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RESEARCH PAPER

PHOTOPERIOD RESPONSE 1 (*PHOR1*)-like genes regulate shoot/root growth, starch accumulation, and wood formation in *Populus*

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Abstract

This study describes functional characterization of two putative poplar *PHOTOPERIOD RESPONSE 1* (*PHOR1*) orthologues. The expression and sequence analyses indicate that the two poplar genes diverged, at least partially, in function. Pt*PHOR1_1* is most highly expressed in roots and induced by short days, while Pt*PHOR1_2* is more uniformly expressed throughout plant tissues and is not responsive to short days. The two *PHOR1* genes also had distinct effects on shoot and root growth when their expression was up- and downregulated transgenically. Pt*PHOR1_1* effects were restricted to roots while Pt*PHOR1_2* had similar effects on aerial and below-ground development. Nevertheless, both genes seemed to be upregulated in transgenic poplars that are gibberellin-deficient and gibberellin-insensitive, suggesting interplay with gibberellin signalling. *PHOR1* suppression led to increased starch accumulation in both roots and stems. The effect of *PHOR1* suppression on starch accumulation was coupled with growth-inhibiting effects in both roots and shoots, suggesting that *PHOR1* is part of a mechanism that regulates the allocation of carbohydrate to growth or storage in poplar. *PHOR1* downregulation led to significant reduction of xylem formation caused by smaller fibres and vessels suggesting that *PHOR1* likely plays a role in the growth of xylem cells.

Key words: Biomass, dormancy, growth, poplar, starch, xylem.

Introduction

Plants generally allocate biomass to organs that assimilate the most limiting resources for their growth and development (Thornley, 1972; Bloom *et al*., 1985). Regulation of biomass growth and allocation is of major importance in response to abiotic stress, shade avoidance, and cyclical seasonal changes (Hermans *et al*., 2006; Forster *et al*., 2011; Ruberti *et al*., 2011). The regulatory mechanisms governing these responses are important for adaptation, and increased nutrient-use efficiency and recycling. However, the underlying molecular mechanisms are still poorly understood.

Previous work has detected significant coordination between shoot and root growth in a *Populus* tree and that the regulatory mechanisms are, at least in part, mediated by gibberellin (GA) signalling and metabolism (Busov *et al*., 2006; Gou *et al*., 2010, 2011). GA-insensitive or -deficient transgenics with severely reduced shoot growth produce extensive root systems, which can be several-fold larger than in wild-type (WT) plants (Gou *et al*., 2010), whereas high GA concentrations lead to increased shoot growth but suppress root proliferation (Gou *et al*., 2011). These responses are typically observed under water or nutrient deficiency or during shade avoidance. For example, insufficient light leads to increased shoot growth but suppressed root development (Ruberti *et al*., 2011). Nutrient and water deficiencies elicit root proliferation but suppress shoot growth (Forster *et al*., 2011).

The mechanism(s) behind these responses are still poorly understood. Uncoupling the morphological responses from their environmental triggers through genetic manipulation provides an

experimental system for understanding their molecular underpinnings. In an attempt to better understand these regulatory mechanisms, a microarray analysis was previously performed on the above-mentioned transgenic poplars. One of the most upregulated transcripts in the transgenic plants was a putative poplar orthologue of *PHOTOPERIOD RESPONSE1* (Pt*PHOR1*) (Gou *et al*., 2010).

PHOR1 was first isolated from potato (*Solanum tuberosum*) in a screen for regulators that promote tuberization (Amador *et al*., 2001). Tuberization is a process associated with switch of elongation from to radial growth and accumulation of massive amounts of reserve substances, such as starch, in modified underground structures known as stolons (Abelenda *et al*., 2011). This process is initiated by short days or cold temperatures, which signal the onset of winter. A screen for transcripts that are upregulated in the leaves of plants exposed to short days led to the identification of *PHOR1*. Plants in which *PHOR1* expression was inhibited by antisense transgenes displayed reduced growth and GA responsiveness, while *PHOR1* overexpression enhanced overall growth and increased GA responsiveness. *PHOR1* encodes an arm-repeat domain and U-box-containing protein. U-box-containing proteins are typically a class of E3 ubiquitin ligases that facilitate ubiquitination of specific proteins targeted for degradation (Pringa *et al*., 2001). As far as is known, the function of *PHOR1* in other species, including the model plant *Arabidopsis thaliana*, has not been reported to date.

Poplars (species within the genus *Populus*) and other woody perennial species from temperate climates survive harsh winter conditions through a temporary cessation of growth known as dormancy. Typically, the process is initiated by sensing critical shortening of the photoperiod, cessation of shoot and vascular cambium growth, and development of buds, which contain the shoot apical meristem (Lang, 1987). In addition, significant changes in metabolism occur. Most importantly, primary metabolism is shifted to increased production of storage compounds, such as starch, particularly in roots (Nguyen *et al*., 1990; Regier *et al*., 2010). The mechanisms that drive these morphological and metabolic changes are still poorly understood.

This study investigates the role of the two poplar *PHOR1* orthologues in controlling vegetative growth and allocation of photosynthate to shoots and roots. It shows that both genes are involved in regulating shoot and root growth, as well as starch accumulation. The results also show functional divergence of the two genes that operates at both gene expression and protein levels.

Materials and methods

Plasmid construction and transformation

For overexpression constructs, the open reading frames of Pt*PHOR1_1* (POPTR_0007s03730) and Pt*PHOR1_2* (POPTR_0005s05880) were amplified via PCR using primers containing the *Hin*dIII and *Bam*HI restriction sites: 5′-GGGGAAGCTTATG GTGAGAGACGTA-3′ and 5′-CCCCGGATCCTC AGAAGGGCATGAT-3′. The amplified fragments were inserted into the shuttle vector pART7 between its *Hin*dIII and *Bam*HI sites, downstream of the cauliflower mosaic virus (CaMV) 35S promoter and upstream of the octopine synthase (OCS) terminator. The expression cassette was transferred from pART7 into the pART27 binary vector using the unique *Not*I restriction site (Gleave, 1992).

An RNA interference (RNAi) construct was assembled using a 377-base pair (bp) region from Pt*PHOR1_1* that was amplified via PCR using the following primers, which contained *att*B1 and *att*B2 sites: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGAGCGTTT CGATCATAGAAAAG-3′ and 5′-GGGGACCACTTTGTACAAGA AAGCTGGGTTCAGAAGGGCATGATATGAGTAGTCTT-3′. The amplified fragment was cloned into the pHellsgate2 vector using the Gateway system (Invitrogen). All constructs were sequence-verified and then transformed into *Agrobacterium tumefaciens* via the freeze–thaw method (Holsters *et al*., 1978). Strain C58 was transformed with the overexpression construct and AGL1 with the RNAi construct. An *Agrobacterium*-mediated protocol (Filichkin *et al*., 2006) was used to introduce constructs into hybrid aspen clone INRA 717–1B4 (*Populus tremula* × *Populus alba*). At least 20 independent events for each constructs were regenerated and PCR-validated for the presence of their respective transgenes.

RNA extraction and reverse-transcription PCR analysis

Total RNA was isolated using the RNeasy Plant Mini Kit with on-column genomic DNA digestion using RNase-Free DNaseI (Qiagen). cDNA was synthesized from 2 µg of DNaseI-treated total RNA using Superscript III reverse transcriptase (Invitrogen) with an oligo-dT primer in a reaction volume of 1 µl. The following primers were used in the reverse-transcription PCR experiments to amplify Pt*PHOR1_1* and Pt*PHOR1_2*: PHOR1_1 (5′-TCCAGACTCAGAAAGACAACC-3′ and 5′-CAACAAGCATTTCAACAAAGCC-3′) and PHOR1_2 (5′-AATGAGAGCCCAGAAGGAG-3′and 5′-CAAGAAGGAGAGA-GAAGACAAAC-3′). Relative expression values were normalized to expression of ubiquitin gene (eugene3.00002054) using the following primers: 5′-AGAGTGTGAGAGAGAGAAGAG-3′ and 5′-CGACGACCATCAAACAAGAAG-3′. Primers used for amplification of IAA genes were as follows: F_PttIAA1 5′-GAGAGAGAGAAGAAGAAGAGGG-3′; R_PttIAA1 5′-AGGATC-ATTAGGGCGAGGAG-3′; F_PttIAA4 5′-AGCATCTCAGCCTCTCA-AC-3′; R_PttIAA4 5′-TCCCCAACAATCAACCAATC-3′; F_PttIAA8 5′-AAACTGCTCCTCCACCCATC-3′; R_PttIAA8 5′-TCCAACCAAC-ATCCAATCTCC-3′.

Auxin measurements

Endogenous indole-3-acetic acid (IAA) was measured on 1-month-old *in vitro*-grown plants. Frozen tissue samples were extracted following the procedure for free IAA determination outlined in Bialek and Cohen (1992). Briefly, tissue samples were homogenized and extracted in isopropanol/0.2 M (pH 7.0) imidazole buffer (65:35, v/v) using a 1.5 buffer-to-tissue volume ratio. Each sample was spiked with 40 ng [$^{13}C_6$] IAA internal standard (Cambridge Isotope Laboratories, Andover, MA, USA) per g frozen weight to determine endogenous IAA levels. After vortexing and centrifuging to remove tissue debris, supernatants were diluted 4.5-fold in preparation for solid-phase extraction. Samples were then loaded onto 3-cc Oasis MAX SPE columns (Waters Corporation, Milford, MA, USA), previously conditioned and equilibrated with equal volumes of methanol and deionized distilled water, respectively. Each sample was washed with equal volumes of 5% ammonium hydroxide in water and in methanol, and then eluted with 2 ml of 2% formic acid in methanol. The solvent was evaporated in a Savant Speedvac (Thermo Scientific, Waltham, MA, USA) at 30 °C for approximately 2.5 h, and then resuspended in 200 µl methanol in preparation for derivatization by methylation. Samples were derivatized by adding 250 µl dichloromethane followed by 5 µl of 2 M trimethysilyl diazomethane in hexanes at room temperature for 30 min. To remove excess diazomethane and stop methylation, 5 µl of 2 M acetic acid in hexanes was added per sample. Samples were then evaporated overnight in a fume hood, washed with 100 µl acetonitrile and dried in a Speedvac at 30 °C twice, and resuspended in 30 µl ethyl acetate for gas chromatography/mass spectrometry (GC-MS) analysis. The GC injector and transfer port temperatures were set to 260 °C. Helium gas was used as a carrier with a flow rate of 1 ml min^{-1}. A DB-5 column (10 m × 0.18 mm

× 0.20 μm) was used. The temperature program for GC consisted of 80 °C for 2 min, 20 °C min^{-1} ramp up to 260 °C, then 260 °C for 2 min. Injection volume was 5 μl with a 1:5 column split ratio. The MS mode had an electron emission of 70 eV and a mass range of 70–200 m/z. The 130/189 and 136/195 m/z ratios were monitored to determine endogenous IAA levels as compared to the internal standard. Absolute amounts of IAA were calculated according to a standard curve developed using the [$^{13}C_6$] IAA standard.

Analysis of root and shoot anatomy

Stem segments at the 15th internode were sampled and processed as previously described (Zawaski *et al*., 2011), using 5-μm sections stained with haematoxylin and eosin. For fibre length analysis, a modified version of Franklin′s maceration technique was used (Franklin, 1945). Stem segments, consisting of the entire section of the 15th internode (approximately 2 cm in length), were incubated at 60 °C for 24 h in equal volumes of glacial acetic acid and hydrogen peroxide. After 24 h of incubation, the samples were washed with deionized distilled water and a small amount of the resulting pulp was stained with Safarinin O. All sections and fibres were observed with a fluorescent microscope (Ernst Leitz, Wetzlar, Germany) with SPOT Insight QC camera and advanced SPOT software (Diagnostic Instruments). Slide measurements were performed in ImageJ version 1.38 (National Institutes of Health). For roots, sections were made approximately 2 mm from the tips of fine roots with a diameter threshold of 4 mm. Root sections were stained with I_2KI solution for visualization of starch, as described in Wargo (1975). Pictures were taken with a Sony 3CCD DKC-5000 microscope and camera system.

Phenotypic characterization and tissue collection

Plants were micropropagated *in vitro* on hormone-free half-strength Murashige and Skoog media containing 2% sucrose and 7% agar. For greenhouse experiments, after 1 month of *in vitro* growth, plants were transferred to a greenhouse and grown in a commercial potting mix (Sunshine Mix #1, Sun Gro Horticulture, Bellevue, WA, USA), under continuous irrigation, fertilization, and pest control. To study the expression of the Pt*PHOR1* genes in different plant tissues, WT plants were grown for 3 months before tissues were harvested. For transgenic characterization experiments, plants were measured and harvested at 105 d for 35S::Pt*PHOR1_1* and 35S::Pt*PHOR1_2*, and 120 d for Pt*PHOR1*-RNAi plants. All leaf and internode numbers refer to measurements made at the respective leaf-plastochron index. Height was measured as the total length of the stem from base to apex. Stem diameter was measured 5 cm from the root-collar and internode distance was calculated as the mean of all internode distances in a given plant. Tissues for dry biomass estimation were dehydrated at 70 °C until a constant weight was achieved. Dormancy experiments were performed in a growth chamber (Conviron, Pembina, ND, USA) on 3-month-old plants. To induce dormancy, the photoperiod under which plants were grown was changed from long days (16/8 light/dark) to short days (8/16 light/dark). Stem elongation was measured and bud formation was observed weekly. Leaves of WT plants subjected to long and short days were collected at the same time weekly for expression analysis.

For all *in vitro* experiments, microshoots were grown for 1 month and then tissues were collect for expression studies and/or final plant measurements made. Expression analysis was performed on shoot and leaf tissue in WT plants grown in media without hormones (control) or supplemented with GA_3 (5 μM) and paclobutrazol (10 μM). The concentrations of GA_3 and paclobutrazol used caused pronounced increases and decreases in plant stem elongation, respectively. Differences in adventitious rooting were measured on overexpressed transgenics and WT plants grown in media with or without indole-3-butyric acid supplement (IBA, 0.5 μM). Data was collected on the number of stem cuttings to root for each treatment. Generation and characterization of GA-deficient (35S::Pc*GA2ox*) and -insensitive (35S::*gai* and 35S::*rgl1*) transgenics have been previously described (Busov *et al*., 2003, 2006). Expression of these transgenes elicit a gradient in phenotypic response ranging from severe dwarfism to nearly WT-like (Zawaski *et al*., 2011). For expression analysis of GA-modified transgenics, leaves were collected from 2-year-old field-grown *gai*-expressing plants with different phenotypic severities, as indicated by their heights. Leaves were collected from 3-month-old greenhouse-grown Pc*GA2ox*- and *rgl1*-expressing plants that had been previously characterized as having dwarf and semidwarf phenotypes (Gou *et al*., 2011; Zawaski *et al*., 2011).

Statistical and bioinformatics analysis

Statistical analysis was performed in SigmaStat (Systat Software). Number of ramets and lines used in each experiment are specified in figure and/or table captions. A Student′s t-test or analysis of variance (ANOVA) followed by Dunnett′s post-hoc test was used to test for significant differences ($P < 0.05$) between WT and transgenic line(s). Identity and similarity among sequences where calculated using the MatGAT program (Campanella *et al*., 2003).

Results

Upregulation of poplar PHOR1*-like genes in GA-insensitive and -deficient transgenics*

Microarray analysis identified a gene, designated Pt*PHOR1_1* (POPTR_0007s03730), that encodes a putative protein with high sequence similarity to the potato *PHOTOPERIOD RESPONSE1* (*PHOR1*) gene (Amador *et al*., 2001) that was highly upregulated in GA-deficient and -insensitive poplar transgenics. The generation, initial characterization, and array analysis of the transgenics have been previously described (Busov *et al*., 2006; Gou *et al*., 2010). In addition, similarity searches in the poplar genome found another *PHOR1* gene (Pt*PHOR1_2*; POPTR_0005s05880). Gene-specific primers were designed to perform reverse-transcription PCR expression analyses for both genes. Pt*PHOR1_1* and Pt*PHOR1_2* were significantly upregulated in both the GA-insensitive and -deficient transgenics (Fig. 1A, 1B). This study also observed a general increase in the expression of the Pt*PHOR1* genes (albeit exceptions to this trend did occur) in relation to the increased severity of the GA-deficient/insensitive dwarf phenotype observed in these transgenics (denoted by height reduction).

Expression patterns of the two PHOR1 *genes*

The expression of the two poplar *PHOR1* genes was studied in a variety of tissues (Fig. 2A). Pt*PHOR1_1* transcript was highly abundant in roots and nearly equally expressed in all other organs tested. Expression of Pt*PHOR1_2* was similar in all tissues analysed. Because potato *PHOR1* was found to be responsive to short-day photoperiods (Amador *et al*., 2001), the present study investigated if this response was conserved in poplar (Fig. 2B). Pt*PHOR1_1* transcript abundance was highly and significantly increased following 3 weeks of short-day treatment, while Pt*PHOR1_2* expression was completely non-responsive to short-day treatment.

*Pt*PHOR1 *overexpression caused declines in adventitious root formation and agravitropic root growth*

To determine their function with respect to poplar growth and development, Pt*PHOR1_1* and Pt*PHOR1_2* were overexpressed in transgenic poplar under the control of the 35S promoter. Six lines (independent insertional events) were selected for each

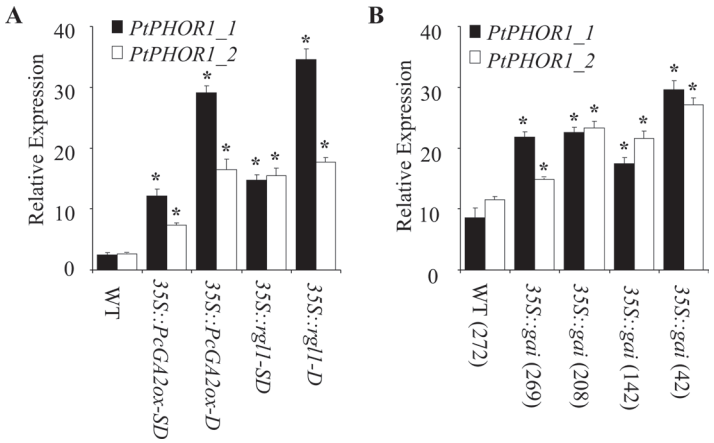

Fig. 1. Modified expression of Pt*PHOR1* genes in GA-deficient (35S::Pc*GA2ox*) and GA-insensitive (35S::*rgl1* and 35S::*gai*) transgenic poplar (genotype 717-IB4). Bars represent event mean ± SE for reverse-transcription PCR expression normalized to *UBQ* for three biological replicates performed on leaf samples collected from 3-month-old greenhouse plants (A: D, dwarf; SD, semi-dwarf) and 2-year-old field-grown plants (B; numbers in parentheses indicate the plant height in cm). Asterisks indicate significant difference ($P < 0.05$) between transgenic and wild-type (WT) events as determined by ANOVA followed by Dunnett's post-hoc test.

gene and upregulation of the respective transcripts confirmed in five of them (Supplementary Fig. S1, available at *JXB* online). All Pt*PHOR1*-overexpressing lines showed a drastic decline in adventitious root formation on hormone-free media, so a rooting test was performed on media with and without auxin (Fig. 3A, B). In the absence of auxin, transgenic stem cuttings had significantly lower rooting than WT segments (Fig. 3A). Because of the strong dependence on auxin, it was hypothesized that Pt*PHOR1* overexpression may affect endogenous auxin levels. Therefore, IAA was measured in transgenic and WT plants but no significant differences were found (Fig. 3C).

This study also observed, after transfer to greenhouse conditions, that many of the transgenic plants exhibited severe agravitropic root growth, which led to a poorly developed, ball-like root system (Fig. 4A), wilting, and low survival (Fig. 4B). The tap roots were either bent upward or completely entangled in the ball-like structure (Fig. 4A). Consistent with its expression in roots, the transgenics overexpressing Pt*PHOR1_1* were much more affected than those containing 35S::Pt*PHOR1_2*, as evidenced by the significantly greater increase in their mortality (Fig. 4B). Despite the initial problems with root initiation and agravitropism, most of the lines were able to overcome these abnormalities and later developed a root system that was significantly larger than the WT (see below).

PHOR1 *overexpression enhances shoot and root growth*

The poor survival and abnormal root development of transgenic plants, following greenhouse establishment (Fig. 4), were likely a result of higher sensitivity to the transplanting stress, as the transgenic plants that survived initial abnormalities in root growth later developed normal root systems. This study found significant increases in several growth parameters of Pt*PHOR1*-overexpressing plants (Table 1). The Pt*PHOR1_2*-overexpressing transgenics produced significantly greater amounts of stem and root biomass, while the 35S::Pt*PHOR1_1* plants showed only a significant increase in root biomass. Neither of the two transgenes had a significant effect on leaf biomass.

RNAi suppression of PHOR1 *inhibits shoot and root growth*

Because of the significant overlap in the expression of the two genes (Fig. 2A), both were targeted for suppression. A 337-bp region of 95% sequence identity between the two genes was used to generate an RNAi construct. Two lines (1 and 2), with significant ($P < 0.05$) suppression of Pt*PHOR1_1* and Pt*PHOR1_2* expression in both roots and leaves (Supplementary Fig. S2), were used in all subsequent experiments. The Pt*PHOR1*-suppressed transgenics showed decreased growth very early in their greenhouse development (Fig. 5), including significant reductions in height, stem diameter, and stem growth (Fig. 5B–E), as well as decreases in leaf, stem, and root biomass (Table 2).

*Pt*PHOR1 *suppression leads to root tip swelling and starch accumulation*

The RNAi plants exhibited lateral roots that were less branched, short, and had swollen tips, and the bulk of the root mass was concentrated toward the bottom of the root system (Fig. 6A–C). The changes in root architecture resulted in significant reductions in root biomass (Fig. 6F, Table 2). To better understand the root phenotype, cross-sections were taken through the affected tissues. Anatomical analysis revealed that cortex cells were significantly larger in diameter (Fig. 6E). Because the root swelling in transgenics resembled a tuber and *PHOR1* is known to be involved in tuber formation in *S. tuberosum* (Amador *et al*., 2001; Monte *et al*., 2003), the present study investigated whether the swelling result from accumulation of starch. Staining root sections with K_2I indeed showed significantly greater starch accumulation in the RNAi plants (Fig. 7A).

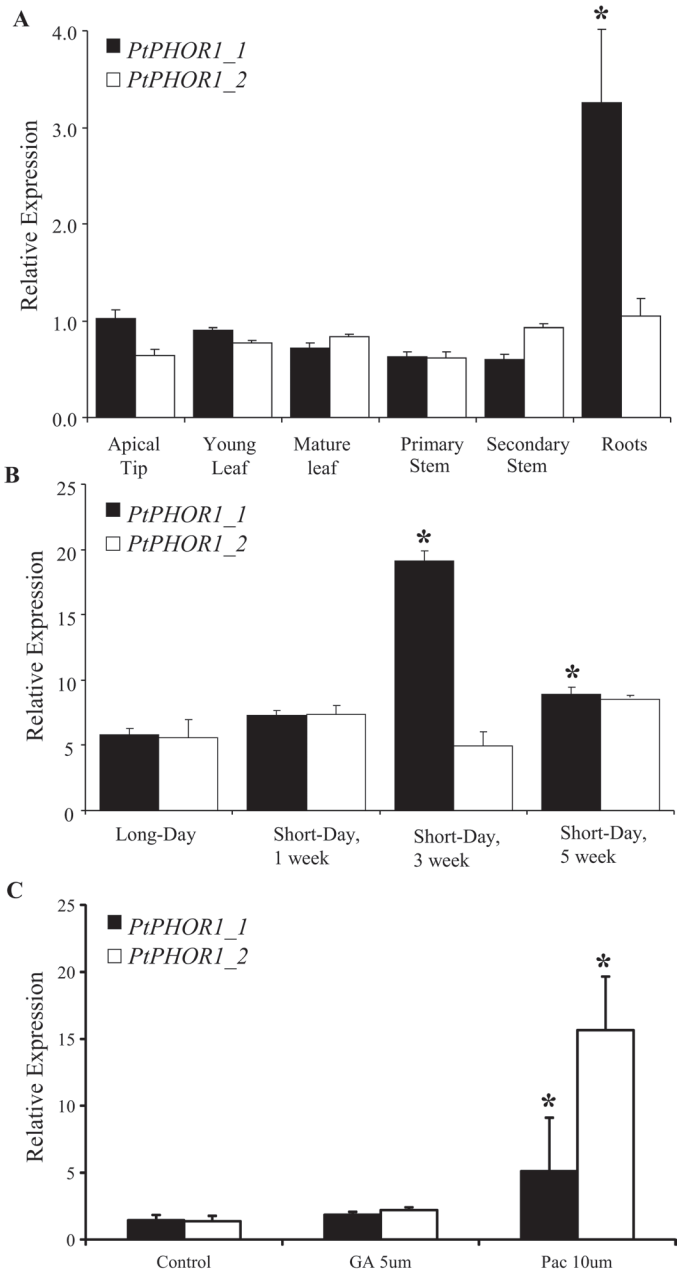

Fig. 2. Expression of Pt*PHOR1* genes in wild-type poplars. Expression was studied in 3-month-old 717-IB4 tissues (A) and leaves subjected to short-day treatment (B). Bars represent mean ± SE for reverse-transcription PCR results normalized to *UBQ* and *CYC*. In (A), means are based on three biological replicates: young leaf, leaf-plastochron index=4–7 and expanding; mature leaf, leaf-plastochron index=15 and fully expanded; primary stem, leaf-plastochron index=2–6 and undergoing primary growth; secondary stems, leaf-plastochron index=15–18 and undergoing secondary (woody) growth and roots, soft non-lignified tips. In (B), expression in plants grown under long days (16/8 light/dark) compared to plants that were subsequently subjected to short days (8/16 light/dark) for 1, 3, and 5 weeks; means are based on two biological replicates (each composed of three technical replicates) on pooled leaf samples (leaf-plastochron index=7–10) collected from five wild-type plants. In (C), hormonal treatments are described in the Materials and methods (GA, gibberellin; Pac, paclobutrazol). All treatments were performed with at least two biological replications. Asterisks indicate significant difference ($P < 0.05$) by ANOVA followed by Tukey's post-hoc test (A), t-test between long-day and short-day values (B), and t-test between control and treatment (C).

RNAi suppression promotes phloem but inhibits xylem formation

Because of the highly significant effect of RNAi suppression on stem growth (Fig. 5C and E), it was suspected that Pt*PHOR1* could modify the pattern of secondary growth. Inspection of cross-sections obtained from stems undergoing secondary growth in the WT and RNAi plants revealed changes in stem morphology (Fig. 8). The amount of phloem was significantly increased, whereas the xylem and pith were significantly

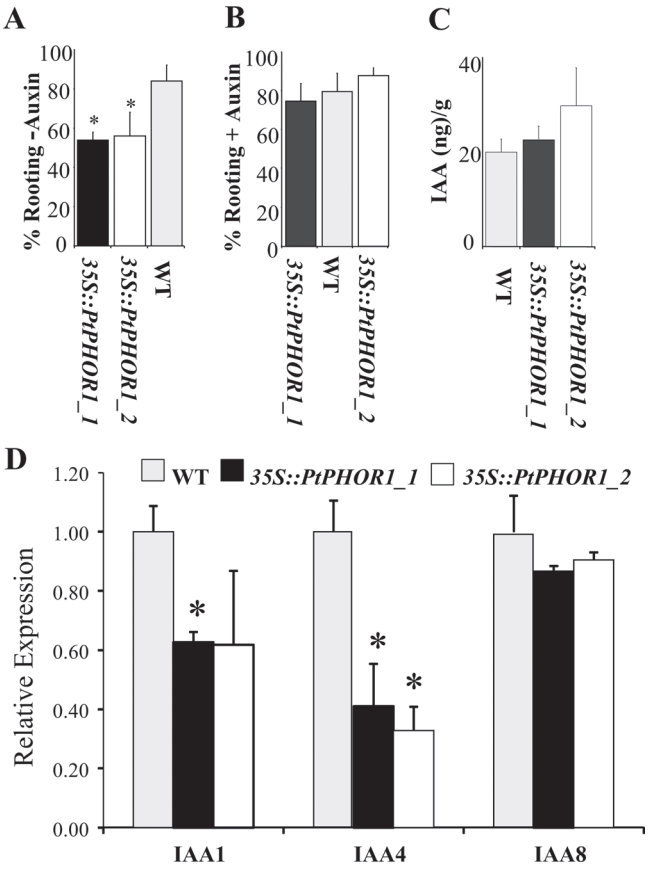

Fig. 3. Overexpression of Pt*PHOR1_1* and Pt*PHOR1_2* impaired adventitious rooting. (A, B) Rooting was impaired in an auxin-dependent manner; microshoots were grown for 1 month in media without (A) or with (B) 0.5 μM indole-3-butyric acid; bars represent mean ± SE of wild-type (WT) and transgenic plants 1 month post-propagation, from two independent experiments with at least 16 WT plants and six independent transgenic events with at least four ramets/event for each transgenic line and treatment; rooting percentage is the percentage of stem cuttings that rooted (as denoted by visible initiation of at least one adventitious root) out of the total number of stem cuttings that were used in the experiment. (C) Endogenous indole-3-acetic acid content in plants propagated *in vitro* from stem cuttings; bars represent mean ± SE based on three events, each with three repetitions, for both transgenic types and three WT replicates, each replicate consisting of shoots and leaves of four plants grown in a single tissue culture box. (D) Expression of *IAA1*, *4* and *8* was studied in three events, for both transgenic types and three WT replicates. Asterisks indicate significant difference ($P < 0.05$) by t-test.

decreased. The reduced xylem in transgenics appeared to be caused by reduced fibre and vessel size rather than fibre and vessel number (Fig. 9). Both xylem fibres and vessel elements were significantly smaller in transgenics than in WT in both their radial and longitudinal axes. Close examination of the phloem cells revealed accumulation of starch granules, similar to what was seen in the roots (Fig. 7B).

*Pt*PHOR1 *modifications do not alter growth cessation and bud set*

Poplars are highly photoperiod sensitive, and a reduction of day length to approximately 8 h causes a cessation of shoot elongation followed by formation of a dormant bud. On a weekly basis, stem elongation was measured and bud formation was observed in WT, overexpressing, and RNAi transgenics. No significant differences were detected in weekly reductions to cumulative percentage height growth (Fig. 10), which lead to the growth cessation and coincided with bud set in both transgenic and WT plants (data not shown).

Discussion

PHOR1 *function is conserved in poplar*

This study provides functional characterization of two putative poplar orthologues of *PHOR1* from potato. Expression of this gene was shown to be induced by short days and to have a strong effect on the timing and amount of tuber formation (Amador *et al*., 2001). As far as is known, this is the first report of *PHOR1* characterization outside its initial discovery in potato. Several lines of evidence suggest that its function is strongly conserved in *Populus*. First, both genes show significant sequence similarity, and, similar to potato, one of the poplar genes is induced by short-day treatment. Second, as in potato, poplar *PHOR1* genes appear to interact with GA signalling, as indicated by their significant upregulation in GA-insensitive and -deficient poplar transgenics. Finally, as with potato, downregulation of the genes in transgenic poplar led to decreased stem elongation while overexpression led to increased stem elongation. The conservation of *PHOR1* function in non-tuberizing plant suggests that *PHOR1* operates within a larger growth and developmental context than just tuber formation.

Partial functional conservation and divergence of poplar PHOR1 *genes*

Because of the absence of whole genome sequence, it is still unclear how many *PHOR1* genes are present in potato; however, Southern blot analysis indicated that there are at least two in the potato genome (Amador *et al*., 2001). Similarity searches identified three *PHOR1*-like sequences in *Arabidopsis*, but to date, little information is available about these genes (Monte *et al*., 2003). The present study found two genes in the most recent version of the *Populus* genome (version 2.0). It should be noted that a previous version of the poplar genome indicated three *PHOR1* genes, two of which were identical in sequence, in a close proximity on the same linkage group, but this appears to have been an assembly artifact.

Expression and sequence analyses indicated that the two poplar genes may have diverged, at least partially, in function. Pt*PHOR1_1* is most highly expressed in roots (Fig. 2A) and is induced by short-day treatment (Fig. 2B). In contrast, Pt*PHOR1_2* is distinct in sequence from *PtPHOR_1*, and is uniformly expressed across the organs studied and not induced by short days (Fig. 2B). Nevertheless, both genes seemed to be

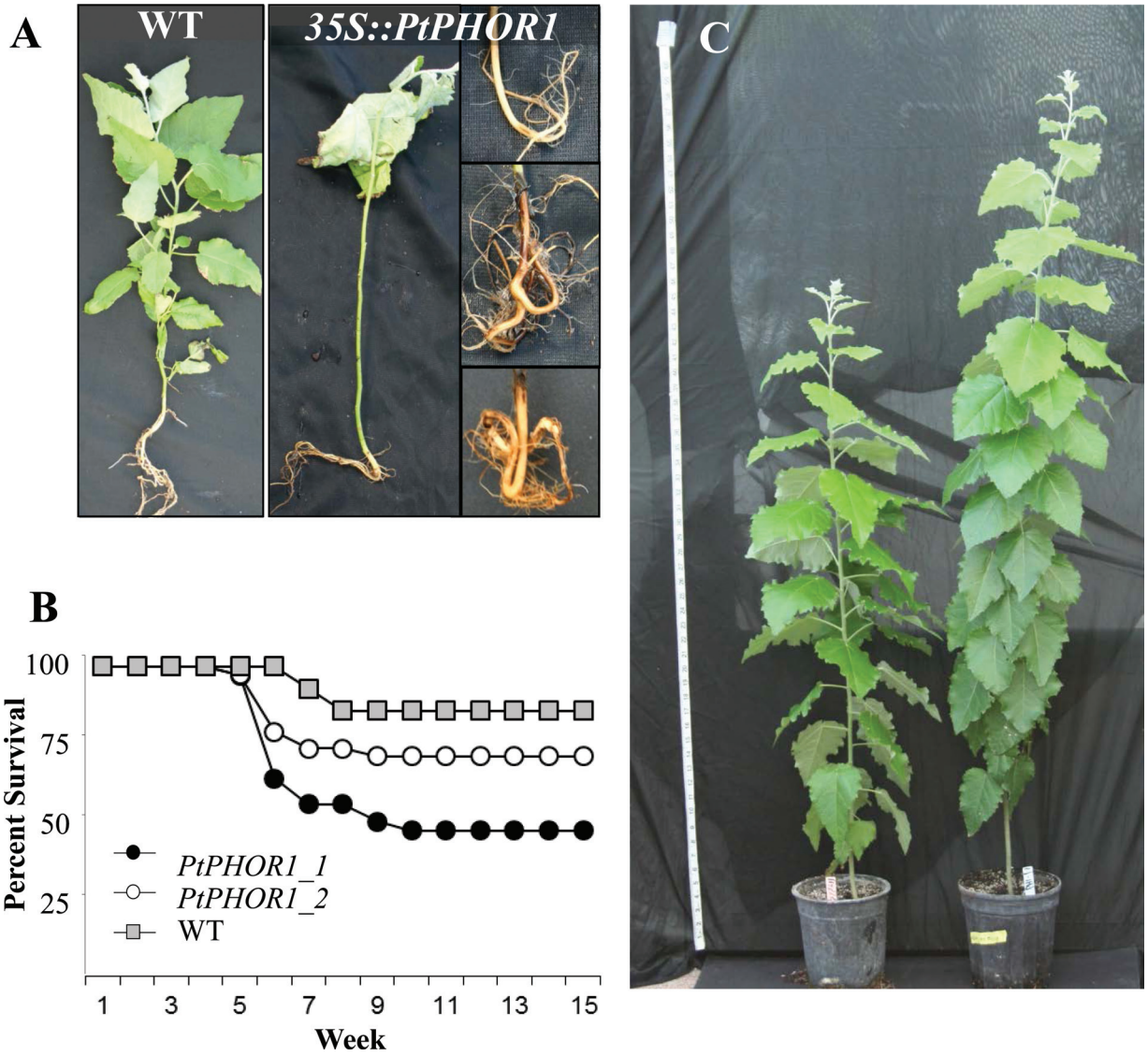

Fig. 4. Abnormal root growth in 35S::Pt*PHOR1* transgenics affected early greenhouse survival. (A) Abnormal root growth, plant wilting, and leaf loss in transgenics compared with wild-type (WT) at 4 weeks post plantlet greenhouse acclimation when plants were transplanted to larger pots. (B) Percentage of plants that survived after growing in a greenhouse for 15 weeks. (C) Greenhouse-grown WT (left) and transgenic (right) plants at 15 weeks. Fifteen WT plants and six transgenic events with at least six ramets/event were used in the experiment (this figure is available in colour at *JXB* online).

upregulated in the poplar GA-deficient and -insensitive transgenics, and the level of upregulation was correlated with phenotypic severity. When their expression was manipulated transgenically, both poplar *PHOR1* genes also had distinct effects on shoot and root growth. Overexpression increased and downregulation inhibited biomass accumulation for both organs in Pt*PHOR1_2*, while Pt*PHOR1_1* effects were restricted to roots.

PHOR1 *role in starch accumulation*

One of the interesting yet unexpected outcomes of this study was the increased starch accumulation in both roots and stems of the *PHOR1*-suppressed poplar plants. Although the effect of the antisense suppression of potato *PHOR1* on starch accumulation was not studied, its downregulation led to stimulation of tuber growth (the main starch storage organ in potato) (Amador *et al*., 2001). The effect of *PHOR1* suppression on starch accumulation is particularly interesting because it was associated with growth inhibition in both roots and shoots. It is widely known that developmental shifts to synthesis of storage compounds are associated with growth inhibition and preparation for unfavourable growth conditions (Hermans *et al*., 2006). Thus, *PHOR1* appears to be part of a mechanism that regulates whether carbohydrate is diverted to growth or storage when plants are exposed to conditions that are unfavourable for growth.

The role of *PHOR1* in relation to starch metabolism may be most readily explained with its connection to the GA signal transduction pathway (Thomas and Sun, 2004). As a major growth hormone in plants that can cause a rapid spurt of growth, GA plays important roles in the metabolism of carbohydrates

Table 1. Pt*PHOR1* overexpression increases biomass

Values are mean ± SE biomass of plants grown in a greenhouse for 105 days. *, $P < 0.05$ by t-test.

Genotype	Stem (g)	Leaf (g)	Roots (g)
35S::Pt*PHOR1_1*	8.79 ± 1.01	13.61 ± 1.47	6.19 ± 0.60*
35S::Pt*PHOR1_2*	12.6 ± 0.86*	17.79 ± 9.46	6.55 ± 0.28*
Wild-type	8.44 ± 1.12	11.65 ± 1.79	4.35 ± 0.36

that can be readily mobilized to sustain growth. Perhaps the best-known example is the effect of GA on α-amylase activity in barley aleurone cells (Gubler *et al*., 2002). Exogenous GA application activates the enzyme to mobilize starch in the endosperm during seed germination. More recently, study of a major GA-deactivating enzyme in rice, ELONGATED UPPERMOST INTERNODE (EUI), led to outcomes very similar to that observed in the present work (Zhang *et al*., 2008). The *EUI* gene encodes a P450 monooxygenase involved in deactivating bioactive GAs (Zhang *et al*., 2008). *EUI* overexpression led to GA deficiency, decreased stem growth, and increased starch granule accumulation. Interestingly, plants with reduced *EUI* expression showed the opposite phenotype: increased shoot elongation and decreased amyloplast accumulation. Thus, the findings of Zhang *et al.* (2008) closely mirror those of the present study that *PHOR1* suppression leads to decreased shoot growth and increased starch granule development. It is unclear if this is a generalized response to modification of GA response and concentrations, as little information is available about amyloplast development in other GA-associated lesions.

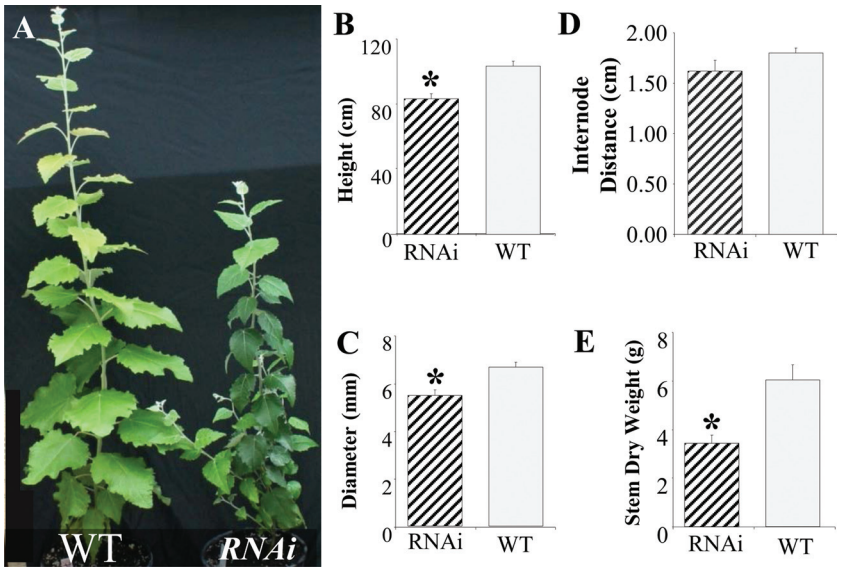

Fig. 5. Suppression of Pt*PHOR1* caused a reduction of shoot growth. Results are for 4-month-old greenhouse-grown Pt*PHOR1* RNA interference (RNAi) transgenics. (A) Above-ground phenotype of representative wild-type (WT) and transgenic plants. (B–E) Shoot growth measurements; bars represent mean ± SE of two RNAi lines, each with at least four ramets, and 10 WT plants: (B) the total length of the stem from base to apex; (C) stem diameter 5 cm above the root-collar; (D) internode distance calculated as the mean distance of all internodes in a plant; and (E) dry biomass estimation at 70 °C. All measurements were taken at 120 days. Asterisks denote significant difference ($P < 0.05$) from WT as determined by ANOVA followed by Dunnett's post-hoc test (this figure is available in colour at *JXB* online).

Table 2. Pt*PHOR1* suppression decreases biomass accumulation

Values are mean ± SE percentage change in biomass expressed as proportion of wild-type plants grown in the same experiment. *, $P < 0.05$ by t-test compared to wild-type.

Genotype	Stem	Leaf	Roots
35S::Pt*PHOR1_1*	104.23 ± 11.98	116.83 ± 18.2	142.38 ± 19.92*
35S::Pt*PHOR1_2*	145.01 ± 10.64*	146.92 ± 16.7	145.84 ± 10.15*
Pt*PHOR1*-RNAi	56.70 ± 5.49*	55.30 ± 5.51*	43.79 ± 5.98*
Wild-type	100.00 ± 11.8	100.00 ± 14.69	100.00 ± 11.29

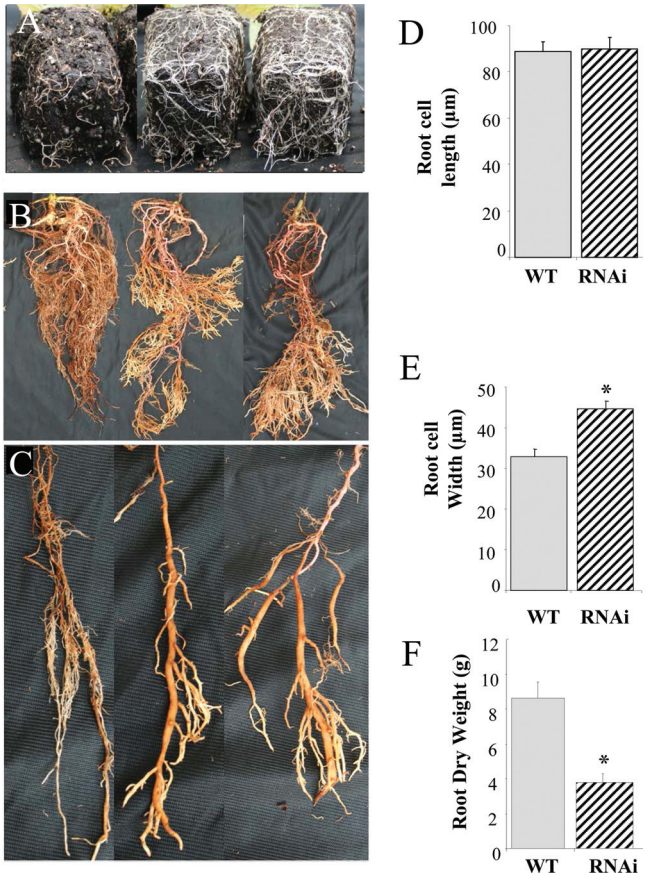

Fig. 6. Downregulation of Pt*PHOR1* changed root architecture. (A–C) Altered root morphology of Pt*PHOR1* RNAi transgenics after 45 days (A) and 90 days (B and C) in a greenhouse; representative wild-type (WT, left) and transgenic roots (middle and right). (D, E) Root cortex cell length (D) and width (E) measured 2 mm from the root tips on radial sections of two RNAi lines, with three ramets each, and three WT plants. (F) Root dry weight of two RNAi lines, each with at least four ramets and 10 WT plants. Bars represent event mean ± SE. Asterisks indicate significant difference ($P < 0.05$) from WT as determined by ANOVA followed by Dunnett's post-hoc test (this figure is available in colour at *JXB* online).

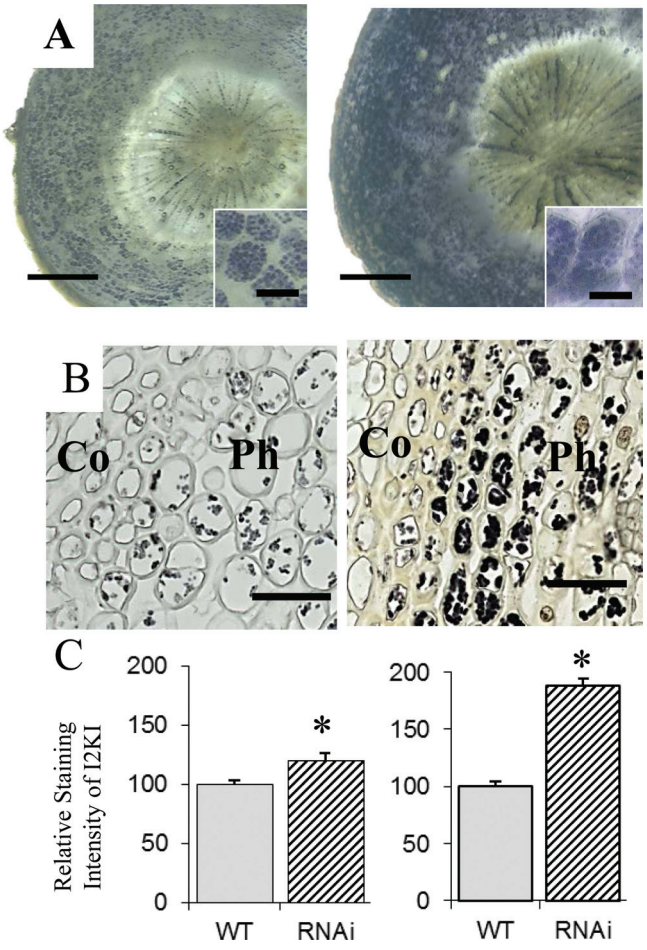

Fig. 7. Starch accumulation was increased in Pt*PHOR1* RNAi transgenics. (A, B) I_2KI stained root cross-sections approximately 6 cm from the root tips, inset showing magnification of cortex cells (A), and stem cross-sections (B) of phloem (Ph) and cortex (Co) cells at the 15th internode in wild-type (WT, left) and Pt*PHOR1*-RNAi transgenic (right) plants. (C) Intensity of I_2KI staining in cortex cells of root sections (left) and phloem cells of stem sections (right) quantified with ImageJ version 1.38. Asterisks indicate significant difference ($P < 0.05$) from WT by t-test. Bars: 0.5 mm (A), 50 μm (A insets), and 25 μm (B).

PHOR1 *and root development*

Several lines of evidence suggest that *PHOR1* genes may play a role in control of root development. First, Pt*PHOR1_1* was found to be more highly expressed in roots than in any other of the organs studied. Similarly, the potato *PHOR1* gene was also most highly expressed in roots. Second, overexpression of Pt*PHOR1_1* led to biomass changes in the roots, but not in leaves and stems. Unfortunately, there is no information on the impact of trangenically altering *PHOR1* expression on root growth in potato. Finally, the present study found dramatically reduced adventitious rooting in the transgenics overexpreressing both Pt*PHOR1_1* and Pt*PHOR1_2* (Fig. 3). The effect of these genes is specific to the initiation stage; once roots developed, they appeared to grow normally. In fact, they produced more root biomass than control plants. Although auxin did improve rooting, measurement of auxin concentrations suggested that its deficiency is not likely to be the cause of the adventitious rooting decline. However, it is also known that, despite the lack of gross changes in the amount of auxin, several genes encoding important regulators of auxin response were downregulated in the transgenic plants. This suggests decreased auxin sensitivity. Furthermore, previous work has shown that GA strongly affects auxin transport (Bjorklund *et al*., 2007; Gou *et al*., 2010; Mauriat *et al*., 2011). It is therefore possible that PHOR1, as part of GA signalling, may interfere with auxin transport and the capacity for the formation of localized auxin concentrations that are important for root initiation. Therefore, the possibility that *PHOR1* expression changes auxin sensitivity and/or transport cannot be discounted.

This study also found a significant impairment of normal gravitropic response in the roots of *PHOR1*-overexpressing plants. It

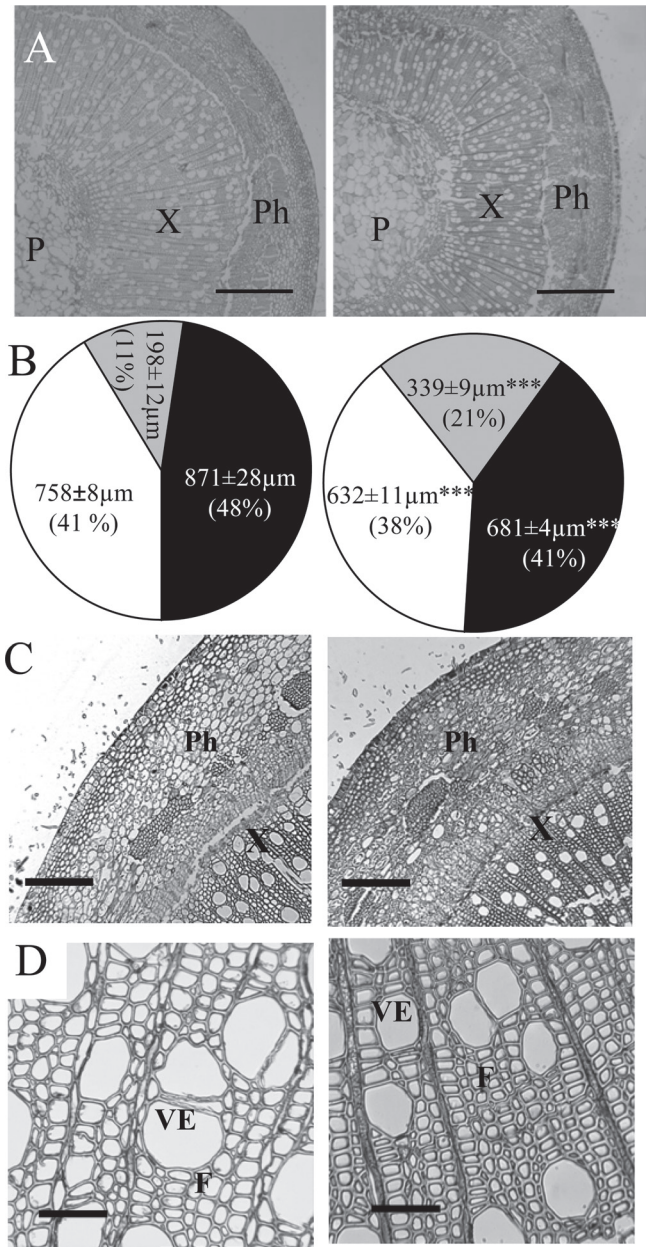

Fig. 8. Pt*PHOR1* suppression led to modified stem morphology. Transverse stem sections and measurements were taken from the 15th internode of Pt*PHOR1*-RNAi events 1 and 2, each with three ramets (right), and three wild-type plants (left). (A, C, D) Representative xylem and phloem (A), phloem (C), and xylem (D). (B) Mean ± SE radial cross-stem widths of phloem (grey), xylem (white), and pith (black); numbers in parentheses are relative percentages. Statistical significance was determined by t-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). F, fibre; P, pith; Ph, phloem; VE, vessel element; X, xylem. Bars, 500 µm (A), 200 µm (C) and 10 µm (D).

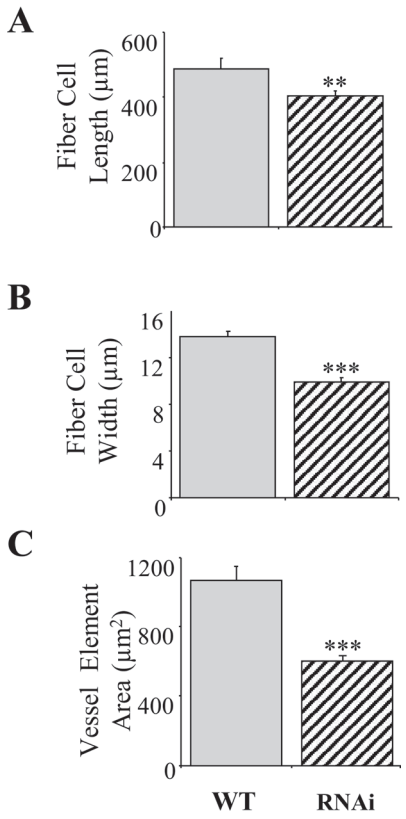

Fig. 9. Pt*PHOR1* suppression caused a decrease in fibre length. Measurements were taken on cells from the 15th stem internode of Pt*PHOR1*-RNAi events 1 and 2, each with three ramets, and three wild-type (WT) plants. Bars represent mean ± SE. Statistical significance was determined by t-test (**, $P < 0.01$; ***, $P < 0.001$).

is well documented that amyloplasts (starch-filled plastids) settle to the bottom of specialized columella cells known as statocytes to direct root tip growth in response to gravity (Chen *et al*., 1999; Boonsirichai *et al*., 2002). Although the present study did not measure amyloplast development in *PHOR1*-overexpressing lines, *PHOR1* suppression led to increased starch granule accumulation in the root tip. This suggests that *PHOR1* overexpression may have led to amyloplast deficiency and, hence, the agravitropic growth observed after initial transplanting to the greenhouse. An above-mentioned study of the function of the *EUI* gene (Zhang *et al*., 2008) supports this explanation. Plants

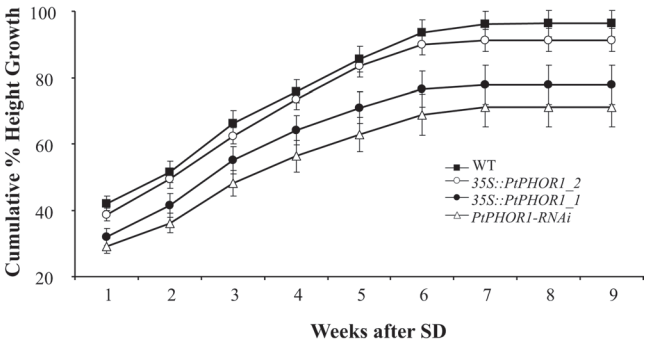

Fig. 10. Short-day (SD) dormancy-inducing conditions led to differences in cumulative percentage height growth. Values are mean ± SE of two transgenic events with five to seven ramets/event and seven wild-type (WT) plants. During weeks 1 and 2, plants were grown under long days (16/8 light/dark) and thereafter subjected to short days (8/16 light/dark).

with reduced *EUI* expression were defective in starch-granule development and gravitropic response, whereas *EUI* overexpression increased starch accumulation and gravitropic response.

PHOR1 *and dormancy*

Freezing and dehydration stress during winter months is one of the main challenges facing plants growing in the temperate zone. The adaptive strategies for winter survival are diverse, but one of the major routes for sensing the approaching winter is the perception of shorter photoperiods. In trees, short days elicit cessation of shoot elongation, bud formation, and bud dormancy (Olsen, 2010). Therefore, the responsiveness of *PHOR1* to short-day treatment, and its role in mediating growth response, suggests that it takes part in development of dormancy. However, the present analyses of transgenic *Populus* strongly suggest a positive effect of *PHOR1* on shoot growth when overexpressed; hence, its induction by short days, which precede cessation of shoot growth, is somewhat puzzling. Furthermore, modification of Pt*PHOR1* expression in transgenic plants had no effect on any aspect of above-ground dormancy induction and/or release (e.g., growth cessation, bud set, or flush). Finally, the expression peak of *PHOR1* occurs at around 3 weeks of short-day treatment (Fig. 2B), while the actual cessation of growth occurs approximately 3 weeks later, after nearly 6 weeks of short days (Fig. 10). Therefore, there is temporal separation of the highest expression of *PHOR1* and the shoot-growth response. Thus, based on the available evidence, it is believed that the induction of *PHOR1* by short-day treatment may serve other function(s) than helping to induce growth cessation and bud set. One possible explanation could be that *PHOR1* induction is associated with a mechanism for triggering foliar degradation of starch into simple sugars, which are then transported via the phloem to the roots where they are incorporated into starch reserves. Similar metabolic responses have been observed in *Populus* in preparation for dormancy (Nguyen *et al*., 1990). Accumulation of starch in roots plays a major role in preparation for winter, but this aspect of dormancy in trees is poorly understood.

PHOR1 *role in wood formation*

One of the most significant effects of Pt*PHOR1* downregulation on above-ground development was the reduction in diameter (woody) growth. Inspection of stem sections showed that the effect was caused by a significant reduction of xylem formation. The reduced size of xylem appeared to be primarily due to smaller fibres and vessels. Thus, the downregulation of Pt*PHOR1* likely reduces the GA response needed to allow normal expansion of xylem cells. Its effect appears to modify all cell types, as suggested by the similar effect on vessels and fibres. Thus, *PHOR1* action may be a major means through which GA effects on wood formation are manifested. In contrast to xylem, downregulation of *PHOR1* genes resulted in increased phloem production. There is very little information on the role of GA in phloem formation; however, it has been demonstrated that low IAA/GA levels stimulate phloem production (Wareing, 1958; Digby and Wareing, 1966). An increase in phloem to xylem ratio was also seen in transgenic *Populus* with aberrant auxin response (Nilsson *et al*., 2008). Thus, an altered GA response in Pt*PHOR1* RNAi plants may have had a misbalancing effect on auxin, which in turn caused a disproportionate increase in phloem growth. Cellular studies of auxin metabolism in *PHOR1*-modified plants are likely to be needed to test this hypothesis.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Overexpression of Pt*PHOR1_1* and Pt*PHOR1_2* in transgenic poplar.

Supplementary Fig. S2. Suppression of Pt*PHOR1_1* and Pt*PHOR1_2* in the leaves and roots of Pt*PHOR1*-RNAi transgenic plants.

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