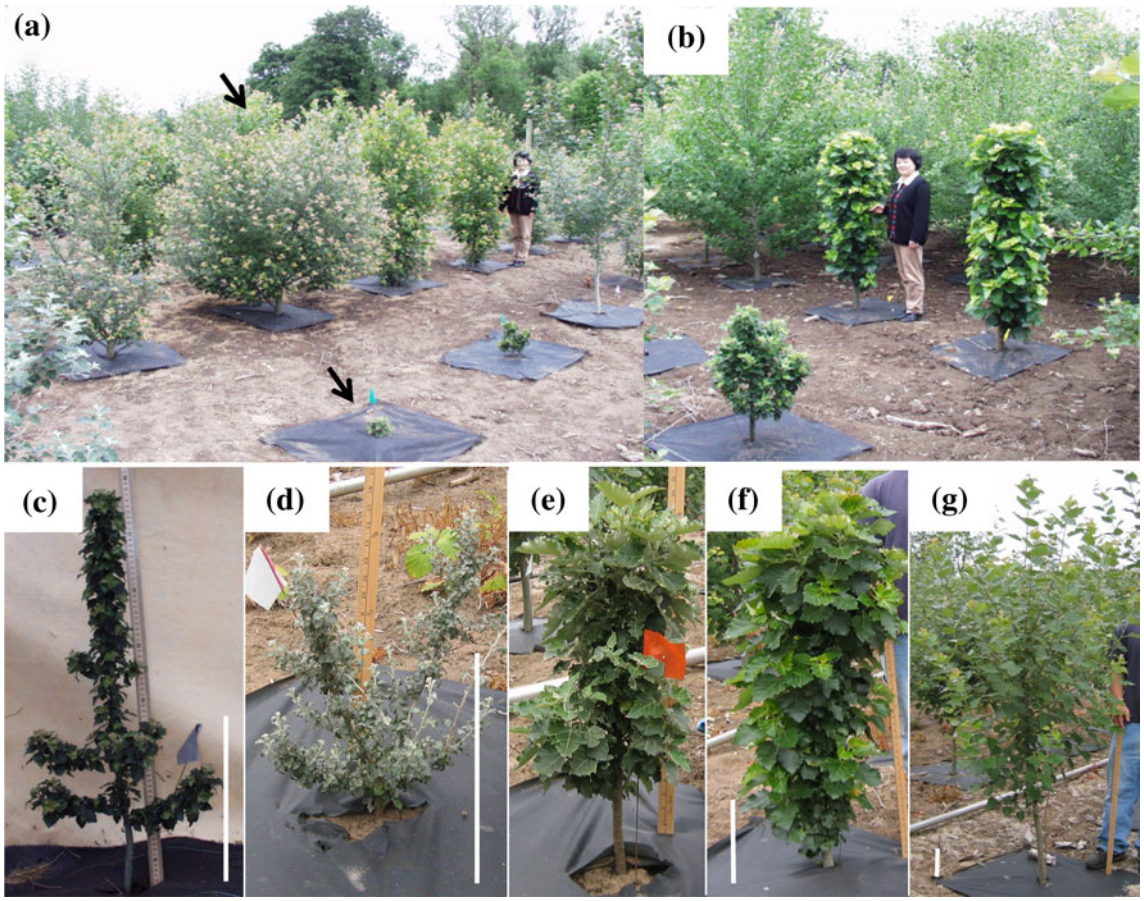


Fig. 1 Representative phenotypes produced in the field by GA deficient or insensitive *Populus* transgenics. **a** Dramatic variation in stature and crown characteristics. *Arrow to the left* points to wide crown observed in *35S::PcGA2ox* transgenics. *Arrow to the right* points to extreme dwarfing in *35S::rgl* expressing transgenics. **b** Narrow compact crowns observed in *pGAI::gai* transgenics. Extreme dwarfing elicited by *35S::PtGA2ox* (**c**), *35S::PcGA2ox* (**d**), *35S::rgl* (**e**), *pGAI::gai* (**f**). **g** WT 717 plant. *All photos* show representative phenotypes from multiple events and ramets of 3-year-old field-grown plants. *White scale bar* corresponds to 30 cm

Regression analysis was used to determine the relationship between transgenes' expression and phenotypic traits. Linear, second-order polynomial, and sigmoidal dose–response (variable slopes) relationships were generated with PRISM (PRISM version 5; Graphpad Software) and best-fit relationships were selected based on comparison of R^2 values.

Results

Growth and form were strongly modified

We studied the growth, form and phenological characteristics of GA-insensitive and deficient poplars in a 3-year field trial. We observed dramatic trait modifications of growth, form, foliage color and shape in the field-grown transgenics (Fig. 1). Some characteristics (e.g., reduction of elongation growth) were similarly affected by the various transgenes. For example, *PtGA2ox*, *PcGA2ox, rgl*, *gai*, elicited severe dwarfism (Fig. 1c–f). However, other phenotypes such as leaf foliage color and texture varied among the different transgenic types. Perhaps the most typical GA-deficient phenotype was observed in the *PtGA2ox* transgenics—severely reduced stature, and small dark green leaves (Fig. 1c). The overexpression of both the poplar and bean GA2ox (*PtGA2ox* and *PcGA2ox*) had a stronger effect on leaf size than any of the DELLA protein expressing poplars, as indicated by high positive correlation between leaf size and total height of the transgenic plants (Fig. 2). In contrast, despite the severe dwarfing observed in *rgl*-overexpressing plants, leaf size was uncorrelated with height, and leaves had nearly normal size and shape. *PcGA2ox*-overexpressing plants often lost apical dominance and produced a ball-like bushy appearance (Fig. 1a) that was not observed in the *PtGA2ox* expressing plants (Fig. 1c). The *gai* and *rgl* expressing plants had very suppressed branch growth and produced a very compact crown form (e.g., Fig. 1b, e, f).

To characterize the observed variability we measured several biometric traits and physiological characteristics (Table 2). A majority of the measured traits showed significant effects (Table 3) (see complete ANOVA results in Supplemental, Table 1). *35S::PtGA2ox* and *35S::rgl* transgenes had the strongest dwarfing effect (Fig. 1c, e). Both transgenes imparted severe reductions in height, volume, and internodal distance (Table 3). In contrast, *pGAI::GAI* transgenics were indistinguishable from wild-type plants (WT-like) and majority of the measured traits were not significantly different from WT (Table 3). Despite being significantly reduced in height, *35S::gai* and *35S::GAI*, transgenics had diameters that were not significantly different from that of WT-controls (Table 3).

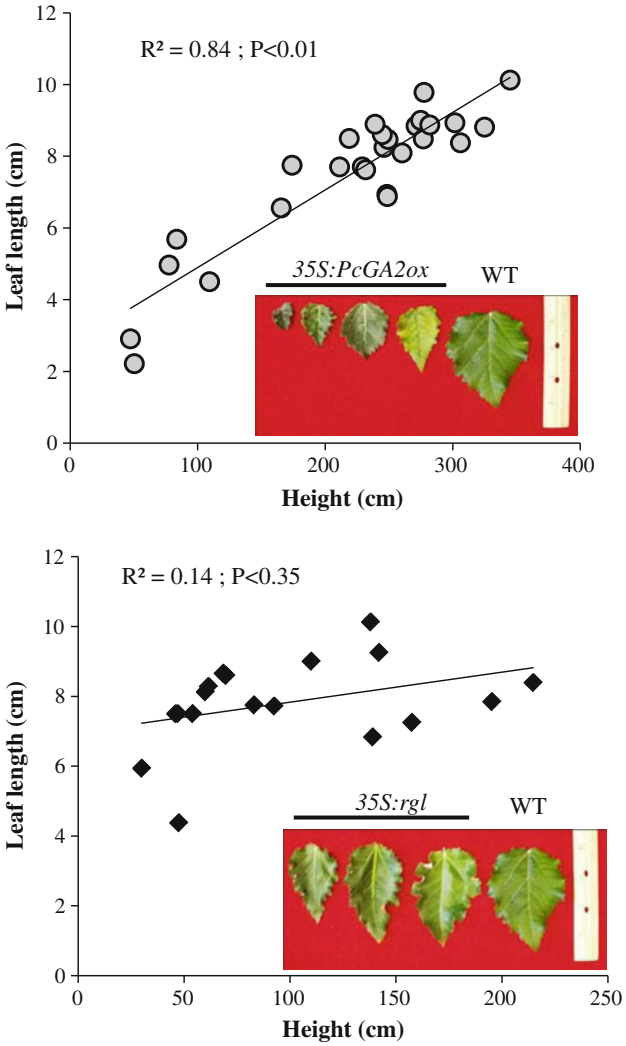


Fig. 2 Association of leaf length with stem height in *35S::PcGA2ox* (**a**) and *35S:rgl* (**b**) poplar transgenics. *Photo insets* show representative leaves from left to right for severely to mildly dwarfed events, respectively. Squared correlation coefficients of the linear relationships between leaf length and height, and the associated probabilities for statistical significance of a linear relationship, are shown at the left top corner of each graph. Note that *35S:rgl* transgenics were much more severely dwarfed as indicated by their heights

Furthermore, the Dia/Ht ratios were significantly increased in *35S::rgl* and *35S::PtGA2ox* transgenics, and above WT-controls in five of the seven constructs tested (Table 3).

We expected high colinearity in the effect of the transgenes on growth traits. We therefore used PCA to determine association between these traits. Indeed, principal component 1 (PC1) captured a large percent of variation of the data (mean of 78%; Table 4). The loadings suggest that PC1 captures nearly all size and growth characteristics. Thus, the severely dwarfed transgenics (*35S::rgl* and *35S::PtGA2ox*) had mainly negative PC1 loadings while

Table 3 Quantitative analysis of growth and form characteristics of transgenic plants

Genotype	*N*	Height	Diameter	Volume	Branch height	Internodal distance	Branch angle	Diameter/height
35S::PtGA2ox	30	**34** e	**58** d	**19** d	**33** d	**49** d	**122** a	**205** e
35S::PcGA2ox	52	89 b,c	82 b,c	82 a,b	89 b,c	**85** b	98 b,c	92 a,b
35S::rgl	26	**39** e	**53** d	**19** d	**45** d	**44** d	**85** d	**150** d
35S::gai	22	**79** c,d	85 a,b	74 b	87 b,c	**79** b	89 c,d	119 c
35S::GAI	52	84 b,c	96 a,b	78 a,b	92 a,b	**83** b	94 b,c,d	119 c
pGAI::gai	60	**68** c,d	**69** c,d	**48** c	**71** c	**62** c	91 c,d	116 c
pGAI::GAI	58	109 a	95 a,b	102 a	110 a	94 a	102 b	**87** a
WT	13	100 a,b	100 a,b	100 a,b	100 a,b	100 a	100 b,c	100 b,c

Mean values of measured traits relative to controls (% of control). ANOVA was performed on each trait ($P < 0.0001$) and differences in letters represent significant construct differences ($P < 0.05$) as determined by Tukeys's HSD test. Construct means that are significantly different from controls are shown in bold

Table 4 Loadings for principle component 1 (PC1) and 2 (PC2) from a PCA on eight measured traits

Variable[a]	PC1	PC2
Height	**0.97**	0.14
Diameter	**0.93**	0.14
Volume (cm^3)	**0.94**	0.19
Internodal distance	**0.85**	0.29
Branch height	**0.96**	0.12
Crown width	**0.93**	0.17
Leaf length	0.68	−0.72
Leaf width	0.74	−0.65
Cumulative %	78	92

All loadings ≥0.80 are shown in bold print

[a] Trait abbreviations are defined in Table 2

WT and the phenotypically indistinguishable from WT *pGAI::GAI* transgenics had predominantly positive PC1 loadings (Fig. 3). In contrast, *35S::gai* transgenics, which showed a wide phenotypic range (see below) in many of the measured traits, showed an almost continuous spread along the PC1 axis (Fig. 3).

Trait expression varied widely among and within constructs

In several of the transgenic types we found a gradient of the phenotypic changes among the multiple transgenic events studied (Fig. 4). The phenotypic changes ranged from severe to almost WT-like. However, not all transgenes elicited continuous phenotypic gradients (Fig. 4). The *35S::PcGA2ox* and *pGAI::GAI* transgenes produced very few events that were significantly different from WT. In contrast, *35S::PtGA2ox* and *35S::rgl* constructs had strong dwarfing effects in the majority of the events, with very few WT or intermediate phenotypes.

Trait variation was strongly associated with transgene expression

To analyze the relationship between trait variation and transgene expression, we performed quantitative real-time PCR (qRT-PCR) on a sub-sample of the whole population. We chose events that represented most of the phenotypic gradient in the corresponding transgenic types. The relationship between *gai* expression and trait variation was generally best fit by a dose–response curve with a sigmoid shape (Fig. 5a–d). The dose–response relationship was independent of promoter (*35S* vs. *pGAI*) and largely dependent on the truncation of the DELLA domain (gai vs. GAI). All traits that showed an association as depicted by PC1 loading scores ≥0.8 were best fit by a dose–response curve (Table 5). The relationship between *PcGAox2* expression and trait variation was best explained by a second-order polynomial (Fig. 5e, f).

Transgenes had significant effects on bud phenology

Dormancy characteristics of transgenic plants were characterized by scoring bud set in the fall of 2004 and bud break in spring of 2005. The effects were highly variable among constructs (Fig. 6). *gai* and *GAI* driven by the *pGAI* promoter caused early bud set and late bud flush but had no effect on both bud set or flush when expressed under the strong *35S* promoter (Fig. 6a, b). Early bud set and late bud flush, however, was observed in the *35S::rgl* transgenics. Overexpression of the native *PtGA2ox* had no effect on bud set but delayed bud flush, while non-native *PcGA2ox1* accelerated bud set and delayed bud flush.

Transgenes affected flowering phenology

We observed early flowering in transgenics that were expressing poplar *PtGA2ox, gai* and *GAI* genes as early as

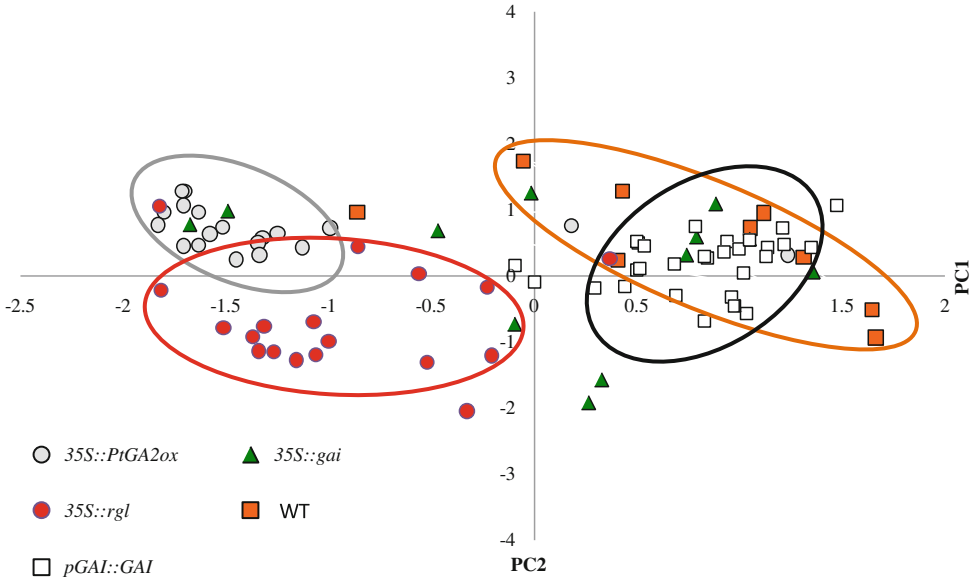


Fig. 3 Principal component analysis of eight morphological traits. *Circles* indicate the area where the majority of the events for their transgenic construct were located. The data points for *35S:gai* were not circled because of the substantial spread over principal component 1 (PC1) range. *Each point* indicates the event means of at least four ramets. For clarity, only four constructs were shown; construct means for all PC1 loadings are shown in Table 4

their second growing season (Fig. 7a). Expression of the *GAI* transgene under the *35S* promoter caused the highest incidence of flowering. All poplar species typically produce flowers early in the spring (March). The observed precocious flowering in the transgenic poplars occurred late in the summer (August). In contrast to the typically pendulous catkins (WT: Fig. 8c), the catkins in transgenic plants were upright (Fig. 8a, b) and resembled short shoots. In many cases the flowers were transformed into leaves (Fig. 8e, f). Finally, poplars are dioecious and clone 717 is female. In contrast, all transgenics produced in the 717 background produced flowers of both sexes on the same catkin (monoecious) (Fig. 8d). Because of the high incidence of flowering in the *GAI* expressing transgenics we compared the GAI expression in the flowering and non-flowering events. The early flowering events showed significantly higher expression level of the *GAI* transgene compared to the non-flowering events (Fig. 7b).

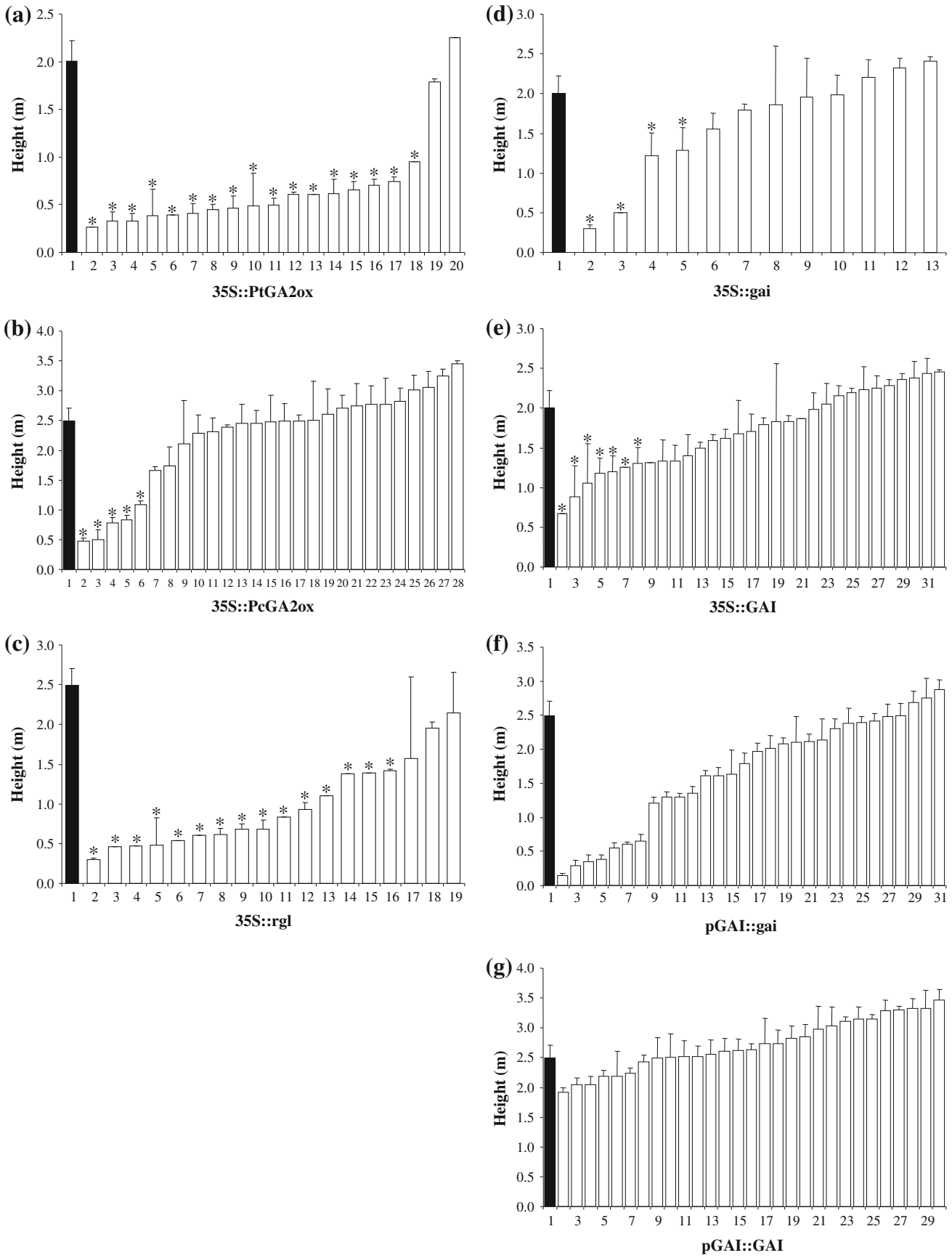
Transgenic phenotypes were stable

To assess the stability of the observed transgenic phenotypes we performed year-to-year correlations of traits that were highly affected by transgene expression. Traits like height, diameter and volume showed very high year-to-year correlations showing stable manifestation of the phenotype (Table 6).

Discussion

To assess the phenotypic consequences of modifying GA level and response, we expressed seven promoter-coding region combinations of GA catabolic (GA2ox) and response repressors (DELLA domain proteins). We recovered a large number of independent events to examine a range of phenotypic responses conditioned by variation in the level and pattern of expression from distinct transgene insertions. In addition, we studied trait expression in a field environment, allowing us to assess the extent to which transgenic modification affected adaptation and interaction with environment.

The transgenes gave a surprisingly large range of phenotypic effects. For example, *gai* and *rgl* affected height and branch elongation growth, but had a small effect on leaf size. Expression of *PcGA2ox* often decreased apical dominance and thus produced short trees with wide crowns. Many of the bean-derived *PcGA2ox* transgenics showed little height reduction, however, overexpression of the same gene derived from poplar (*PtGA2ox*), under the same 35S promoter, produced a very strong dwarfing effect and a proportional decrease of all aerial organs, including leaves and branches. The bean *PcGA2ox1* also caused more variation among events, with phenotypes spread more evenly across a gradient from severe dwarfing to WT-like (Fig. 4b). The differences in phenotypic consequences from the expression of the bean and poplar GA2oxes are somewhat puzzling. Both enzymes belong to the C_{19} GA2oxes and therefore different enzyme specificities cannot explain the different phenotypes. In a separate study we have characterized the GA spectrum in a subset of events which show similar trends in the changes caused by the overexpression of the two enzymes but the magnitude was much higher in *35S:PtGA2ox* plants (Rood and Pearce, in preparation). Therefore it appears that the native poplar enzyme is much more efficient in the stem and leaf tissue where we observed a much stronger effect. It is also possible that the bean enzyme may be more active in other


(a)
Height (m)
35S::PtGA2ox
(b)
Height (m)
35S::PcGA2ox
(c)
Height (m)
35S::rgl
(d)
Height (m)
35S::gai
(e)
Height (m)
35S::GAI
(f)
Height (m)
pGAI::gai
(g)
Height (m)
pGAI::GAI

◀ **Fig. 4** Gradient of height reduction within the different transgenic constructs. *Black bars* to the left in each graph represent controls (WT); *white bars* are means of independent transgenic events. Heights represent mean values and standard errors from up to four field-grown ramets. *Significant differences relative to WT as determined by ANOVA ($P = <0.0001$) using Fisher's protected least significant difference test

tissues like meristems and thus caused a loss of apical dominance and proliferation of sylleptic branches.

The phenotypic consequences of the heterologous expression of most transgenes in this study support conservation in large of their function among *Populus* and *Arabidopsis*. However some clear differences suggest

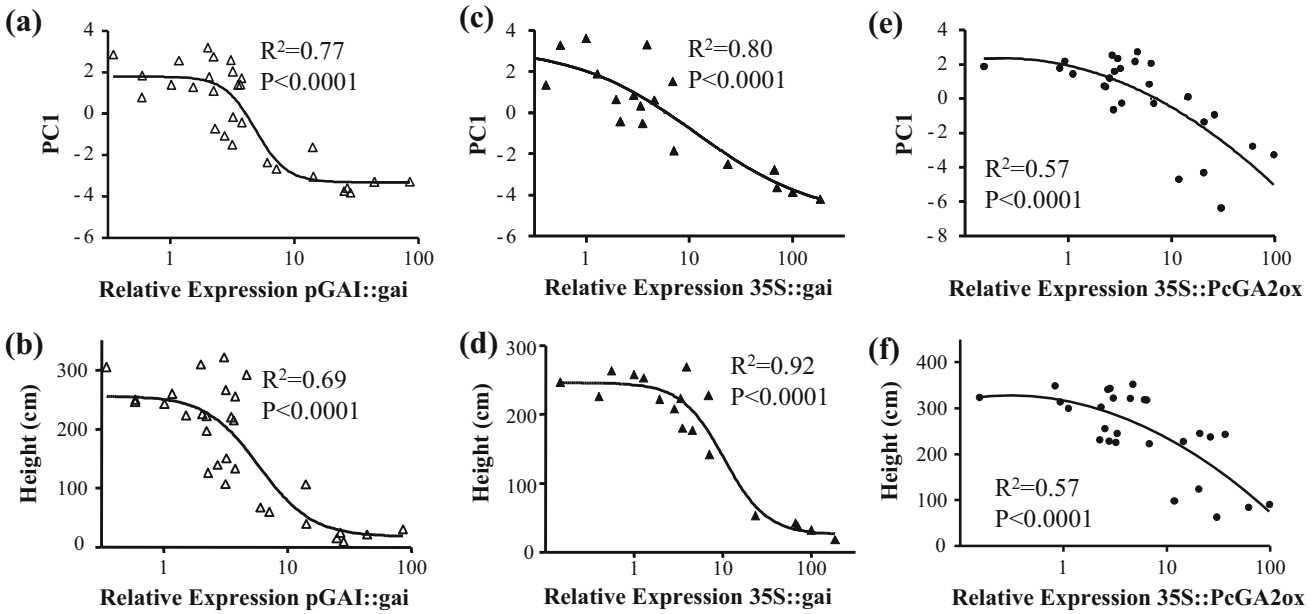


Fig. 5 Effect of transgene expression on trait variation. Dose–response, sigmoidal relationships between PC1, height and relative expression were used for *pGAI::gai* (**a**, **b**), and *35S::gai* (**c**, **d**) transgenic poplars. A second-order polynomial was used to describe the relationship between PC1, height and relative expression for *35S::PcGA2ox* (**e**, **f**)

Table 5 Coefficient of determination (R^2) from regression models for significant relationships between transgene expression and 11 traits/parameters

Trait/parameter	Transgene				
	35S::PcGA2ox	*pGAI::gai*	*35S::gai*	*pGAI::GAI*	*35S::GAI*
PC1	0.5703***,a	0.7651***,b	0.8039***,b	0.8022***,c	0.2145 ns[c]
Height	0.5665***,a	0.6927***,b	0.9226***,b	0.8355*[c]	0.636**,c
Diameter	0.6405***,a	0.7019***,b	0.731***,b	0.7275***,c	0.4748 ns[c]
Volume (cm^3)	0.5040***,a	0.537***,b	0.7565***,b	0.8135***,c	0.2486*[c]
Branch height	0.5580***,a	0.692***,b	0.8503***,b	0.8668***,c	0.4313**,c
Internodal distance	0.3600**,a	0.6066**,b	0.6394**,b	0.6762**,c	0.0522 ns[c]
Leaf length	0.1820 ns[a]	0.2077**,c	0.4588**,b	0.3317**,c	0.2541 ns[c]
Leaf width	0.1856 ns[a]	0.2917**,c	0.4727**,b	0.2570*[c]	0.0692 ns[c]
Leaf L/W	0.0710 ns[a]	0.1507*[c]	0.4396**,b	0.0004 ns[c]	0.072 ns[c]
Crown width	0.3551**,a	0.6683***,b	0.5618***,b	0.4253*[c]	0.2428*[c]
Dia/Ht	–	0.7511***,a	0.6689***,a	0.3168*[c]	0.5636**,c

ns non-significant

* $P \leq 0.05$, ** $P \leq 0.01$,*** $P \leq 0.0001$

[a] Second-order polynomial

[b] Dose–response

[c] Linear

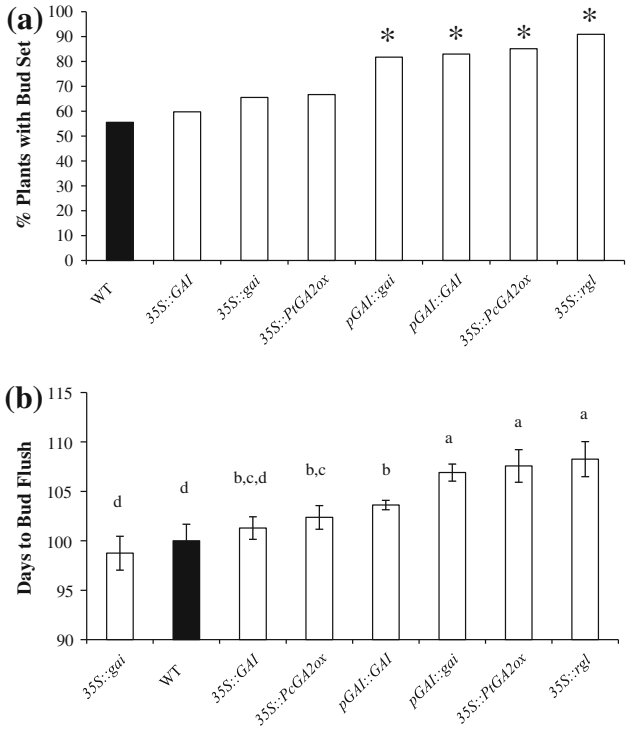


Fig. 6 Dormancy characteristics of the transgenic plants. **a** Percent of plants with bud set. *Statistically significant differences ($P < 0.05$) compared to WT as determined by Fisher's exact test. **b** Days to bud flush. *Different letters* denote significant ($P < 0.05$) differences as determined by ANOVA following Tukey's post-hoc test. Bud set was recorded on 1 October 2004. Days to bud flush were counted from 1 January 2005

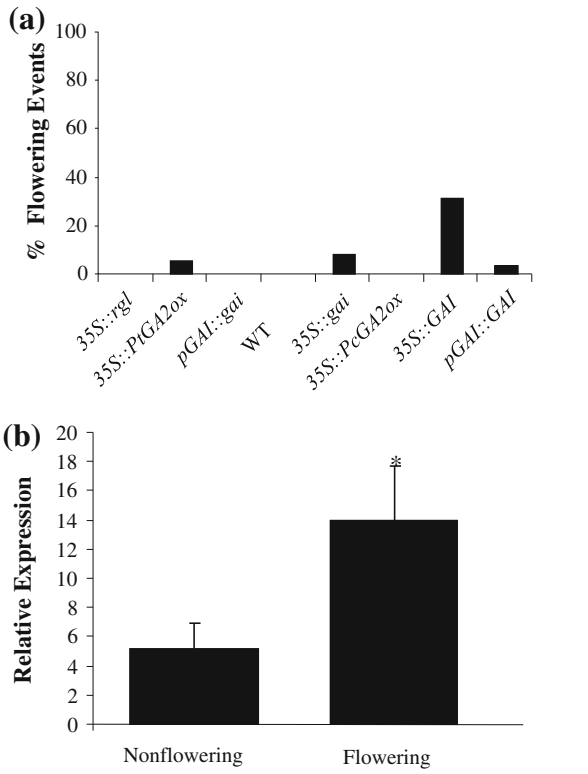


Fig. 7 Flowering behavior of the transgenic plants. **a** Proportion of flowering events in each transgenic construct. **b** Level of *GAI* expression in flowering and non-flowering lines (mean of *GAI* expression in both *35S::GAI* and *pGAI::GAI* transgenics). *Significant differences ($P < 0.05$) as determined by Student's *t* test. Flowering was recorded during the 2005 growing season

functional divergence. For example, DELLA proteins affected stem elongation but had only modest effect on leaf growth (Fig. 4b). Because both *gai* and *rgl* cause a significant reduction in leaf size in *Arabidopsis* (Peng et al. 1997; Wen and Chang 2002) it appears that the two proteins have diverged roles with respect to control of leaf size. Furthermore, *rgl* and *gai* transgenes produce severely dwarfed phenotype in *Arabidopsis* (Peng et al. 1997; Wen and Chang 2002). However, in poplar trees the two transgenes produced very different phenotypic alterations. When modifications with the same *35S* promoter are compared, *rgl* had a much stronger dwarfing effect than *gai*. Finally, the native form of GAI (e.g., without the DELLA truncation) was more effective in promoting flowering, whereas, in *Vitis*, a DELLA domain mutation (similar to *gai*) caused early flowering.

We found that expression of the transgene was a useful predictor of phenotype for most constructs, and thus could enable early selection. Traits that were controlled by the *gai* expression, irrespective of promoter, had a sigmoidal dose–response relationship between transgene expression and phenotype (Fig. 5). A threshold of expression was required for a phenotypic effect to be observed, and then its effects seemed to saturate when expression became very high. This type of dose–response plateau has been previously documented between levels of GA concentration and the phenotypic severity of affected traits (Chandler and Robertson 1999). In contrast to *gai,* the expression of *PcGA2ox* transgene had a largely continuous polynomial relationship with phenotype, and we did not detect saturation under the conditions of our experiment.

In addition to growth retardation, changes in bud dormancy and reproductive phenology were observed. Most of the transgenes affected vegetative bud phenology, while the native GAI accelerated the onset of flowering during an unusual mid-summer flowering event that also affected other transgenic modifications of flowering time genes in the same 717 poplar clone, growing on the same field site (Mohamed et al. 2010). The transgenics expressing the native GAI protein showed the highest incidence of flowering, and the events with the highest expression of GAI had the most advanced flowering. The role of GA in flower induction is well-established; however, it varies widely. In annual plants, GA promotes the transition to flowering, while in angiosperm woody perennials, GA inhibits flowering (Mutasa-Gottgens and Hedden 2009).

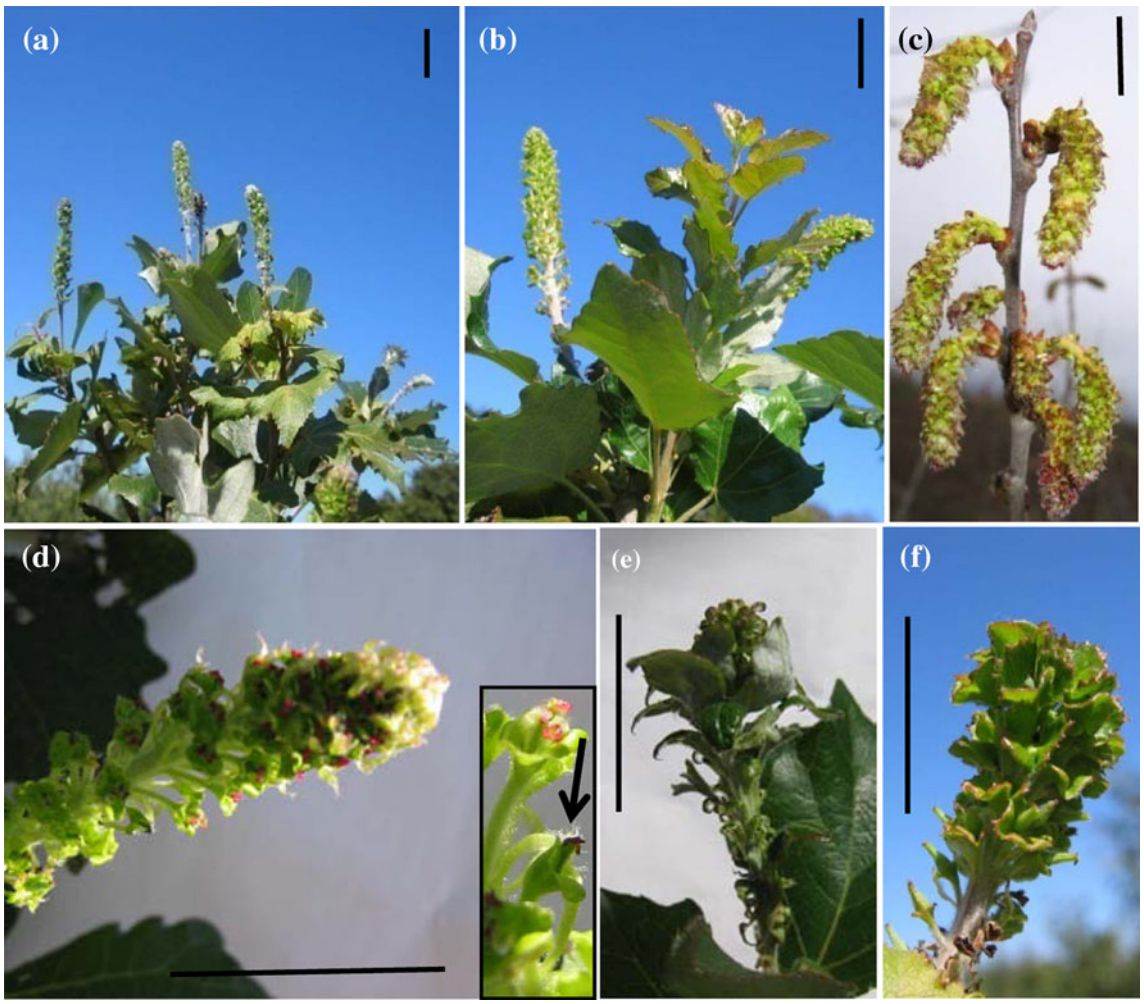


Fig. 8 Precocious flowering in *GAI* and *PtGA2ox* expressing transgenic plants. Upright flower catkin in event 62 of the *35S::GAI* (**a**) and event 95 of *35S::PtGA2ox* (**b**) transgenics. **c** Normal pendulous catkins. **d** Close-up of an erect flower catkin showing more leafy appearance of the perianth cup (see also *inset*). *Inset* shows male and female flowers on the same catkin and leafy appearance of the perianth cup. Proliferation of leafy structure in the catkins in *PtGA2ox* (**e**) and *GAI* (**f**) expressing transgenics. *Arrow* in **d** points to a female flower observed in the same catkin as male flowers. *Scale bar* approximately corresponds to 5 cm

Table 6 Year-to-year correlations for height, diameter and volume

Genotype	Height (cm)		Diameter (cm)		Volume (cm^3)	
	1 vs. 2	2 vs. 3	1 vs. 2	2 vs. 3	1 vs. 2	2 vs. 3
35S:PtGA2ox	NA	0.989	NA	0.914	NA	0.930
35S:PtGA2ox	0.815	0.967	0.903	0.970	0.868	0.961
35S::rgl	0.927	0.990	0.765	0.948	0.913	0.995
35S::gai	NA	0.974	NA	0.931	NA	0.882
35S::GAI	NA	0.975	NA	0.934	NA	0.916
pGAI::gai	0.873	0.979	0.788	0.968	0.807	0.954
pGAI::GAI	0.500	0.910	0.766	0.900	0.754	0.912
WT	0.787	0.876	0.892	0.966	0.817	0.980

All correlation coefficients have $P < 0.05$

NA data not available because planted in the fall of the same year

Sprays of GA inhibitors like paclobutrazol can be used to promote flowering of developmentally mature poplar propagules (Yuceer et al. 2003). Mutations in a GAI-like gene in grapevine that affected the DELLA domain and blocked GA responses caused dwarfing and precocious flowering (Boss and Thomas 2002). We found that

expression of GAI gene from *Arabidopsis* caused the highest occurrence of early flowering; however, in contrast to the *Vitis* study, the non-modified GAI (e.g., with intact DELLA domain) was most successful in causing the early flowering phenotype. One possible explanation of this phenomenon is that GAI expressing plants were generally as large as WT, and plant size has been found to affect the time of first flowering. However, that is most likely not the only factor as some of the *PtGA2ox* plants that produced extremely dwarfed phenotypes also flowered early. A more plausible explanation is the difference in ontogenetic development of flowers in the two species. In *Vitis* flowers develop from modified tendrils while in poplar flowers develop from axillary buds. Because these plants flowered at the end of the summer, not the usual time of flowering in poplar, we hypothesize that GAI presence allowed transformation of the vegetative into a floral meristem, but the susceptibility of the protein to degradation allowed bud outgrowth at the end of the summer. The vegetative to floral meristem transition was not complete as many catkins showed proliferation of leaf-like structures, suggesting that GA signaling alone is likely not sufficient for a complete transition from vegetative to reproductive growth.

It has been long known that GAs play significant role in vegetative bud phenology of forest trees (Olsen 2010). Specifically, modulation of GA levels at the onset of dormancy has been linked to the short day (SD)-induced cessation of shoot elongation which precedes bud formation and eventual onset of dormancy and endodormancy (Olsen et al. 1995). Transfer from LD to SD reduces the level of GA_1 in aspen and causes accumulation of $GA_{19/20}$, suggesting a reduction in GA 20-oxidase activity (Olsen et al. 1997). Consistently, poplar *PtGA20-oxidase1* transcript levels were reduced in young expanding leaves during the transition from LD to SD (Eriksson and Moritz 2002). In support of these previous observations, we show that GA deficient and/or insensitive poplar transgenics have extended dormancy periods due to early bud set and late bud flush. The early bud set phenology is a logical response as increased sensitivity to the SD-induced shoot growth cessation is increased through a muted GA signal or response. The mechanism by which GAs affect bud flush is still poorly understood although some recent evidence suggest role in activation of glucanases that open the apoplastic flow to the meristem (Rinne et al. 2011). However, other roles in reinitiating bud outgrowth are very likely given its growth promoting function.

Many of the phenotypic consequences, particularly those related to growth retardation, were predictable as they were previously observed in greenhouse environments (Busov et al. 2003, 2006). However, testing under field conditions allowed us to observe traits related to tree form and phenology which are not readily approachable under greenhouse conditions. In addition, trees are perennials, and our studies have showed that the transgenes' effects on the affected traits are indeed stable over multiple years.

Our results suggest that GA-inhibiting transgenes such as those studied could have a variety of uses in horticulture and other applications. We found that flowering was accelerated by GAI overexpression. Early flowering is a desirable trait in many fruit-producing horticultural species, particularly when coupled with growth retardation. It might also be used to speed breeding. Semi-dwarf trees are useful in a variety of ornamental applications, where small sizes are desired for backyards, street trees, and decks. The great diversity in leaf and crown morphology that was generated would provide a means to produce novel varieties with less breeding effort, and without the need to use exotic species and hybrids—with their attendant ecological risks. However, semi-dwarfism transgenes could also be used to mitigate the risk of spread of exotic species, especially when transgenics with multiple semi-dwarfism gene insertions are employed. The extent of stem height reduction was considerably less than the extent of diameter reduction, and semi-dwarf trees also appear to produce a higher proportion of root relative to shoot growth (Busov et al. 2006; Gou et al. 2010). This suggests that biomass production or carbon storage in the soil might be increased or at least not reduced, especially in high density plantings with mild levels of semi-dwarfism. The modification in vegetative phenology observed could reduce growth rates on favorable sites, however, it also may increase stress tolerance for plantings on cold or arid sites. The increased root allocation would also be expected to promote tolerance of drought and soil nutrient deficiency. For commercial applications of transgenic perennial plants, traits must be stable for many years. Our study agrees with several others showing very high levels of stability in trait, and thus transgene, expression (reviewed in Brunner et al. 2007) and/or associated phenotypes (Leple et al. 2007; Li et al. 2009; Mohamed et al. 2010).

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