Tissue-specific expression of *Populus* C_{19} GA 2-oxidases differentially regulate above- and below-ground biomass growth through control of bioactive GA concentrations

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Summary

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• Here, we studied the poplar C_{19} gibberellin 2-oxidase (GA2ox) gene subfamily. We show that a set of paralogous gene pairs differentially regulate shoot and root development.
• *PtGA2ox4* and its paralogous gene *PtGA2ox5* are primarily expressed in aerial organs, and overexpression of *PtGA2ox5* produced a strong dwarfing phenotype characteristic of GA deficiency. Suppression of *PtGA2ox4* and *PtGA2ox5* led to increased biomass growth, but had no effect on root development. By contrast, the *PtGA2ox2* and *PtGA2ox7* paralogous pair was predominantly expressed in roots, and when these two genes were RNAi-suppressed it led to a decrease of root biomass.
• The morphological changes in the transgenic plants were underpinned by tissue-specific increases in bioactive GAs that corresponded to the predominant native expression of the targeted paralogous gene pair. Although RNAi suppression of both paralogous pairs led to changes in wood development, they were much greater in the transgenics with suppressed *PtGA2ox4* and *PtGA2ox5*. The degree of gene suppression in independent events was strongly associated with phenotypes, demonstrating dose-dependent control of growth by GA2ox RNA concentrations.
• The expression and transgenic modifications reported here show that shoot- and leaf-expressed *PtGA2ox4* and *PtGA2ox5* specifically restrain aerial shoot growth, while root-expressed *PtGA2ox2* and *PtGA2ox7* promote root development.

Introduction

Gibberellins are a major class of growth regulators with > 126 members that play critical and diverse roles in plant growth and development (Olszewski *et al.*, 2002; Sun & Gubler, 2004; Schwechheimer & Willige, 2009). Because of their essential regulatory roles, the concentrations of bioactive GAs are precisely controlled by several mechanisms, including regulation of the genes encoding enzymes from both biosynthetic and catabolic pathways (Olszewski *et al.*, 2002). In higher plant, GAs are produced in three consecutive steps that occur in the plastid, endoplasmic reticulum (ER) and cytoplasm (Hedden & Phillips, 2000; Olszewski *et al.*, 2002; Yamaguchi, 2008). During the first stage in the plastids, geranylgeranyldiphosphate (GGPP) is converted to *ent*-kaurene by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). In the second stage, *ent*-kaurene in the ER is converted to GA_{12} by cytochrome P450-

dependent monooxygenases *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). GA_{12} can be further hydrolyzed by GA 13-oxidase to produce GA_{53}. The GAs from this stage are known as C_{20} GAs. GA_{12} and GA_{53} are substrates for the final steps of GA biosynthesis which take place in the cytosol, and result in bioactive GAs (GA_1, GA_4 and GA_3). This third stage of the biosynthetic pathway is carried out by 2-oxoglutarate-dependent dioxygenases (GA 20-oxidase and GA 3-oxidase).

The main route for GAs inactivation is through 2-oxidation catalyzed by 2-oxoglutarate-dependent GA 2-oxidases, although other inactivation reactions involving methylation (Varbanova *et al.*, 2007) and epoxidation (Zhu *et al.*, 2006) have been described. Originally described in runner bean (*Phaseolus coccineus*) (Thomas *et al.*, 1999), GA 2-oxidases have now been characterized in many plant species from different taxonomic groups and growth habits, including trees (Martin *et al.*, 1999; Sakamoto *et al.*, 2001b; Busov *et al.*, 2003; Lee & Zeevaart, 2005; Dayan *et al.*, 2010). The gibberellin 2-oxidase (GA2ox) gene families have been most thoroughly studied in *Arabidopsis* and rice (Thomas *et al.*, 1999; Sakamoto *et al.*, 2001a; Ogawa *et al.*, 2003; Schomburg *et al.*, 2003; Wang *et al.*, 2004; Lo *et al.*, 2008; Rieu *et al.*, 2008). In relation to their substrate specificity GA2oxes can be subdivided in two groups. The first-discovered and larger group catalyzes the 2-oxidation of C_{19} GAs. Subsequently, work in spinach (Lee & Zeevaart, 2005) and *Arabidopsis* (Schomburg *et al.*, 2003), and more recently in rice (Lo *et al.*, 2008), has shown that specific GA2oxs can inactivate C_{20} GAs (GA_{12} and GA_{53}). The C_{20} GA2oxs are significantly divergent from the C_{19} GA2oxs and display a characteristic C-terminal-conserved protein domain known as Domain III.

Gibberellin 2-oxidases have diverse functions in plant growth and development that are still poorly understood (Lo *et al.*, 2008; Rieu *et al.*, 2008). In *Arabidopsis*, comprehensive expression, physiological and genetic studies of the five C_{19} *GA2oxs* showed largely overlapping expression patterns, and knockout-related phenotypes could be only observed in quintuple mutations. The loss-of-function of all five C_{19} *GA2oxs* revealed negative effects on seed germination, delayed vegetative to floral transition, and defects in flower development associated with suppression of pistil elongation before fertilization. Small increases in root elongation were reported but lateral root development was not studied. Because in many organs, including leaves and inflorescences, GAs are near to saturation concentrations, the effect of the quintuple mutations could only be revealed under GA-limiting conditions generated by either treatment with paclobutrazol (PAC) or in a *GA20ox* mutant background. Under such conditions, the quintuple mutant showed effects on elongation of the main stem and side branches.

Similar studies in rice showed significant differences in the expression patterns of the GA2ox genes (Lo *et al.*, 2008). One larger group of GA2ox genes was expressed in vegetative tissues and another smaller group, comprising two genes, was expressed during the reproductive stage. Furthermore, gene-specific differences were detected during tillering. Gain-of-function activation tagging mutations in the C_{20} *GA2oxs* revealed that GA deficiency had a much stronger effect on shoot development than on root elongation. Furthermore, activation of *GA2oxs* promoted adventitious rooting, a process very similar to lateral root formation.

Outside *Arabidopsis* and rice, very little is known about the physiological and developmental roles of the GA2ox gene family. In garden pea, mutation in a *GA2ox* caused the *slender* phenotype associated with increased stem elongation (Martin *et al.*, 1999). We have previously identified the first C_{19} GA2ox in trees from the *stumpy* poplar mutant (Busov *et al.*, 2003). Activation of the gene caused severe dwarfism that could be rescued via exogenous GA_3 application. Here we report the isolation of the remainder of the poplar GA2ox gene family as well as the expression patterns and loss-of-function phenotypes for the C_{19} GA2ox genes. Our results suggest that several of these genes play major roles in regulating coordination of root/shoot growth.

Materials and Methods

Plant material

In all experiments we used hybrid clone INRA 717-IB4 (*Populus tremula* × *Populus alba*). All plants were grown in hormone-free ½MS (Sigma) media containing 0.7% Phytagar (Gibco–BRL, Carlsbad, CA, USA) and 1% sucrose, and maintained at 26°C under 16 : 8 h photoperiod. Plants were transplanted in the glasshouse and grown under standard fertilization, watering and pest control conditions. Tissues for expression analysis were sampled from 1-month-old glasshouse-grown plants and stored at −80°C until processed. For biomass measurement, tissues were sampled from 3-month-old glasshouse-grown plants. Dry biomass was determined after incubation in an oven at 70°C for 3 d.

Generation of RNAi binary vectors and transformation

Fragments of high sequence homology between the two targeted genes which were not present in the other genes were selected for down-regulation of both genes (Supporting Information, Figs S1, S2). The fragments were PCR-amplified using primers with attB1 and attB2 sites (Table S1) and inserted into binary RNAi vector *pHELLSGATE* using BP clonase (Wesley *et al.*, 2001). The integrity of each RNAi construct was sequence-verified using P27-5 (5′-GG GATGACGCACAATCC) and P27-3 (5′-GAGCTACA CATGCTCAGG) primers, respectively. All RNAi 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 (Filichkin *et al.*, 2006) into the same genetic background – a hybrid aspen clone INRA 717-IB4 (*P. tremula* × *P. alba*). All putative transgenic plants that rooted on selection media were PCR-verified for the presence of the transgene using attB1 (5′-G GGGACAAGTTTGTACAAAAAAGCAGGCT) and att-B2 primer (5′-GGGGACCACTTTGTACAAGAAAGC TGGGT). Validation of RNAi suppression was performed using gene-specific primers (Table S2).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Reverse transcription was performed using 3 μg DNaseI-treated total RNA, SuperScript III reverse transcription kit (Invitrogen) and oligo-dT primers. Gene-specific primers were used for PCR amplification of each gene (Table S2). Poplar elongation factor 1α (gw1.X.2539.1) and ubiquitin4 (eugene3.00011726) expression levels were used to correct for small variations in loadings. The number of cycles for each gene was varied to confirm that the PCR amplification was in the linear range (Fig. S3). Relative expression was estimated by measuring band intensity using GelDoc-It Imaging System and analyzed via LaunchVisionWorksLS software (Ultra-Violet Products Ltd., Cambridge, UK). 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, Reading, PA, USA).

Biometric measurements

To measure lateral root development, 5 cm cuttings were grown *in vitro* in hormone-free ½ MS media. Digital images of roots were obtained in the presence of a scale bar and quantified using ImageJ 1.63 software (http://rsb.info.nih.gov/ij/download.html). We counted the number of emerged roots and measured lateral root length in a 2 cm section starting at the root tip. Each experiment was repeated three times with at least five plants per replication. Biomass was measured after the end of the trial. Leaves and stems were removed and separated. Roots were carefully cleaned to remove soil. For each fraction (tissues/organs), FW and DW were determined (see earlier 'Plant material' section).

Microscopy

Stems from wild-type (WT) and transgenic plants were sampled at the same respective positions (internodes) at an equal distance from the adjacent leaves and immediately fixed in FAA solution (containing 5% (v/v) formalin, 5% (v/v) acetic acid and 50% (v/v) ethanol). Embedding and staining were performed using Shandon Excelior and Histocentre 2 (Thermo Scientific Co., Waltham, MA, USA). Five-micrometer-thick sections were stained with haematoxylin and eosin. Numbers of cells in the cambium zone as well as phloem and xylem widths were measured at five different randomly selected positions in each stem section.

For xylem fiber and vessel measurements, bark and phloem were first peeled from the stem sections. The remaining woody stem sections were then macerated in the mixture of equal volumes of glacial acid and hydrogen peroxide for 24 h at 60°C followed by thorough rinse with cold water. Cells were separated by vortexing and stained using Safranin O. Images of stained cells were captured using a Nikon microscope equipped with Sony 3CCD DKC-5000 camera and measured using ImageJ 1.63 software (http://rsbweb.nih.gov). A total of 10 images/line and three lines/transgenic modification were used in the analyses.

Phytohormone analysis

Three grams of roots and expanding leaves were harvested from 3-month-old glasshouse-grown transgenic plants, each line represented by three independent plants. The samples were immediately weighed (fresh), frozen, powdered in liquid nitrogen and later lyophilized. The internal standards used for hormone quantifications were as follows: $[^2H_2]$ GAs (provided by Professor L. Mander at Australian National University, Canberra, Australia), $[^{13}C_6]$ IAA (C/D/N Isotopes Inc., Quebec, Canada). Each replicate sample was extracted in 80% MeOH with internal standards overnight and reduced to aqueous phase by vacuum. The aqueous phase was adjust to pH 3.0 and partitioned with EtOAc (Merck, Darmstadt, Germany), and then the organic phase was repartitioned with K-Pi buffer (pH 8.5) (Merck), and the aqueous phase was adjusted to pH 3.0 and partitioned again with EtOAc. Further purification was performed on C_{18} Sep-Pak and MCX SPE columns (Waters Ltd, Milford, MA, USA). The eluate was dried and redissolved with high-performance liquid chromatography (HPLC) initial solution and filtered through a 0.22 μm filter and analyzed with an LC-MS system (LCQ Deca MAX, HPLC-ESI-MS, Thermo-Finnigan, Ringoes, NJ, USA). MS–MS data were then analyzed using the software Xcalibur 2.1 (Thermo-Finnigan) and quantified by reference to ratios of specific ions of phytohormones in natural samples over those of internal standards using equations for isotope dilution analysis.

Results

Populus GA2ox gene family

Using homology searches in the poplar genome sequence, we identified 11 putative *Populus* GA2oxs (Table S3,

Fig. S4a). Phylogenetic analysis with all Arabidopsis proteins indicates a clear separation of the C_{19} and C_{20} GA2oxs. The group of poplar C_{19} GA2oxs comprised seven members and the C_{20} GA2oxs comprised four members. The seven C_{19} GA2ox proteins group in four distinct lineages comprised three pairs of closely related family members (PtGA2ox1 and 6; PtGA2ox2 and 7, and PtGA2ox4 and 5), likely representing paralogous genes and showing very clear relationships to their three *Arabidopsis* orthologous proteins (AtGA2ox1, AtGA2ox6 and AtGA2ox4). PtGA2ox3 formed a separate lineage with no apparent paralog and showing homology to two *Arabidopsis* proteins (AtGA2ox2 and 3). Interestingly, the RNAi suppression of *PtGA2ox3* precluded regeneration of transgenic plants, suggesting an essential role in the regeneration process (discussed later).

The C_{20} GA2oxs were clearly separated from the C_{19} group, and alignment of their protein sequence clearly showed the conservation of the C-terminal Domain III, which is a characteristic feature of C_{20} GA2oxs in *Arabidopsis*, spinach and rice (Lee & Zeevaart, 2005; Lo *et al.*, 2008; Rieu *et al.*, 2008) (Fig. S4b, Table S4). The four proteins were grouped in two lineages each with one *Arabidopsis* orthologous protein.

Lineage-specific divergent expression patterns of poplar C_{19} GA2-oxidases

We first studied the expression of all seven C_{19} GA2ox genes in various tissues and developmental stages. *PtGA2ox1* and *PtGA2ox6* showed similarly low transcript abundance in all tissues tested (Fig. 1a). By contrast, all other genes showed highly divergent and usually stronger expression. *PtGA2ox2* and *PtGA2ox7* were predominantly expressed in root tissue (Fig. 1b) while *PtGA2ox4* and *PtGA2ox5* were expressed in leaves (Fig. 1c). The *PtGA2ox3* transcript was most abundant in shoot apex and younger leaves (Fig. 1c). The paralogous pairs inferred by sequence homology analysis showed nearly identical expression levels and patterns, which further supports their paralogous relationships.

GA2ox overexpression elicits severe dwarfism

We previously recapitulated the *stumpy* mutant phenotype by overexpressing bean GA2ox (*PcGA2ox1*) (Busov *et al.*, 2003). However, only 10% of the transgenic insertion events displayed obvious dwarf phenotypes. The tagged gene in the *stumpy* mutant corresponds to *PtGA2ox5*. We therefore generated 35S promoter-driven overexpression constructs with the native *PtaGA2ox5* gene (Fig. 2). Among the multiple events that were recovered and expressed the transgene, > 70% (10 of 14 events) were severely dwarfed (Fig. 2c,d). Their average heights were the same or lower
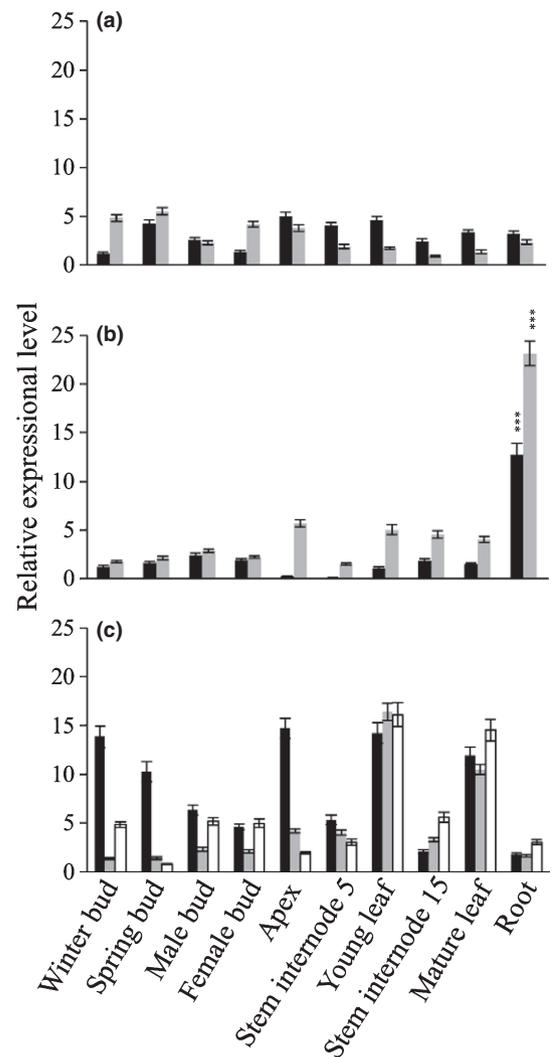

Fig. 1 Expression of seven *Populus* C_{19} gibberellin 2-oxidase (GA2ox genes). (a) Low and ubiquitous expression of *GA2ox1* (black bars) and *GA2ox6* (gray bars). (b) *GA2ox2* (black bars) and *GA2ox7* (gray bars) are highly and predominantly expressed in roots (highly significant differences against any of the aerial organs). Significance as determined by Student's *t*-test: ***, $P < 0.001$. (c) *GA2ox3* (black bars), *GA2ox4* (gray bars) and *GA2ox5* (white bars) are expressed in above-ground organs. The different tissues were harvested from the same 3-month-old plants. Total RNAs were isolated and analyzed by reverse transcription-polymerase chain reaction using gene-specific primers (see Supporting Information, Table S2). Bars represent means ± SE of three independent biological replications.

than that of the original mutant (Fig. 2f). Many events were near phenocopies of the original mutant, displaying greatly reduced stature and dark green foliage (Fig. 2e). The expression of the phenotype was observed over 4 yr under both glasshouse and field conditions. Therefore the effect of the overexpression of the native poplar GA2ox gene was much stronger than had been observed in the *PcGA2ox1* overexpressing poplar transgenics.

Fig. 2 Phenotypes of *Populus* plants transformed with *35S:PtaGA2ox5*. (a, b) Wild-type (WT) control plants. (c–e) Plants transformed with *35S:PtaGA2ox5*. Pictures and measurements were from plants grown for 2 yr in the field. Phenotypes depict typical morphological characteristics observed in more than five lines and four ramets (clonally propagated plants) per line. (f) Height of *stumpy*, *35S:PtaGA2ox5* and WT plants after 2 yr of field growth. Bars represent means ± SE of more than four ramets per event and 12 recapitulation events from *35S:PtaGA2ox5*. Significance as determined by Student's *t*-test: ***, *P* < 0.001.

RNAi modification of poplar C$_{19}$ *GA2-oxidases*

Because of the strong indications of paralogous relationships and hence functional redundancy which may obscure phenotypic differences, we simultaneously suppressed the two paralogous genes inferred from sequence and expression analyses. We selected sequences that were conserved for the paralogous pair but different from all other members and used these to generate RNAi constructs (Figs S1, S2). In total we generated four constructs (RNAi 1–6; RNAi 4–5; RNAi 2–7 and RNAi 3) with the numbers indicating the targeted paralogous genes. We recovered > 20 independent transgenic events from each construct with the exception of RNAi 3. Although we do not know the exact reason, it is possible that the suppression of *PtGA2ox3* may interfere with the regeneration process. We therefore proceeded with the characterization of only the remaining three transgenic types. We first randomly selected 10 independent events and screened for the degree of suppression of the targeted genes (Fig. 3). We then selected events with significant and > two-
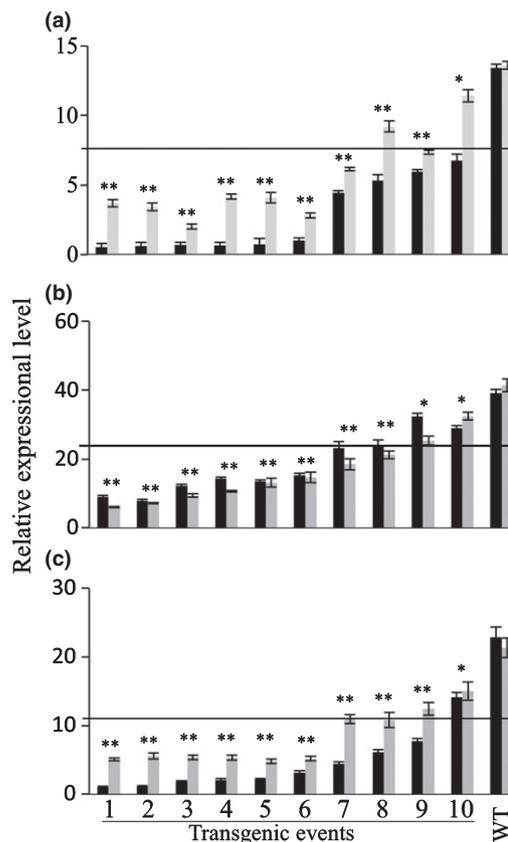

Fig. 3 RNAi suppression of *GA2ox1*, (black bar) and *GA2ox6* (gray bar) (a); *GA2ox2* (black bar) and *GA2ox7* (gray bar); (b) and *GA2ox4* (black bar) and *GA2ox5* (gray bar) (c) in 10 independent *Populus* transgenic events. Bars represent means ± SE of three independent biological replications. Significance as determined by Student's *t*-test: **, *P* < 0.01; *, *P* < 0.05. The line in each graph corresponds to half of the expression level of the corresponding genes in wild-type (WT) plants.

fold suppression of both genes compared with nontransgenic, WT plants. The selected events were subjected to further observations and measurements as described below.

Suppression of *PtGA2ox1* and *6* does not produce detectable phenotypes

In vitro and glasshouse observations of seven selected transgenic events with a strong (2.5- to 20-fold) reduction of both genes did not show any observable or statistically significant differences between the measured parameters (Fig. 4). Based on these results we concluded that suppression of the two genes does not produce detectable phenotypes under our experimental conditions, or the phenotypic consequences are so specific and/or subtle that they were not detected. The low expression of these two genes supports the latter interpretation.

RNAi down-regulation of *PtGA2ox4* and *5* promotes aerial shoot growth

Phenotypic differences were evident as early as during their *in vitro* growth and development (Fig. 5a). Events with severe suppression of the two targeted genes showed increased shoot growth. We next transferred the plants to the glasshouse and studied their development for 3 months in two independent trials. Consistent with the *in vitro* observations, the transgenic plants showed increases in many aspects of their above-ground organ growth, and

particularly in leaf size and biomass (Fig. 5b–e). Stem height, diameter and biomass were also significantly increased (Fig. 6). The numbers of internodes and root biomass, however, were unaffected (Fig. 6c,f).

Silencing of *PtGA2ox2* and *7* affected root biomass in the glasshouse

We have previously described the *in vitro* root phenotypes of these RNAi transgenic plants which showed decreased lateral root proliferation (Gou *et al.*, 2010). Here we studied the development of these plants under glasshouse conditions (Fig. 7b). Consistent with the *in vitro* measurements and observation (Fig. 7a), root biomass was significantly reduced in the suppressed plants (Fig. 7g,h). In addition, we found an increase in height and diameter growth in a limited number of transgenic events in which expression of the two genes was most severely reduced (Fig. 7e,f).

Changes in wood development and properties

Gibberellins play an important role in the regulation of wood formation (Bjorklund *et al.*, 2007; Mauriat & Moritz, 2009). We therefore studied wood development in the transgenic plants (Fig. 8a). Cross-sections through stems undergoing secondary woody growth showed significant increases in cell number in the cambium zone in both *PtGA2ox4/5-* and *PtGA2ox2/7-*suppressed transgenics (Fig. 8a,b). The changes were detectable in stems in the transition zone between
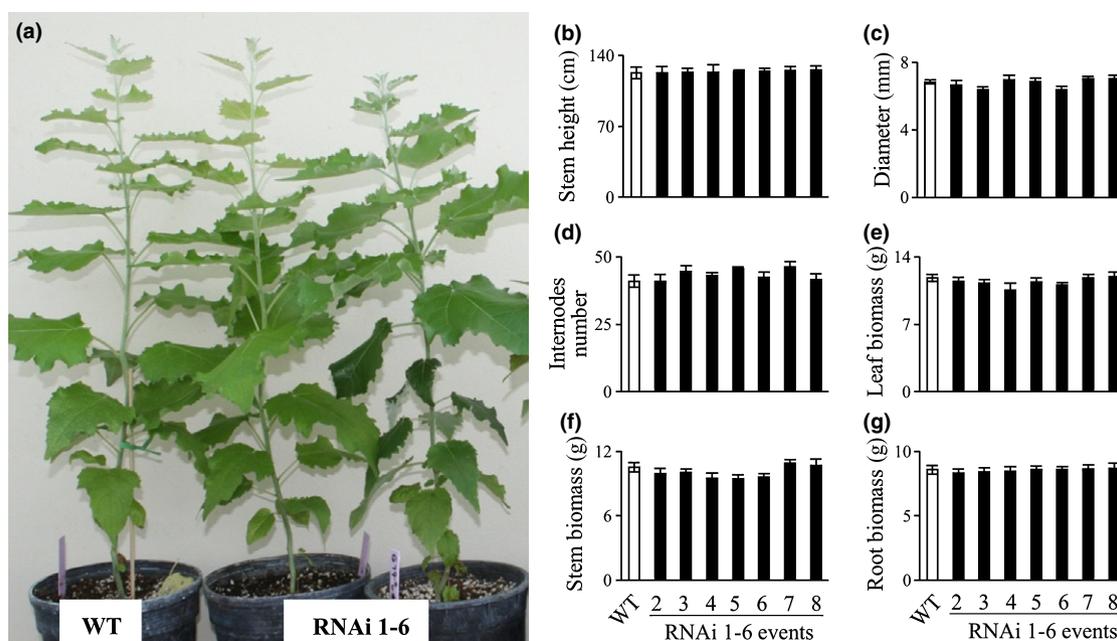

Fig. 4 Suppression of *GA2ox1* and *6* did not produce detectable phenotypes in glasshouse grown *Populus* plants. (a) Wild-type (WT) control and RNAi 1–6 transgenic plants (RNAi 1–6); (b) stem height (cm); (c) stem diameter (mm); (d) number of internodes; (e) dry leaf biomass; (f) dry stem biomass; (g) dry root biomass. Bars show means ± SE over three independent experiments, and each experiment was performed with at least six ramets per event. Black bars, transgenic; white bars, WT control plants. Event numbers correspond to these in Fig. 3.
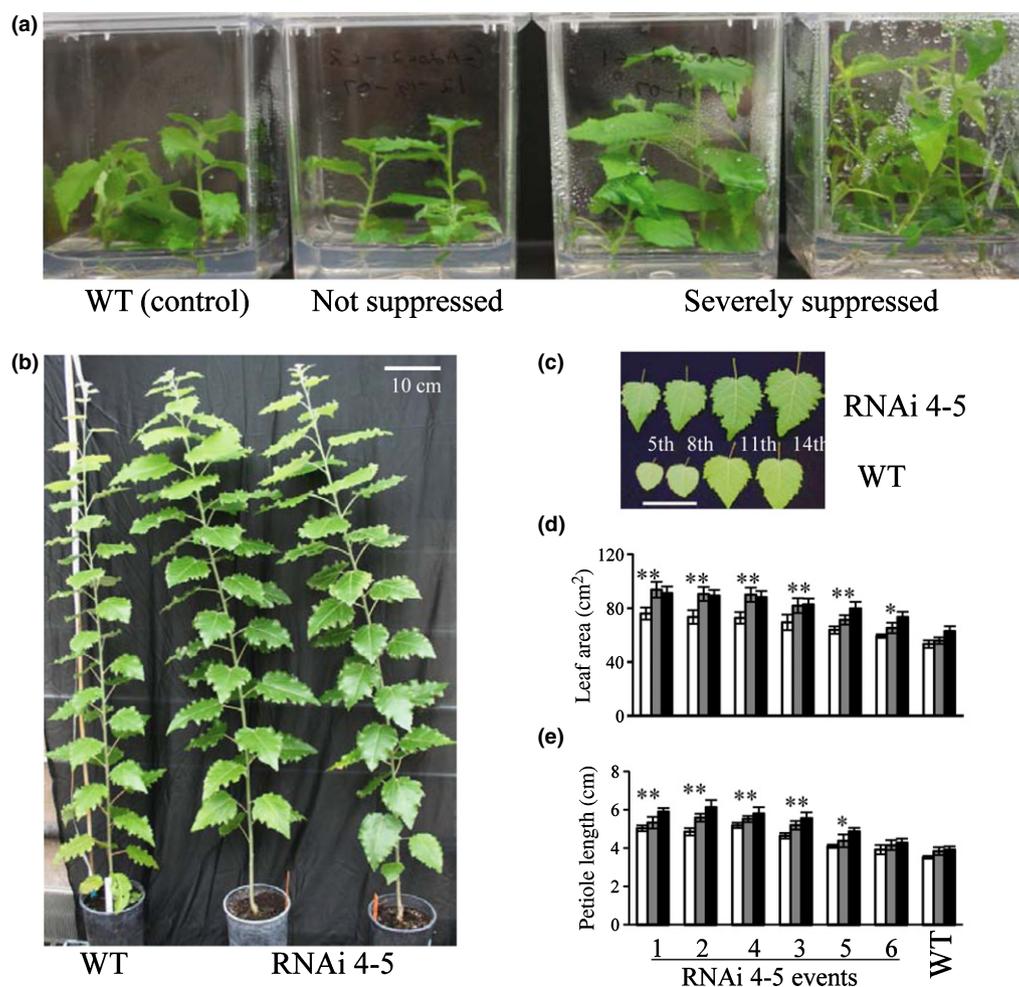
Fig. 5 Suppression of *GA2ox4* and *5* promotes leaf growth. (a) Four-week-old *in vitro*-grown *Populus* plants. RNAi 4–5, *PtGA2ox4/5* RNAi transgenics. (b) Two-month-old glasshouse-grown plants. (c) Leaves from different leaf plastochron index (LPI)s (fifth, eighth, 11th and 14th) of transgenics (upper) and wild-type (WT) (lower). All photos were taken at the same time and are representative of multiple ramets (+15) of the same transgenic events. (d) Leaf area of different LPIs (white bar, eighth; gray bar, 14th; and closed bar, 20th). (e) Petiole length of different LPIs. Significance as determined by Student's *t*-test: **, $P < 0.01$; *, $P < 0.05$. Event numbers correspond to these in Fig. 3.

primary and secondary growth (10th internode) but were much more pronounced in stem sections from wood-producing stem tissues (15th internode) (Fig. 8b). Consequently, phloem and, most prominently, xylem width were increased (Fig. 8c,d). In addition, significant increases in fiber length, but no changes in fiber width or vessel length or width, were found (Fig. 9). All changes were much greater in the transgenics with suppressed *PtGA2ox4* and *PtGA2ox5* genes that are predominantly expressed in stem tissues (Figs 8, 9).

Phenotypes were correlated with target gene suppression

To assess the degree of correspondence between suppression of the target genes and growth phenotypes, we performed regression analysis. The expression of two targeted genes (*GA2ox2* and *GA2ox7*) in the RNAi 2–7 transgenics was highly correlated to lateral root length and density, especially

to the lateral root density (Fig. 10a), but was largely uncorrelated with the other morphological traits (Fig. S5). Moreover, in RNAi 4–5 transgenics, expression of the two targeted genes (*GA2ox4* and *GA2ox5*) was highly correlated with height and leaf size (area) (Fig. 10b), as well as other stem and leaf traits (Fig. S5). The expression of the five untargeted genes was largely uncorrelated ($R^2 > 0.6$; $P < 0.05$) with any of the studied morphological traits in both RNAi 2–7 and RNAi 4–5 transgenics (Figs S6, S7, respectively). Mild cross-silencing of GA2ox2/7 in RNAi 4–5 transgenics may slightly contribute to the leaves and petiole phenotypes, but the correlations were not significant at $P < 0.05$ (Fig. S7).

Changes of GAs and IAA concentrations in transgenic plants

To test if the phenotypic and expression changes observed in transgenic plants correspond to changes in GA concentrations,
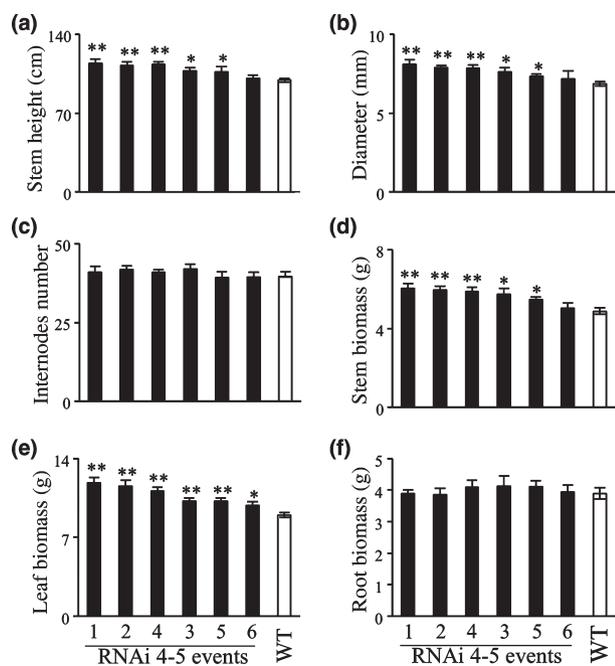
Fig. 6 Suppression of *GA2ox4* and *5* increased above-ground stem and leaf biomass in *Populus*. (a) Stem height. RNAi 4–5, *PtGA2ox4/5* RNAi transgenics; (b) stem diameter; (c) number of internodes; (d) dry stem biomass; (e) dry leaf biomass; (f) dry root biomass. Bars show means ± SE over three independent experiments, and each experiment was performed with at least six ramets per event. Black bars, transgenic; white bars, wild-type (WT) control plants. Significance as determined by Student's *t*-test: **, $P < 0.01$; *, $P < 0.05$. Event numbers correspond to these in Fig. 3.

we measured the concentrations of the bioactive GA_1 and GA_4. Consistent with the expression pattern of the two genes, concentrations of bioactive GA_1 and GA_4 were increased in *PtGA2ox4*- and *5*-suppressed transgenics leaves but not roots (Fig. 11a,b). By contrast, *PtGA2ox2*- and *7*-suppressed RNAi transgenics showed significantly increased bioactive GA_1 and GA_4 in roots but not in leaves (note that GA_4 was also increased in leaves but not to the extent observed in RNAi 4–5 transgenics, Fig. 11b). Therefore, the changes in GAs closely mirrored the observed phenotypic changes in the respective organs of predominant expression.

Because of the well-known crosstalk between auxin and GA signaling at both the molecular and phenotypic levels (Ross *et al.*, 2001; Bjorklund *et al.*, 2007; Weiss & Ori, 2007), and because some of the observed phenotypes may also result from altered auxin concentrations, we tested if IAA concentrations were changed in the transgenic plants. We did not measure any significant changes (Fig. 11c).

Discussion

Poplar GA2ox gene family

Gibberellin concentrations and responses are tightly controlled in plants (Yamaguchi, 2008) and the GA metabolic

pathway has been well characterized (Hedden & Phillips, 2000). Although GA synthesis and inactivation involve many enzymes and are localized to three intracellular compartments, the flux of GAs is determined by the action of three enzymes that are localized in the cytoplasmic stage of GA metabolism. GA2ox catalyzes the main inactivation step through 2-oxidation of both the bioactive forms and their precursors (Sakamoto *et al.*, 2001b; Schomburg *et al.*, 2003).

In many plant species, GA2ox genes are encoded by small gene families of various sizes. In *Arabidopsis* the GA2ox family comprises seven members, of which five show C_{19} and two C_{20} GA2ox activities (Rieu *et al.*, 2008). *Arabidopsis*' five C_{19} GA2ox genes are nearly ubiquitously expressed in different organs and developmental stages and very high-order mutations (quintuple) are needed before phenotypes can be detected (Rieu *et al.*, 2008). In contrast to *Arabidopsis*, the gene family in rice is larger (10 members) and the different members show more diversified expression patterns (Lo *et al.*, 2008). A systematic study of the effects of loss-of-function mutations for these genes has not been reported to our knowledge; however, expression data suggest that the expansion of the gene family has been accompanied by functional diversification of family members. The poplar gene family consists of 11 members, with seven showing homology to C_{19} and four to C_{20} GA2oxs. The expansion of poplar gene family has likely occurred during the genome-wide salicoid duplication. Sequence analyses indicate that eight of the 11 poplar GA2oxs belong to four pairs of highly similar proteins. These include PtGA2ox1 and 6, PtGA2ox2 and 7, PtGA2ox4 and 5, and PtGA2ox9 and 10. Moreover, each pair shows clear homology, as supported by high bootstrap confidence in its close relationship to an *Arabidopsis* homolog. This strongly suggests that the expansion of the family has occurred through genome-wide duplication and retention of duplicated copies. In addition to high sequence conservation, the duplicated copies show highly similar expression patterns. The similar sequence and expression patterns strongly point to functional redundancy. However, the level and pattern of expression among the different pairs can vary substantially. Perhaps more strikingly, *PtGA2ox4* and *PtGA2ox5* are predominantly expressed in shoots but not in roots, while *PtGA2ox2* and *PtGA2ox7* transcripts are most abundant in roots and barely detected in shoots. This indicates that these gene pairs are highly specialized in function – a result that was further supported by our transgenic analyses.

GA2ox loss-of-function phenotypes

There is considerable information on GA2ox biochemistry and *GA2ox* overexpression phenotypes. Gain-of-function mutations are typically dwarfs with classical signs of GA deficiency (i.e. reduced stature and dark green foliage). By

comparison, little is known about *GA2ox* loss-of-function phenotypes. In pea, mutation in a single *GA2ox* gene confers the *slender* phenotype – pale green leaves and thin elongated stems (Martin *et al.*, 1999). In tobacco, RNAi suppression of a single gene increases growth but without signs of the *slender* phenotype; leaves were normal-colored and the stems were thicker than WT plants (Dayan *et al.*, 2010). However, single gene mutations in *GA2ox* genes in *Arabidopsis* are aphenotypic; only when all five C_{19} GA2oxs (quintuple) are knocked-out were phenotypes observed (Rieu *et al.*, 2008). In poplar, suppression of paralogous gene pairs showed distinct phenotypes (discussed later), but not to the extent described in tobacco and pea.

The causes of the large interspecific differences in knock-out phenotypes are still hard to understand. The strong effects of single gene knockouts in pea and tobacco might result from very small gene families, thus with dominating functions of one of their genes. Alternatively, these differences may be indicative of the strength of the feedback mechanism in each species that adjusts the metabolic homeostasis in hormone concentrations. However, a lack of information about the *GA2-oxidase* family in these species, as well as about the behavior of the genes encoding the biosynthetic enzymes, precludes more definitive inferences. Nevertheless, these studies suggest substantial differences in the evolved roles of *GA2-oxidases* among different dicotyledonous plant species.

Specialized and divergent functions of poplar *GA2oxs* in regulation of shoot/root growth

Our expression analysis and transgenic manipulations showed that there is functional specialization of GA2ox with respect to control of shoot and root growth. *PtGA2ox4* and *5* were expressed predominantly in shoots and the simultaneous down-regulation of the two genes led to increased GA concentrations and more leaf and stem biomass. However, *PtGA2ox4* and *5* suppression had no effect on either root GA concentrations or biomass. The phenotypic effect from *PtGA2ox4* and *5* down-regulation observed in poplar were similar to that found in tobacco. Plants had increased leaf biomass, stem diameter and height growth. We did not observe characteristics of the *slender* phenotype (i.e. thinner stems and pale leaves) described in the pea GA2ox mutant. By contrast, *PtGA2ox2* and *7* were expressed predominantly in roots and their suppression increased GA concentrations in roots and caused a decrease in root biomass. The suppression of *PtGA2ox2* and *7* had a small effect on leaf bioactive GA concentrations and no effect on biomass.

These results are consistent with our previous findings of a negative effect of GA on lateral root formation (Gou *et al.*, 2010). Under *in vitro* conditions overexpression of GA2ox led to increased lateral root proliferation and transcriptome changes consistent with increased growth and
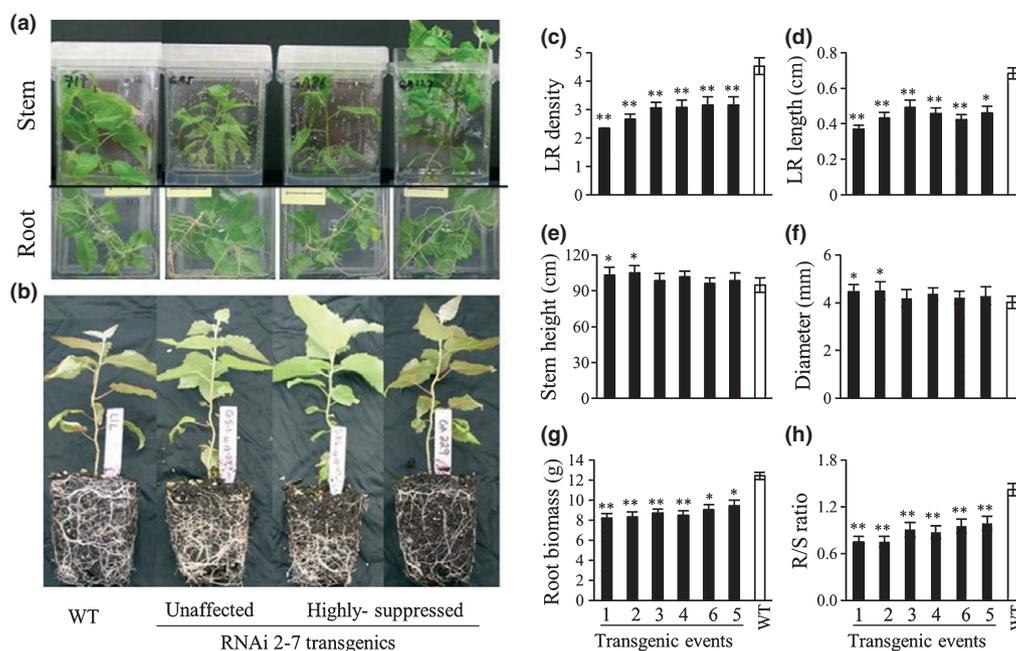

Fig. 7 Suppression of *GA2ox 2* and *7* decreased root production. (a) Four-week-old *in vitro*-grown *Populus* plants. Upper and lower panels represent photos of the same plants taken from the side and bottom of the magenta box. RNAi 2–7, *PtGA2ox2/7* RNAi transgenics. (b) Two-month-old glasshouse-grown plants. The root plug was carefully removed from the pot before taking the picture. All photos were taken at the same time and are representative of multiple ramets (+15) of the same transgenic events. (c, d) Lateral root (LR) density (LR number per 1 cm of primary root) and length of 4-wk-old *in vitro*-grown plants. (e, f) Stem height and diameter. (g) Dry root biomass. (h) Dry root : shoot (g : g) ratio. Significance as determined by Student's *t*-test: **, $P < 0.01$; *, $P < 0.05$.
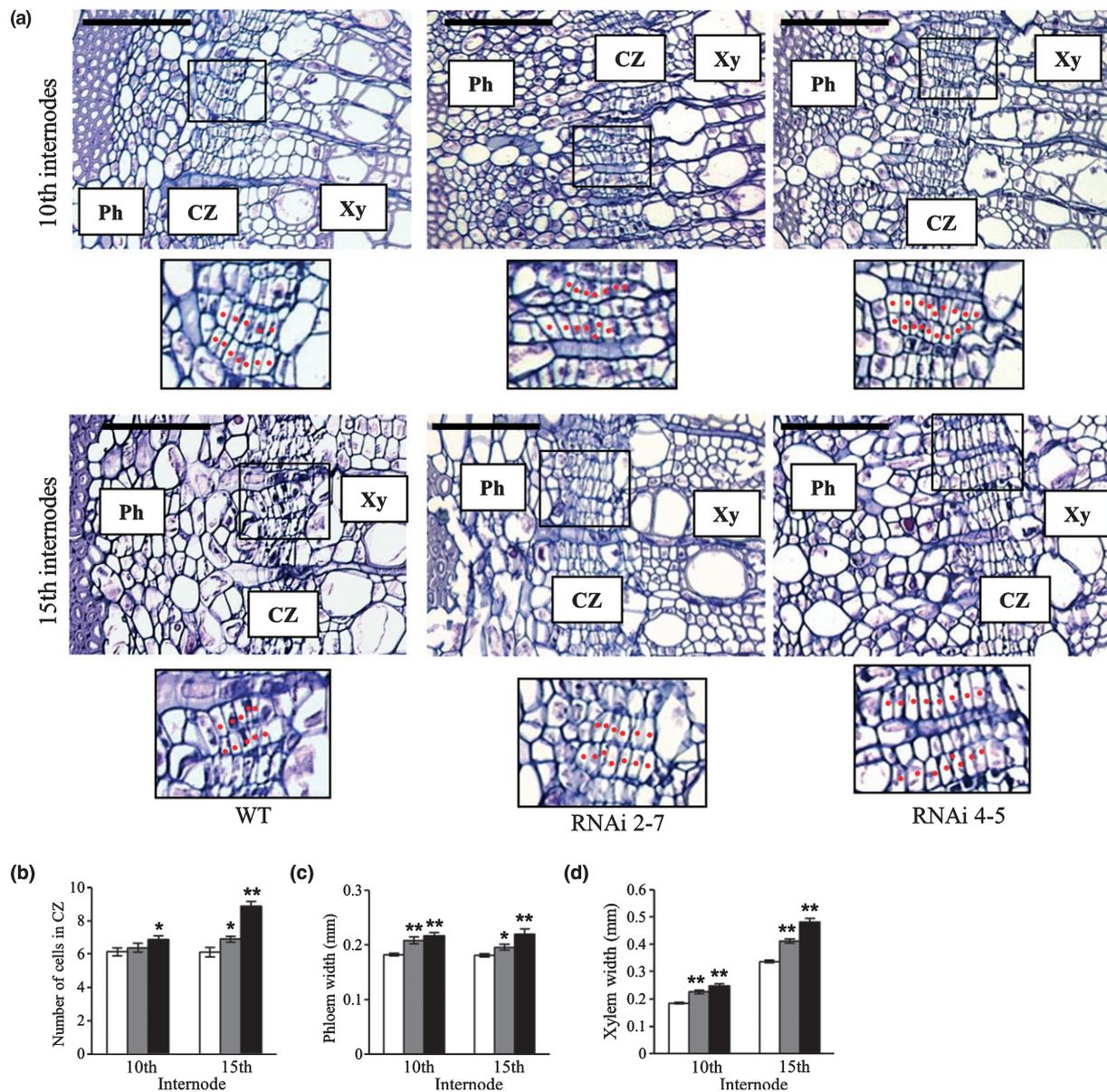
Fig. 8 Gibberellin 2-oxidase (GA2ox) suppression affects wood development. (a) Cross-sections of stems from the 10th (top row) and 15th (bottom row) internodes of *Populus* wild-type (WT; left column), RNAi 2–7 (*PtGA2ox2/7* RNAi transgenics, middle column) and RNAi 4–5 (*PtGA2ox4/5* RNAi transgenics, right column) transgenics. Blown-up framed sectors are shown under each photograph. Red dots indicate individual cells in the cambium zone. Xy, xylem; Ph, phloem; CZ, cambium zone. Bars, 50 μm. (b) Number of cells in the cambium zone in WT and different RNAi transgenics. White bars, WT; gray bars, RNAi 2–7; black bars, RNAi 4–5. Bars represent means ± SE of at least three independent transgenic events (see the Materials and Methods section for more details). (c) Phloem width. (d) Xylem width. Significance as determined by Student's *t*-test: **, $P < 0.01$; *, $P < 0.05$. Internodes number from the top (apex) of the stem.

lateral root initiation in the transgenic roots. Furthermore, in support to our findings, ovrexpression of *Arabidopsis GA20-oxidase* (*GA20ox*) in poplar led to increased bioactive GA concentrations and reduced adventitious rooting (Eriksson *et al.*, 2000). However, in that study, root development was not affected during later stages of glasshouse growth, in contrast to our findings. This may be the result of the ubiquitous and much stronger effect of GA20ox on GA concentrations, which in roots may have

led to severe feedforward and feedback regulation, as described in other species (Olszewski *et al.*, 2002), or from tissue-specific cosuppression. Two other paralogous genes, *PtGA2ox1* and *6*, were expressed in the studied organs at low levels. Their RNAi suppression had no detectable phenotypic consequences under the conditions of this study.

Interestingly, suppression of the only gene that had no redundant paralog (e.g. *PtGA2ox3*) led to an inability to
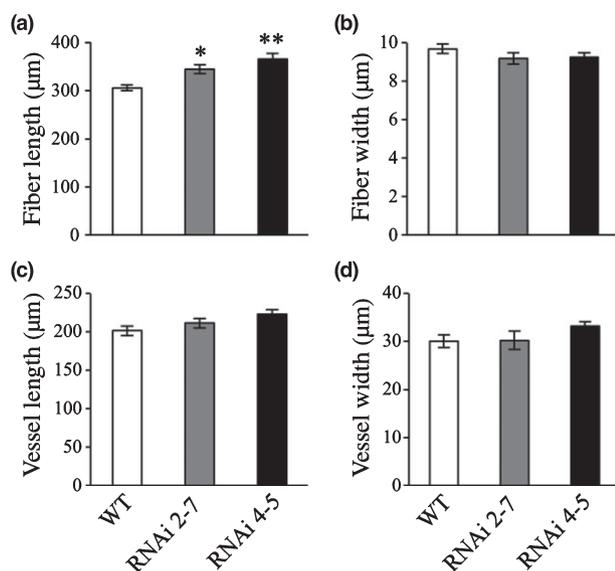
Fig. 9 Gibberellin 2-oxidase (GA2ox) suppression promoted fiber elongation in *Populus*. (a, b) Xylem fiber length and width. (c, d) Vessel length and width. WT, wild-type control. RNAi 2–7, *PtGA2ox2/7* RNAi transgenics. RNAi 4–5, *PtGA2ox4/5* RNAi transgenics. Bars show means ± SE over three independent experiments. Significance as determined by Student's *t*-test: **, $P < 0.01$; *, $P < 0.05$.

Fig. 10 Associations of gene suppression and phenotypes in *Populus* RNAi transgenic plants. Correlation of morphological traits with the expression levels of *GA2ox2* and *7* (a) and *GA2ox4* and *5* (b). Correlation coefficient (R^2) of the linear relationships between expression and trait variation and the respective probabilities (*P*) from a two-sided test are shown in each panel. See associations of expression with all measured traits in Supporting Information, Fig. S3. Bars indicate means ± SE of two independent glasshouse trials. Each experiment was performed with at least four ramets per event. LR, lateral root.

regenerate transgenic plants, suggestive of a lesion in an essential cellular process for callus and/or shoot production. Among all poplar *GA2oxs*, *PtGA2ox3* was the most highly expressed in the apex (including the shoot apical meristem) which may explain the poor regeneration of the transgenic plants. Reduced GA concentrations are required for the normal development and maintenance of the shoot apical meristem because this deficiency promotes their growth and differentiation (Sakamoto *et al.*, 2001a). GA is selectively excluded from shoot apical meristem via down-regulation of *GA20ox* in the meristem dome and up-regulation of *GA2ox* at the boundary with emerging leaf primordia (Sakamoto *et al.*, 2001a,b). It is thus possible that suppression of *PtGA2ox3* interfered with organization and development of new shoot apical meristems during *in vitro* regeneration. By contrast, in *Arabidopsis* the knockout of all C_{19} *GA2-oxidases* did not completely arrest development. This could be explained by the different transformation systems for producing transgenic plants in *Arabidopsis* (a lack of *in vitro* regeneration) vs poplar (organogenic *in vitro* regeneration, with induced development of new shoot meristems).

In summary, when comparing poplar and *Arabidopsis* it appears that the gene families and their functions are organized in a contrasting manner. In *Arabidopsis* there is nearly complete functional redundancy of the five C_{19} GA2oxs (Rieu *et al.*, 2008). The five genes were expressed in all tissues tested and quintuple knockout was needed to expose loss-of-function phenotypes. In poplar, we also observe
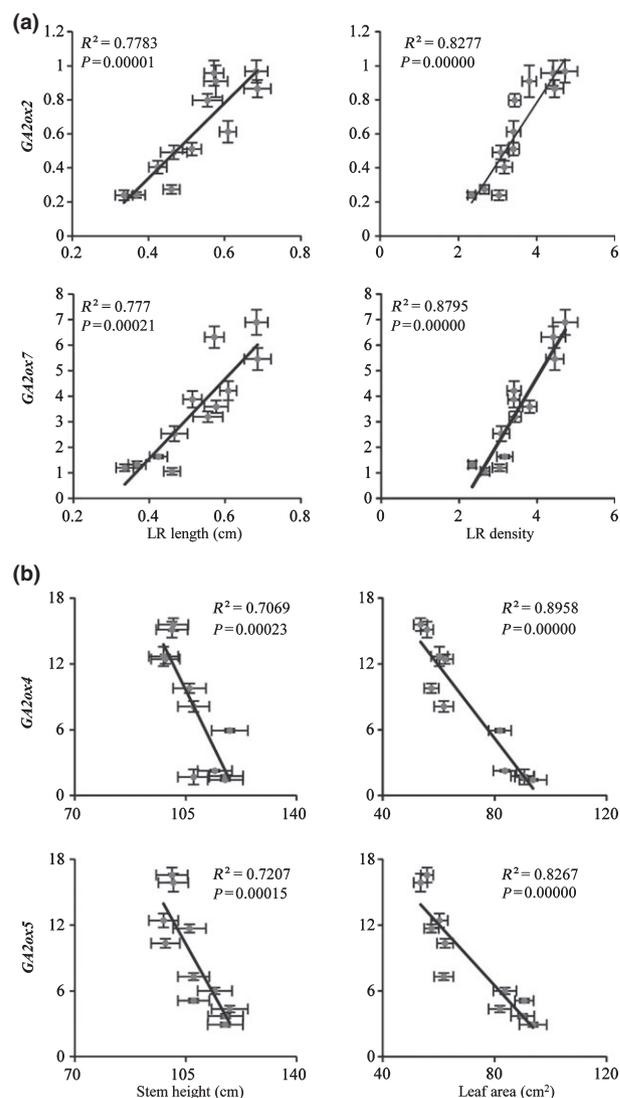
functional redundancy but organized in a very different way. The genome-wide duplication has relieved selection pressure and allowed specialization of each pair for a specific process, specifically for the control of root and shoot development. This is likely to be much more important for trees, which must maintain a tight coordination of root and shoot size and function over decades to centuries, compared with short-lived herbaceous plants. It also appears that the level of expression of the redundant and paralogous genes is linked to the strength of the phenotype; the suppression of
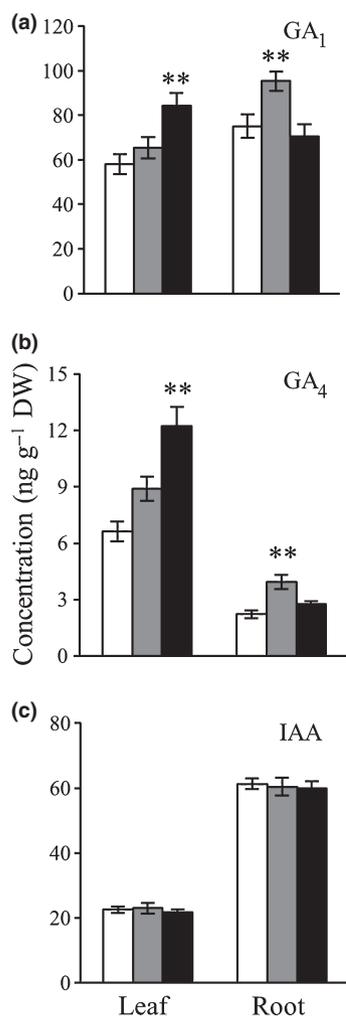
Fig. 11 Gibberellin (GA) and auxin concentrations in leaves and roots of wild-type (WT; white bars) and transgenic *Populus* plants (RNAi 2–7, gray bars; RNAi 4–5, black bars). Concentrations of GA_1 (a), GA_4 (b) and IAA (c). WT, wild type control. RNAi 2–7, *PtGA2ox2/7* RNAi transgenics. RNAi 4–5, *PtGA2ox4/5* RNAi transgenics. Significance as determined by Student's *t*-test: **, $P < 0.01$. Bars represent means ± SE of at least three independent biological replications.

PtGAox1 and *6* genes, which are expressed at very low levels, did not detectably affect any of the measured traits.

Effect of *GA2ox* down-regulation on secondary growth

We did not find a high level of expression in woody tissues, nor a phloem/xylem-predominant expression pattern, for any of the GA2ox genes. Nevertheless the suppression of both the root- and shoot-specific GA2ox gene pairs did affect wood development. This is likely to have been manifested via GA transport to the woody tissues, or through small and highly cell type-localized changes in GA concentrations. The role of GAs in wood development is still poorly understood, and is primarily based on inferences from exogenous applications (Bjorklund *et al.*, 2007). Nevertheless, transgenic manipulation through overexpression of *Arabidopsis GA20ox* in transgenic aspen showed that increased endogenous increases of GA concentration can cause increased fiber length (Eriksson *et al.*, 2000). However, exogenous application of GA through the apex, or through local application, led to increased proliferation in the cambium zone but delayed or prevented vascular differentiation, for which an external supply of auxin was needed (Bjorklund *et al.*, 2007). The increase in fiber length that we observed indicates that, in addition to promotion of cambial cell division, GAs are also involved in xylem cell growth and development as well.

GA2ox down-regulation as a tool for modification of biomass growth

Manipulation of biomass growth and wood properties has been a long-standing goal of forest tree breeding. These goals have become increasingly important with the renewed emphasis on using woody biomass as a feedstock for biofuel production (Somerville *et al.*, 2010). The potential value of genetic means for GA concentration manipulation with the goal of increased biomass growth has been reviewed elsewhere, and will not been discussed here (Busov *et al.*, 2008). The current study shows that RNAi-mediated *GA2ox* suppression of specific gene family members may provide useful tools for improving biomass production in poplar and related species. The *GA2ox* family in poplar comprises functionally redundant gene pairs which are specialized with respect to control of root vs shoot development. The effects of GA deactivation in roots and shoots are opposite; *GA2-oxidase* suppression in shoots has a growth-promoting effect while the same manipulation suppresses root development. Thus selection of the appropriate gene target and change in activity is essential, depending on the environment and crop management system, and whether the goal is improved shoot growth for harvest or increased root growth for drought tolerance, phytoremediation or carbon sequestration.

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Sequence fragments used in generation of RNAi 1–6, RNAi 2–7, RNAi 3 and RNAi 4–5.

Fig. S2 Sequence alignment and position of the RNAi-targeted sequences.

Fig. S3 Selection of optimal PCR cycles for the RT-PCR quantification of gene expression.

Fig. S4 Phylogenetic analysis of the GA2ox families in *Populus* and *Arabidopsis*.

Fig. S5 Associations of gene suppression and phenotypes in RNAi transgenic plants.

Fig. S6 Associations of phenotypes and expression of untargeted GA2ox genes in the RNAi 2–7 transgenics.

Fig. S7 Associations of phenotypes and expression of untargeted GA2ox genes in the RNAi 4–5 transgenics.

Table S1 Primers used to amplify the 4 fragments for generation of the RNAi constructs

Table S2 Primers used for RT-PCR expression analyses

Table S3 Gene models used in the phylogenetic analysis

Table S4 Protein sequences used in the phylogenetic analyses

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