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Research paper

Constitutive expression of the poplar WRKY transcription factor PtoWRKY60 enhances resistance to *Dothiorella gregaria* Sacc. in transgenic plants

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WRKY proteins are involved in various physiological processes in plants, especially in coping with diverse biotic and abiotic stresses. However, limited information is available on the roles of specific WRKY transcription factors in poplar defense. In this study, we reported the characterization of *PtoWRKY60*, a Group IIa WRKY member, from *Populus tomentosa* Carr. The gene expression profile of *PtoWRKY60* in various tissues showed that it significantly accumulated in old leaves. Phylogenetic analyses revealed that PtoWRKY60 had a close relationship with AtWRKY18, AtWRKY40 and AtWRKY60. *PtoWRKY60* was induced mainly by salicylic acid (SA) and slightly by *Dothiorella gregaria* Sacc., jasmonic acid, wounding treatment, low temperature and salinity stresses. Overexpression of *PtoWRKY60* in poplar resulted in increased resistance to *D. gregaria*. The defense-associated genes, such as *PR5.1*, *PR5.2*, *PR5.4*, *PR5.5* and *CPR5*, were markedly up-regulated in transgenic plants overexpressing *PtoWRKY60*. These results indicate that *PtoWRKY60* might be partly involved in the signal transduction pathway initiated by SA in *Populus*.

Keywords: pathogen, *Populus*, salicylic acid.

Introduction

Pathogen-induced hypersensitive response (HR) is often associated with activation of salicylic acid (SA)-regulated defense mechanisms in plants, leading to systemic acquired resistance (SAR) (Zheng et al. 2006). The role of SA in plant defense had been widely researched (Chaturvedi et al. 2008). Salicylic acid-mediated signaling is primarily involved in plant defense against pathogens that exhibit a biotrophic phase during their life cycle (Glazebrook 2005). By triggering the downstream signaling processes, SA has been found to activate a number of the defense-associated genes and establish SAR (Klessig et al. 2000, Maleck et al. 2000, Schenk et al. 2000). Compared with wild-type (WT) plants, the SA-associated *npr1* mutant could reduce the number of fungal conidiophores (Fabro et al. 2008). In *Arabidopsis pen3* mutant, which lacks an ABC transporter, SA-associated genes were hyper inducible (Stein et al. 2006). Finally, a detailed transcriptional profile of host responses towards *Golovinomyces orontii* infection defined a set of genes that require SA for inducing expression during later stages of fungal growth and reproduction (Chandran et al. 2009). Notably, SA plays a positive role in resistance against biotrophic and hemibiotrophic pathogens (Thaler et al. 2012).

Several classes of transcription factors, including DREB, bZIP, NAC and WRKY, have been reported to be associated with plant

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defense (Eulgem et al. 2000, Maleck et al. 2000, Dong et al. 2003, Ulker and Somssich 2004). These transcription factors have been shown to be able to interact with the promoter *cis*-elements of various plant defense-related genes and induce their expression to enhance resistance to biotic stresses (Agarwal et al. 2006). In *Arabidopsis*, a lot of *WRKY* genes were induced by both pathogen infection and SA treatment (Dong et al. 2003), leading to increasing attention for their roles in plant defense. A common feature of the WRKY proteins was the presence of one or two conserved DNA-binding domains (called the WRKY motif) followed by a unique zinc-finger pattern of Cys and His residues at the C-terminus of these proteins (Rushton et al. 1996). According to the number of WRKY domains and the pattern of the zinc finger motif, the WRKY superfamily proteins were divided into three major groups (Eulgem et al. 2000). Group I contained two $CX_4CX_{22-23}HXH$ zinc-finger motifs, Group II contained one domain with a $CX_{4-5}CX_{23}HXH$ zinc-finger motif and the unique domain of Group III members consisted of a $CX_7CX_{23}HXC$ zinc finger. Mutations in either the WRKYGQK motif or the zinc finger of WRKY domains will result in the loss of their DNA binding ability (Maeo et al. 2001). Most of the WRKYs seemed to interact specifically with a DNA motif termed W box (TTGACC/T). Most plant defense and defense-related genes, such as pathogenesis-related (*PR*) genes and the regulatory *NPR1* gene, contain W box sequences in their promoter regions (Yu et al. 2001). It has been demonstrated that specific recognition and binding of W box by WRKY proteins are necessary for inducing expression of these genes (Kalde et al. 2003, Turck et al. 2004). An increasing number of studies has shown that WRKY proteins play important roles in plant defense responses to pathogen infection. Pathogen infection and treatments with pathogen elicitors or SA have been shown to be able to induce rapidly expression of *WRKY* genes in a number of plants (Rushton et al. 1996, Chen and Chen 2000, Hara et al. 2000, Asai et al. 2002, Dong et al. 2003). For example, in *Arabidopsis*, AtWRKY46, AtWRKY53 and AtWRKY70 positively regulated basal resistance to *Pseudomonas syringae* (Hu et al. 2012). AtWRKY7 as a repressor was negatively involved in resistance to the bacterial pathogen *P. syringae* (Kim et al. 2006), while AtWRKY51 might have an additive function as a positive regulator of basal defense against *P. syringae* (Gao et al. 2011). Disruptions of the structurally related AtWRKY40 and AtWRKY60 were shown to enhance resistance to biotrophic pathogens *P. syringae* and *G. orontii* (Xu et al. 2006, Shen et al. 2007), indicating that they played overlapping and synergistic roles in plant basal defense. Likewise, AtWRKY11 and AtWRKY17 functioned as negative regulators of plant resistance against *P. syringae* (Journot-Catalino et al. 2006). In addition, AtWRKY25 and AtWRKY72 were also plant defense regulators in response to biotrophs *P. syringae* pv. *maculicola* strain ES4326 and *Hyaloperonospora arabidopsidis* (Zheng et al. 2007). Recently, Hwang et al. (2011) found that heterologous expression of *OsWRKY6* in *Arabidopsis* enhanced disease resistance to the biotrophic pathogen *Xanthomonas campestris*.

In *Arabidopsis* and rice, the functions of WRKY members in the SA signaling pathway have been well characterized (Hwang et al. 2011, Hu et al. 2012). However, there are only limited studies on the identification and functional characterization of *WRKY* genes in poplar. A recent study reported the molecular cloning and functional characterization of *PtWRKY23* in poplar (*Populus tremula* × *Populus alba*), which was induced rapidly by *Melampsora* infection and SA treatment (Levee et al. 2009). Either overexpression or silencing of this gene in poplar led to increased susceptibility to *Melampsora* infection compared with WT. However, the role of PtWRKY23 in the SA signaling pathway is still unknown.

In this study, we reported the isolation and characterization of *PtoWRKY60*, a Group IIa member of WRKY family, from *Populus tomentosa* Carr. Transcripts of *PtoWRKY60* mainly accumulated in old leaves and SA could induce a significant increase of its expression. Phylogenetic analysis showed that PtoWRKY60 had a close relationship with AtWRKY18, AtWRKY40 and AtWRKY60. Constitutive expression of *PtoWRKY60* in poplar resulted in increased disease resistance to *Dothiorella gregaria* Sacc. The SA-responsive genes, *PR5* and *CPR5*, were markedly up-regulated in *PtoWRKY60* overexpression lines. These results indicated that PtoWRKY60 might be involved in the SA-mediated defense pathway in *Populus*.

Materials and methods

Plant materials

Populus tomentosa (clone 741) was grown in the greenhouse at 25 °C under a 14-/10-h light/dark cycle with supplemental light (4500 lux). *Arabidopsis thaliana* (ecotype Columbia-0) was grown in an illumination incubator at 22–23 °C with a 16-/8-h light/dark cycle, ~80% relative humidity. This work was finished in Southwest University, which is located in Tiansheng Road, Beibei, Chongqing, China (29°40′N–29°61′N, 106°17′E–106°29′E).

Cloning of PtoWRKY60

The complementary DNA (cDNA) fragment encoding *PtoWRKY60* was amplified with gene-specific primers (*PtoWRKY60*-F: 5′-AACCATGGATGTCTTTCCCTC-3′; *PtoWRKY60*-R: 5′-TTCACAGCATTCTTGACGAAGA-3′) based on the gene sequence (POPTR_0018s02480) from the *Populus trichocarpa* genome by PCR. The PCR was carried out with pfu DNA polymerase (Takara, Dalian, China) in a total volume of 50 µl at 94 °C for 3 min; 32 cycles of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 90 s, followed by a final extension of 72 °C for 10 min. The PCR product was cloned into the plant binary vector pCXSN (Chen et al. 2009). The resulting vector, p*35S:PtoWRKY60*, with the *PtoWRKY60* open reading frame (ORF) driven by the cauliflower mosaic virus (CaMV) 35S promoter and the hygromycin phosphotransferase (*Hpt*) gene, which conferred resistance to hygromycin, were transferred into *Agrobacterium tumefaciens* strains EHA105 by the freeze–thaw method.

Sequence comparisons and phylogenetic analysis

Amino acid sequence alignments of five WRKY proteins were performed with Clustal X 1.81. The parameters of alignment were used as follows: gap penalty, 10.00 (both in pairwise alignment and multiple alignment); gap extension penalty, 0.20 (both in pairwise alignment and multiple alignment); protein weight matrix, gonnet; residue-specific penalties, ON; hydrophilic penalties, ON; gap separation distance, 0; end gap separation, ON; use negative matrix, OFF; and delay divergent cutoff (%), 30. A total of 17 WRKY proteins were used to construct phylogenic trees through the neighbor-joining (NJ) method using program MEGA4.1 (http://www.megasoftware.net/mega.html). The parameters of the constructed trees were: phylogeny test and options: bootstrap (1000 replicates; random seed = 9928); gaps/missing data: complete deletion; model: amino: Poisson correction; substitutions to include: all; pattern among lineages: same (homogeneous); and rates among sites: uniform rates.

Transformation of P. tomentosa plants

Agrobacterium tumefaciens strains EHA105 containing p*35:PtoWRKY60* were incubated in liquid yeast extract peptone medium supplemented 100 μmol l^{-1} acetonesyringone at 28 °C with constant shaking (200 rpm) until the culture reached an optical density of 0.8 at OD 600 nm. The *A. tumefaciens* culture was then diluted with one volume of liquid woody plant medium (WPM) (Lloyd and McCown 1980).

Poplar transformation methods were described previously by Jia et al. (2010). Leaves of Chinese white poplar (*P. tomentosa*) were excised from in vitro plantlets, cut into disks and dipped in the diluted *Agrobacterium* culture for 8–10 min. After excess liquid on the surface was absorbed by sterilized paper, the leaf disks were transferred to WPM (2.0 mg l^{-1} zeatin, 1.0 mg l^{-1} 1-naphthalene acetic acid [NAA]). The infected disks were co-cultivated in the dark for 2 days and then transferred to callus-inducing medium containing 2.0 mg l^{-1} zeatin, 1.0 mg l^{-1} NAA, 400 mg l^{-1} cefotaxime, 9.0 mg l^{-1} hygromycin and 0.8% (w/v) agar. After 2 to 3 weeks of culture without light, these leaf disks with induced calli were subcultured on screening medium (2.0 mg l^{-1} zeatin, 0.1 mg l^{-1} NAA, 400 mg l^{-1} cefotaxime, 9.0 mg l^{-1} hygromycin and 0.8% [w/v] agar) to induce adventitious buds. Regenerated shoots were transferred to the rooting medium, containing 0.1 mg l^{-1} NAA, 400 mg l^{-1} cefotaxime and 9.0 mg l^{-1} hygromycin. Transgenic plants were selected with 9.0 mg l^{-1} hygromycin. Rooted plantlets were acclimatized in pots placed inside a humid chamber (16 h photoperiod, 25 °C, 70% relative humidity) for 2 weeks and finally transferred to the greenhouse.

Molecular analysis of transgenic plants

Genomic DNA was extracted from poplar leaf material (300 mg) of untransformed control and transgenic plants using a CTAB method (Jia et al. 2010). Each PCR mixture (10 μl) contained 5.5 μl GoTaqGreen Master Mix (Promega, Beijing, China), 0.25 μl each primer, 0.5 μl cDNA and 3.5 μl nuclease-free water. The PCR analysis was carried out employing gene-specific primers: *Hpt* (F: 5′-ATCGGACGATTGCGTCGCATC-3′; R: 5′-GTGTCACGTTGCAAGACCTG-3′). PCR conditions were 94 °C for 3 min: 30 cycles of 94 °C for 45 s, 56 °C for 30 s, 72 °C for 1 min, followed by a final extension of 72 °C for 8 min. PCR products were resolved on a 1% (w/v) agarose gel and visualized after ethidium bromide staining.

Transformation of A. thaliana

The *GUS* reporter gene was employed to study the spatial and temporal expression pattern of *PtoWRKY60*. The 1.2-kb upstream sequence of *PtoWRKY60* was amplified by the primers: F: 5′-AAGAATGATCAGGGACGGTC-3′; R: 5′-GCAAGAGGGAAAGACATCCATG-3′. The PCR-amplified genomic DNA fragments were cloned into the plant binary vector pCX-GUS-P (Chen et al. 2009) to produce the vector p*PtoWRKY60:GUS*. The resulting construct was transformed into WT *Arabidopsis* plants by the floral dip method (Clough and Bent 1998). Transformants were selected on MS plates supplemented with 30 μg ml^{-1} of hygromycin. Six-week-old transgenic plants were examined for the expression of the *GUS* reporter gene (Zhong et al. 2005).

RNA extraction and RT–PCR analysis

Total RNA was extracted from plants using TRIzol Reagent (Invitrogen, Beijing, China) according to the manufacturer's instructions. For reverse transcription (RT)–PCR, 2.0 μl RNA was reverse transcribed in a total volume of 20 μl, using a PrimeScriptRT reagent Kit with gDNA Eraser (Takara) according to the manufacturer's instructions. To determine *PtoWRKY60* expression, PCR amplification was performed for 30 cycles, with each cycle consisting of 94 °C for 1 min, 58 °C for 30 s, respectively, 72 °C for 1 min and finally 8 min at 72 °C. The constitutively expressed *18S* gene was used to confirm equal amount of cDNA in each reaction.

Quantitative real-time PCR

Quantitative real-time PCR was performed on a Takara TP800 real-time PCR detection system. The specific primers for *PtoWRKY60* were qt-*PtoWRKY60*-F: 5′-GTGGAAGCTCTTCAA TTCGA-3′ and qt-*PtoWRKY60*-R: 5′-GCTCTTGTCATTGGAGT GAC-3′; and primers for *Populus 18S* gene were qt-*18S*-F: 5′-GGCATGGAAGGTGATGCAGATC-3′ and qt-*18S*-R: 5′-CTGTGTCAAACAAGAACTTGTCC-3′. Quantitative real-time PCR and data analysis were performed as described by Tsai et al. (2006) in a 25-μl reaction volume containing 12.5 μl of SYBR Premix ExTaq™ (Takara). Differences in gene expression, expressed as fold change relative to control, were calculated using the $[\Delta][\Delta]C_t = 2^{[\Delta]C_t,18S-[\Delta]C_t}$ method. Each measurement was carried out in triplicate, and the error bars represent SE of the mean of fold changes for the three biological

replicates. Analysis of defense-associated genes was similarly performed using gene-specific primers (Table S1 available as Supplementary Data at *Tree Physiology* Online).

Subcellular localization

The PCR-amplified cDNA of *PtoWRKY60* was ligated into pCX-DG (Chen et al. 2009) to generate the *GFP-PtoWRKY60* construct. The resulting vector was induced into onion epidermal cells by a Gene Gun (GJ-1000; SCIENTZ, Beijing, China). The onion skin was stained with 4′,6-diamidino-2-phenylindole (DAPI), and photographed under a light microscope (Olympus BX53, Tokyo, Japan).

Transactivation assay

The yeast single-hybridization system was used with full-length transcription factors in $pGBKT_7$ (Clontech) and introduced into the yeast strain *Saccharomyces cerevisiae* Gold2. Transformants were grown on SD medium lacking tryptophan (Trp) for positive clone selection and then on SD medium lacking Trp, histidine (His) and adenine (Ade) for the transactivation assay, according to the manufacturer's instructions.

Inoculations and treatments of Populus and transgenic Arabidopsis

For all treatments, 1-month-old transgenic *Arabidopsis* were used. After all the treatments, the leaves that were similar in size and age were chosen to GUS stain.

Fungal inoculation The inoculation of poplar leaves was performed with *D. gregaria* Sacc. Leaves of 3-month-old plants were selected for inoculation. Mycelial plugs (6 mm) were placed on excised leaves (three leaves for each plant) and the leaves incubated in Petri dishes with humid filter paper in a humid chamber for 3 days (Huang et al. 2012). Proportion of the lesion area to the total leaf area was computed by Adobe Photoshop software. Each experiment was performed with at least three replicates, and contained WT controls. All data were analyzed by *t*-test at $P \leq 0.05$, using the Origin 6.1 software version v6.1052 (B232) (OriginLab Corp., Northhampton, MA, USA). The leaves from 1-month-old transgenic *Arabidopsis* were inoculated with *D. gregaria* based on the same method as *Populus*.

Hormone treatments Salicylic acid and methyl jasmonate were treated as described by Li et al. (2004). The treated plants were immediately covered with a transparent lid. The leaves were collected 24 h after treatments.

Low temperature stress The healthy, 4-week-old *Arabidopsis* plants were transferred to a growth chamber at 4 °C under the same light and photoperiodic conditions for 1 h. After treatments, plants were recovered at 20 °C for 1 h.

Salinity stress The 4-week-old *Arabidopsis* plants were subjected to salt stress. Saline treatments had an NaCl concentration of 100 mM added to full-strength Hoagland's solution for 2 days. The method was as described previously (Yang et al. 2009).

Wounding stress For wounding treatment, the leaves of *Arabidopsis* plants were harvested after being punctured with sterile needles and placed at 20 °C for 2 h.

Statistical analysis

The Student's *t* test program (http://www.graphpad.com/quickcalcs/ttest1.cfm) was used for statistical analysis of the data in the experiments of quantitative RT-PCR. In all these experiments, it was found that the quantitative differences between the two groups of data for comparison were statistically significant ($P < 0.001$).

Accession numbers of WRKYs from different species

The accession numbers of the *WRKY* genes are: *AtWRKY18* (At4g31800), *AtWRKY40* (At1g80840), *AtWRKY60* (At2g25000), *AtWRKY46* (AT2G46400), *AtWRKY25* (At2g30250), *AtWRKY26* (At5g07100), *AtWRKY33* (At2g38470), *NaWRKY3* (AY456271), *NaWRKY6* (AY456272), *HvWRKY34* (DQ863118), *HvWRKY41* (DQ863124), *TcWRKY53* (EF053036), *GmWRKY13* (DQ322694), *OsWRKY08* (05g50610), *OsWRKY11* (01g43650), *OsWRKY72* (11g29870).

Results

Isolation and characterization of the PtoWRKY60 gene from P. tomentosa

We isolated the *PtoWRKY60* cDNA encoding a putative Group IIa protein of the WRKY family, by RT–PCR using gene-specific primers based on the sequences deposited in the *Populus* genome database. *PtoWRKY60* appeared to be a full-length cDNA of 819 bp encoding a protein of 272 amino acid residues. Homology searches against the NCBI database revealed that PtoWRKY60 with a typical WRKY domain was highly conserved with the *Arabidopsis* WRKY Group IIa members AtWRKY18 and AtWRKY40. Multiple sequence alignment of the deduced amino acid sequence of PtoWRKY60 and other WRKY proteins showed that they possessed the same type of potential zinc ligands ($CX_{4-5}CX_{23}HXH$) and contained potential leucine zippers (LZs), which was known to allow protein dimerization (Eulgem et al. 2000) (Figure 1a). A phylogenetic tree was constructed based on the amino acids of PtoWRKY60 and other known WRKYs from different species, such as rice (*Oryza sativa*), soybean (*Glycine max*), *Hordeum vulgare*, *Nicotiana attenuata*, *A. thaliana* and *Thlaspi caerulescens*. In the phylogenetic tree, PtoWRKY60 showed high similarity

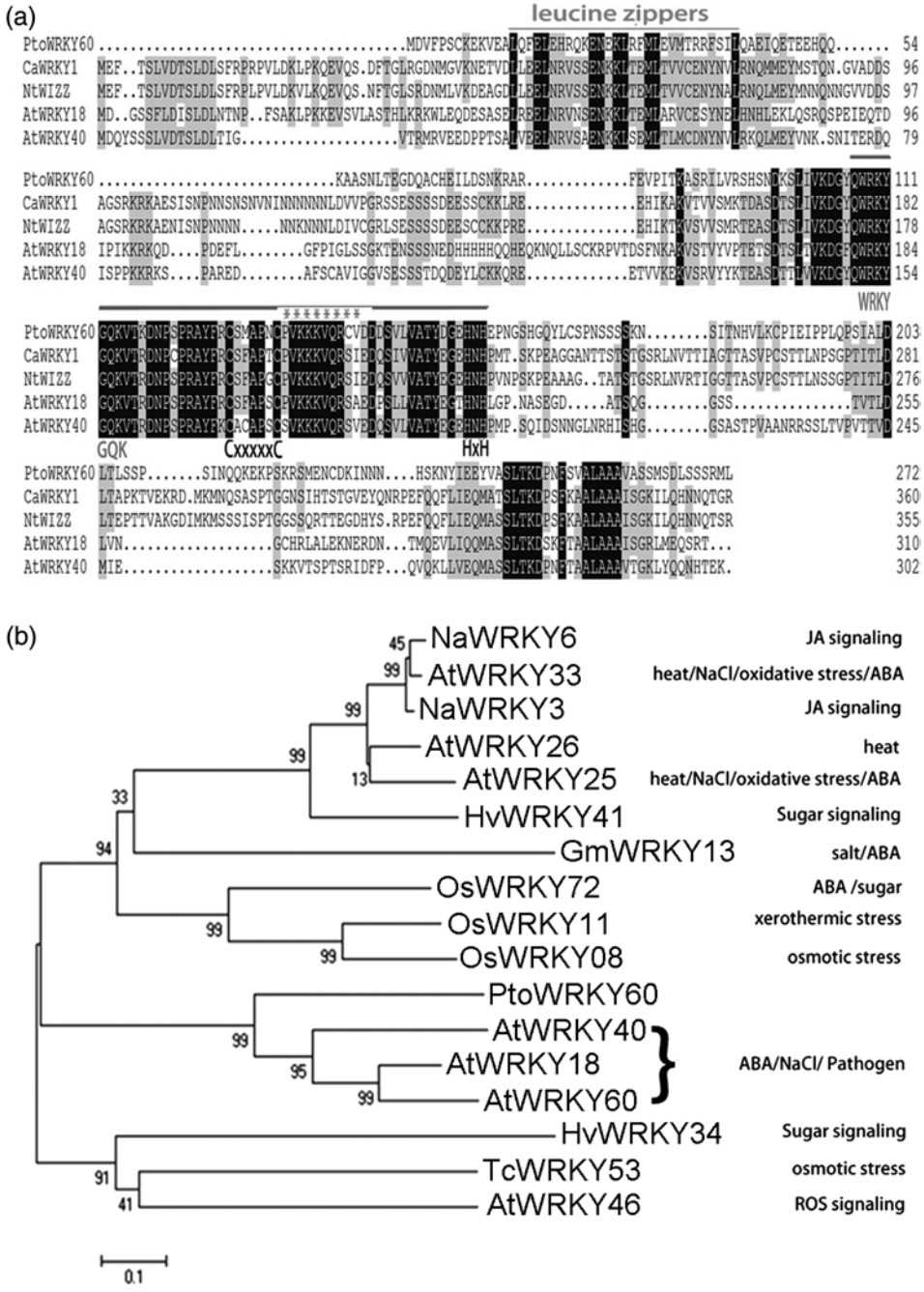

Figure 1. Comparison of PtoWRKY60 with other WRKY amino acid sequences. (a) Sequence alignment between PtoWRKY60 and the other WRKYs. Sequences are from *Capsicum annuum* (CaWRKY1), *Nicotiana attenuata* (NtWIZZ) and *A. thaliana* (AtWRKY18, AtWRKY40). Identical amino acids are indicated by white letters on a black background, conservative amino acids by black on a dark gray background. The subscript fonts indicate the leucine zippers, WRKY domain (WRKYGQK) and zinc fingers ($CX_5CX_{23}HXH$). Peptide sequences were aligned with the software of DNAMAN. (b) Phylogenetic relationships of WRKY proteins from *P. tomentosa* Carr. and selected species. Sequences are from *N. attenuata* (NaWRKY3, NaWRKY6), *A. thaliana* (AtWRKY18, AtWRKY25, AtWRKY26, AtWRKY33, AtWRKY40, AtWRKY46, AtWRKY60), *P. tomentosa* (PtoWRKY60), *G. max* (GmWRKY13), *O. sativa* (OsWRKY08, OsWRKY11, OsWRKY72), *H. vulgare* (HvWRKY34, HvWRKY41) and *T. caerulescens* (TcWRKY53). Phylogenetic analysis was performed by the NJ method using MEGA version 4. Bar, 0.1 substitutions per site.

in the WRKY domain with three pathogenesis-related WRKY proteins, AtWRKY18, AtWRKY40 and AtWRKY60, from *Arabidopsis* which were involved in SA-mediated defense (Figure 1b). In *Arabidopsis*, it has been extensively described that a complex interaction between AtWRKY18, AtWRKY40 and AtWRKY60 is required for the resistance to a variety of pathogens such as *Botrytis cinerea* (Chen and Chen 2002, Xu et al. 2006) and *G. orontii* (Shen et al. 2007, Wenke et al. 2012). Therefore, the phylogenetic analysis indicated that PtoWRKY60 might correlate to the SA

signaling networks which are involved in regulation of plant defense.

Spatio-temporal expression profiles of PtoWRKY60 in poplar

PtoWRKY60 expression in various organs of Chinese white poplar was analyzed by quantitative real-time PCR (Figure 2a). Under normal growth conditions, *PtoWRKY60* was detected in all tissues tested, but the highest accumulation was observed in the old leaves compared with other tissues.

To further determine the expression profiles of *PtoWRKY60*, a 1.2-kb upstream sequence of *PtoWRKY60* was amplified and constructed into the pCXGUS-P vector (Chen et al. 2009), and then transformed into the WT *Arabidopsis*. In 7-day-old seedlings, GUS expression was detected in cotyledons and stems (Figure 2b) but not in root tips. In 4-week-old *Arabidopsis*, the GUS activity was stronger in the old leaves than in the young ones (Figure 2d and e), which is consistent with the results of quantitative real-time PCR above. In addition, GUS expression was also found in the inflorescence (Figure 2c).

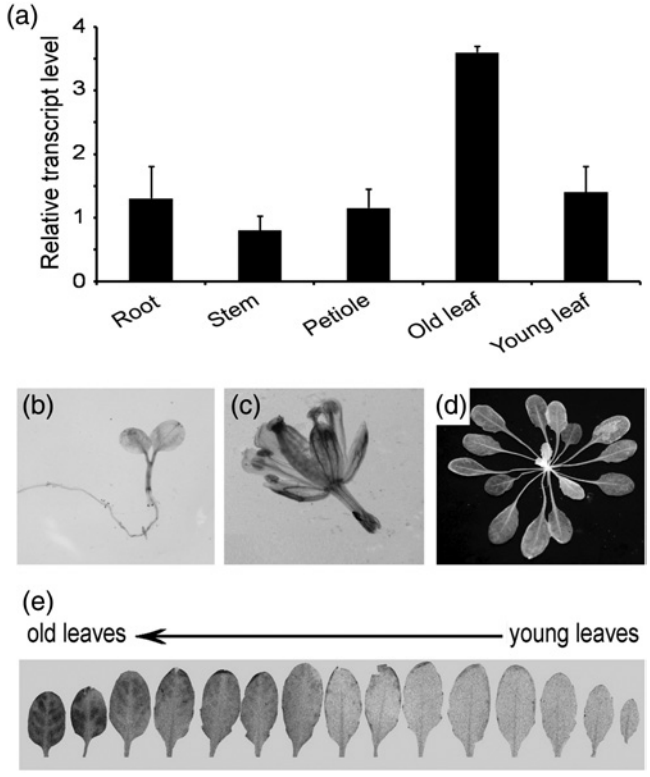

Figure 2. Expression profiles of *PtoWRKY60* in poplar. (a) Quantitative real-time PCR analysis of *PtoWRKY60* transcript levels in various tissues of *P. tomentosa* Carr. Poplar *18S* expression was used as a control. Total RNA was isolated from root, stem, petiole, old leaf (the sixth leaf from the apex) and young leaf (the first leaf). The *PtoWRKY60* gene promoter-driven GUS expression vector was engendered and introduced into *A. thaliana*. Gus staining pattern of transgenic plants: (b) 1-week-old seedling; (c) inflorescence; (d) 4-week-old seedling; (e) leaves detached from 4-week-old seedlings.

Expression patterns of PtoWRKY60 under different treatments

Expression patterns of *PtoWRKY60* were analyzed by semi-quantitative RT-PCR with various treatments. As shown in Figure 3a, *PtoWRKY60* was strongly induced by SA treatment and slightly by salinity stress, whereas *PtoWRKY60* expression was not changed under jasmonic acid (JA), fungal infection, wounding and cold stresses.

To determine the expression patterns of *PtoWRKY60* in response to biotic and abiotic treatments, the transgenic *Arabidopsis* plants containing *PtoWRKY60:GUS* were treated by SA, JA, low temperature, salinity and wounding, and inoculated with *D. gregaria*, respectively. The GUS activity could be clearly detected in leaves after various treatments (Figure 3b). Obviously, the strongest GUS activity was detected in leaves of transgenic plants after SA treatment.

Subcellular localization and transcriptional activity of PtoWRKY60

As shown in Figure 1a, there was a nuclear location signal 'PVKKKVQR' in the amino acid sequence of PtoWRKY60, suggesting that it might be a nucleus-localizing protein. To examine this reasoning, the ORF of *PtoWRKY60* was fused to 3′-terminus of green fluorescent protein (GFP) under the control of the constitutive cauliflower mosaic virus 35S promoter (CaMV 35S) and delivered into onion epidermal cells. The green fluorescence was exclusively observed in the nucleus of the onion epidermal cells when the recombinant construct was

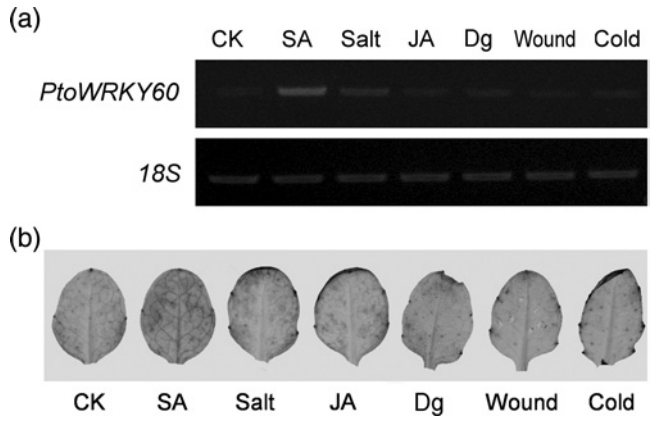

Figure 3. The expression patterns of *PtoWRKY60* under different treatments. (a) Semi-quantitative RT-PCR analysis of *PtoWRKY60* expression by treatments of salicylic acid (SA), salt, jasmonic acid (JA), *D. gregaria* Sacc. (Dg), wounding and cold. (b) The *PtoWRKY60* gene promoter-driven GUS expression vector was introduced into *A. thaliana*. The 4-week-old transgenic plants were treated by SA, salt, JA, Dg, wound and cold. CK represents transgenic plants without any treatment.

used, while when the p*35S* :: *GFP* vector was transformed as a control, green fluorescence was detected in the whole cell (Figure 4), indicating that PtoWRKY60 was specifically localized to the nucleus.

To identify the possible transcriptional activation function of PtoWRKY60, we fused it with the GAL4 DNA-binding domain for transactivation analysis in yeast. As shown in Figure 5, all transformants grew well on SD/Trp medium. However, only transformants containing GAL4BD + PtoMYB216 could grow on SD/Trp–/His–/Ade medium and these cells containing GAL4BD or GAL4BD + PtoWRKY60 could not. In a previous study, it has been demonstrated that PtoMYB216 functions as a transcriptional activator (Tian et al. 2013). The result above suggested that *PtoWRKY60* was not a transcriptional activator.

Overexpression of PtoWRKY60 enhanced resistance to D. gregaria in poplar

Dothiorella gregaria could cause poplar canker that decreased the wood quality and production of timber forest, whereby the economy would suffer a heavy loss (Huang and Su 2003). To investigate whether *PtoWRKY60* is involved in the regulation of pathogen resistance, a plant binary vector containing the *PtoWRKY60* ORF under the control of the CaMV 35S promoter was introduced into Chinese white poplar by *A. tumefaciens*-mediated transformation. A total of 10 hygromycin-resistant putative transformants were obtained and grown in the greenhouse. The generated transgenic plants did not show phenotypic change compared with the WT (Figure S1 available as Supplementary Data at *Tree Physiology* Online). The PCR analysis using gene-specific primers was employed to confirm the presence of the transgenes in transformed plants. An expected amplification product for the *HPT* gene was obtained from all transgenic lines tested, whereas no signal was detected from untransformed plants (Figure S2 available as Supplementary Data at *Tree Physiology* Online), indicating the successful integration of the transgene into the poplar genome. From all of the independent hygromycin-resistant transgenic lines harboring the *35S:PtoWRKY60* construct, three lines (3, 7 and 10) with high *PtoWRKY60* transcript levels were selected for further analysis (Figure 6a).

To determine the effect of *PtoWRKY60* overexpression on disease resistance in poplar, leaves excised from transgenic and control plants were inoculated with agar plugs containing hyphae of *D. gregaria*, a hemibiotrophic fungus. Compared with the severe disease symptoms that appeared on the control leaves at 3 days post inoculation, only slight necrotic lesions existed on the leaves of the transgenic *35S:PtoWRKY60* lines (Figure 6b). Quantification assays showed that lesions of *35S:PtoWRKY60* lines were significantly ($P < 0.05$) smaller than those of control plants (Figure 6c). The lesion area of WT plants was close to 30%. However, it was <15% in *PtoWRKY60* transgenic plants (L3, 11.63%; L7, 14.52%; L10, 14.36%). These results indicated that overexpression of *PtoWRKY60* resulted in an elevation of the basal resistance to infection by hemibiotrophic fungal pathogens in the transgenic poplar.

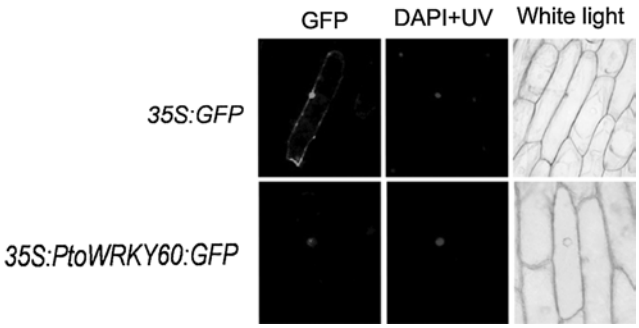

Figure 4. The *35S:PtoWRKY60:GFP* was transformed into onion epidermal cells by gene gun particle bombardment. The position of nucleus was ensured by DAPI staining and bright-field images were compared. In this experiment, *35S:GFP* served as control.

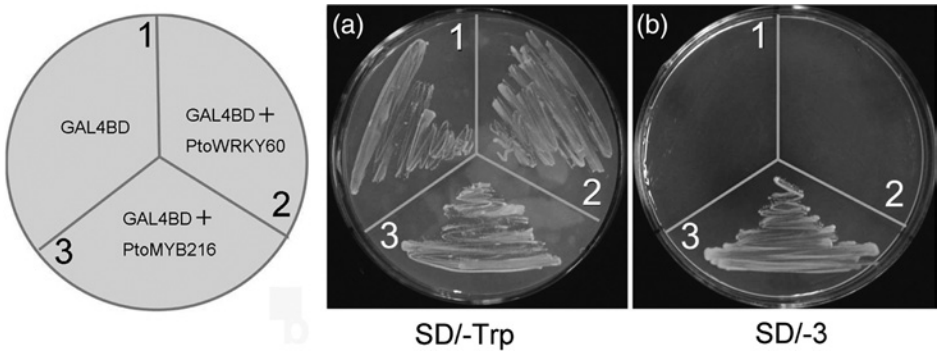

Figure 5. Transactivation assay of *PtoWRKY60*. Transformants were grown on SD medium lacking tryptophan (Trp) for positive clone selection (a) and then on SD medium lacking tryptophan (Trp), histidine (His) and adenine (Ade) for the transactivation assay (b), according to the manufacturer's instructions. (1) GAL4BD is a negative control. (2) GAL4BD + *PtoWRKY60*. (3) GAL4BD + *PtoMYB216* represents a positive control.

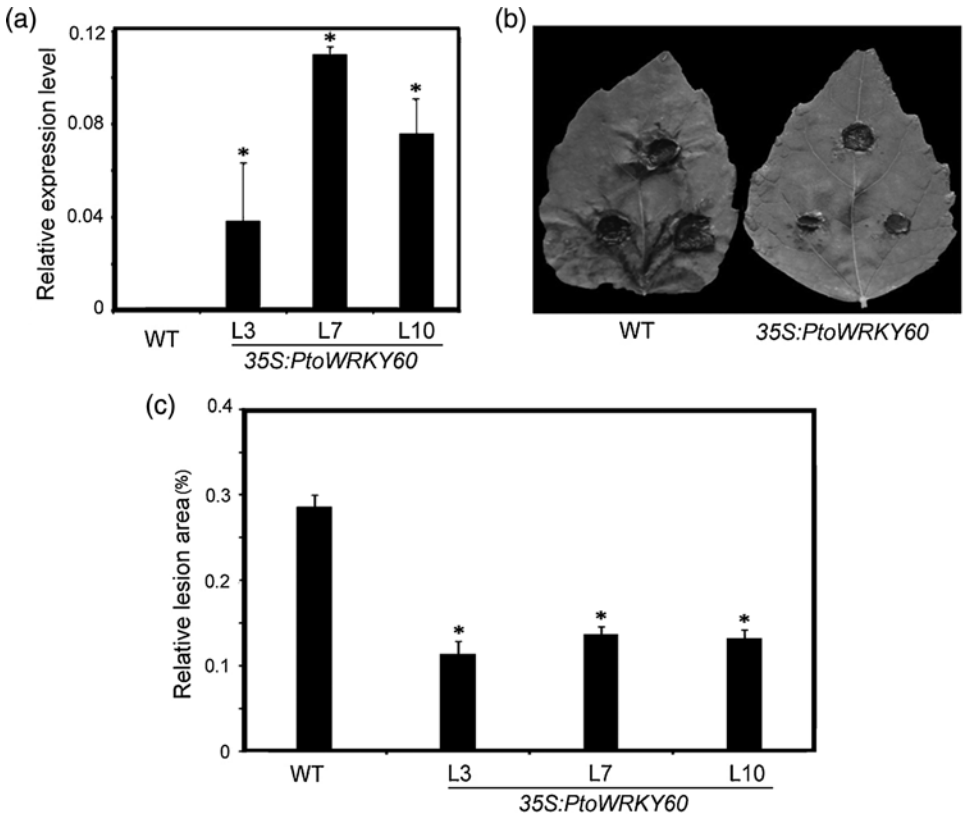

Figure 6. Resistance of transgenic poplar plants inoculated with *D. gregaria*. (a) Quantitative real-time PCR analysis of *PtoWRKY60* transcript levels in leaves of transgenic poplar plants. (b) The leaves from WT and transgenic plants infected with *D. gregaria* Sacc. were photographed 3 days after inoculation. (c) Lesion area of transgenic lines to the fungal pathogen. Values are means of three replications. Error bars indicate standard deviation. Lines 3, 7 and 10 were transgenic plants with high expression levels of *PtoWRKY60*. Asterisks indicate a statistically significant difference between WT and transgenic plants ($P < 0.05$ by Student's *t*-test).

PtoWRKY60 regulated the expression of plant disease resistance genes

Increased disease resistance in plants is usually accompanied by the enhanced expression of *PR* genes associated with the SA-mediated defense pathway (Uknes et al. 1992, Dong 2004, Loake and Grant 2007). Because expression of *PtoWRKY60* was triggered by exogenous SA and overexpression of *PtoWRKY60* in poplar enhanced the resistance to pathogens, we further determined whether *PtoWRKY60* was involved in controlling the expression of *Populus PR* genes. Using the genome sequence of *P. trichocarpa*, we identified candidate *Populus* orthologs of *PR* genes, including *PR1.1*, *PR2.1* and *PR5.1*, based on a co-phylogenetic approach. Semi-quantitative RT-PCR analysis showed that *PR5.1* expression was activated in *PtoWRKY60*-overexpressing lines (Figure 7a). However, the transcript levels of other *PR* genes were not changed in *PtoWRKY60* overexpressors. In *Arabidopsis*, non-expressor of *PR1* (*NPR1*) was a key regulator of the SA signal pathway. There was no visible difference in the expression of *NPR1* gene between the WT and *PtoWRKY60*-overexpressing lines (Figure 7a). In *Arabidopsis*, the constitutive expression of *PR* genes (*CPR5*) was known to be a defense mechanism regulator (Bowling et al. 1997, Yoshida et al. 2002, Orjuela et al. 2013). We tested the expression level of *CPR5* gene and found that the transcript level of *CPR5* was obviously increased in transgenic *35S:PtoWRKY60* lines. In addition, JASMONATE ZIM-domain (JAZ) proteins acted as repressors of JA signaling (Chung and Howe 2009). We determined the expression levels of two *Populus JAZ* genes, *JAZ8* and *JAZ10*, in *PtoWRKY60* overexpressed plants. But no obvious difference could be detected between all transgenic and WT plants. Overall, these results indicated that *PtoWRKY60* might be a regulator of SA-mediated defense marker genes in poplar.

Constitutive expression of PtoWRKY60 activated the expression of Populus PR5 genes

In the previous experiments, we had demonstrated that overexpression of *PtoWRKY60* resulted in increased pathogen resistance and elevated expression of *PR5.1*. We further searched against the *Populus* genome database and found several candidate genes with high similarity to *Arabidopsis PR* genes. The expressions of all the paralogs of *PR*s were detected by quantitative real-time PCR in WT and transgenic plants. However, no change was found in expressions of all *PR1s* (*PR1.1* and *PR1.2*)

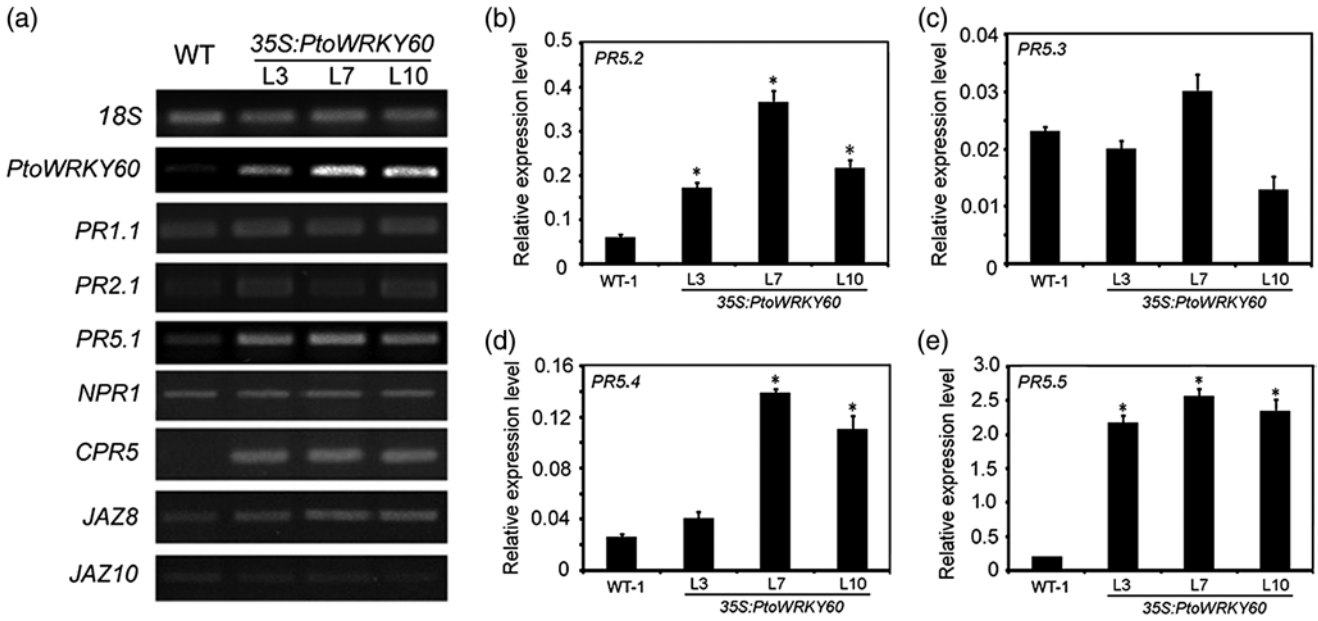

Figure 7. Gene expression analysis of SA-responsive marker genes in transgenic plants overexpressing *PtoWRKY60*. (a) Transcript levels of *PR*s, *NPR1*, *CPR5*, *JAZ8* and *JAZ10* in WT and transgenic plants were tested by semi-quantitative RT-PCR. Poplar *18S* expression was used as a control. (b) Expression level of *PR5.2*. (c) Expression level of *PR5.3*. (d) Expression level of *PR5.4*. (e) Expression level of *PR5.5*. Asterisks indicate significant differences using Student's *t*-test ($P < 0.05$).

and *PR2s* (*PR2.1*, *PR2.2*, *PR2.3*, *PR2.4* and *PR2.5*) between the WT and transgenic *35S:PtoWRKY60* plants (data not shown). Interestingly, the expressions of *PR5.1*, *PR5.2*, *PR5.4* and *PR5.5* but not for *PR5.3* were significantly up-regulated in transgenic lines overexpressing *PtoWRKY60* (Figure 7b–e). The results suggested that *PtoWRKY60* might confer enhanced disease resistance by regulating the expression of *Populus PR5* genes.

Discussion

The plant defense mechanisms against pathogens have been well studied in various plants. Many defensive genes and signal transduction pathways are activated when plants perceive invading pathogens. Plants often employ distinct recognition mechanisms and signaling pathways for different pathogen elicitors. These responses invite the accumulation of various endogenous signal molecules, such as SA, JA and ethylene (ET), which form a network of synergistic and antagonistic interactions to modulate plant defense (Glazebrook 2001, Kunkel and Brooks 2002, Spoel et al. 2003, Robert-Seilaniantz et al. 2011). Previous studies demonstrated that WRKY transcription factors participate in plant responses to various biotic and abiotic stresses such as pathogen invasion, freezing, drought and nutrient deficiency (Eulgem et al. 2000, Pandey and Somssich 2009, Zhou et al. 2011), and in several developmental and physiological processes (Rushton et al. 2010). Here, we isolated a poplar WRKY transcription factor PtoWRKY60, which is a Group IIa member (Figure 1a). A phylogenetic analysis of 16 plant WRKY transcription factors associated with different functions revealed that PtoWRKY60 was clustered into the clade with *Arabidopsis* AtWRKY18, AtWRKY40 and AtWRKY60 (Figure 1b). Recently, it had been demonstrated that AtWRKY18, AtWRKY40 and AtWRKY60 negatively regulate the basal defense response to *P. syringae* (Xu et al. 2006). We found that *PtoWRKY60* expression was induced by SA and the poplar fungal pathogen *M. brunnea* (Figure 3). Constitutive overexpression of *PtoWRKY60* led to enhanced resistance to a virulent pathogen (Figure 6). These results suggest that pathogen- and SA-induced PtoWRKY60 plays an important role in plant defense against these fungal pathogens.

Salicylic acid is produced by plants in response to challenge by a diverse range of phytopathogens and is essential to the establishment of both local and SAR (Loake and Grant 2007). It plays an important role in defense signaling but distinct from that mediated by JA (Feys and Parker 2000, Durrant and Dong 2004). Salicylic acid accumulation can elevate the expression of defense-related genes including the *PR* genes, *PR1*, *PR2* and *PR5*, resulting in enhanced disease resistance against pathogens (Gaffney et al. 1993, Delaney et al. 1994). Therefore, a few of the SAR marker genes likely to be involved in pathogen defense are constitutively expressed in the overexpression plants and might account for the enhanced resistance phenotypes (Clarke et al. 2000, Petersen 2000). For example, overexpression of *Arabidopsis WRKY70* resulted in constitutive expression of SA-regulated *PR* genes and enhancing resistance to *P. syringae* (Li et al. 2004). In our study, interestingly, constitutive expression of *PtoWRKY60* only up-regulated

expression of *PR5s* but did not affect accumulation of *PR1* and *PR2* mRNAs (Figure 7a). Further analysis by quantitative real-time PCR demonstrated that *PR5.1*, *PR5.2*, *PR5.4* and *PR5.5* but not *PR5.3* were highly expressed in *PtoWRKY60* overexpressed lines (Figure 7b–e). Similar results have been reported in previous studies (Rogers and Ausubel 1997, Nawrath and Métraux 1999, Clarke et al. 2000, Li et al. 2004). These results revealed that PtoWRKY60 could enhance the resistance to *D. gregaria* via activating expression of *PR5s* in *Populus*.

Many WRKY proteins act as positive transcriptional regulators (Eulgem et al. 2000, Hara et al. 2000) and the W box (TTGACC/T) elements in the promoters of several defense-regulated genes, including *PR1*, *PR2*, *PR5* and *NPR1*, are necessary for their induction by WRKY proteins (Rushton et al. 1996, Yang et al. 1999, Yu et al. 2001, Robatzek and Somssich 2002). It has also been reported that the W box could act as a negative regulatory element in the promoter of *Arabidopsis WRKY18* gene, which is induced by pathogen and SA (Chen and Chen 2002). In addition, a number of transcription factors have been identified as transcriptional repressors due to a conserved repression domain containing the LxLxL amino acid motif (Ohta et al. 2001, Hiratsu et al. 2002, Tiwari et al. 2004). In this study, overexpression of *PtoWRKY60* enhanced resistance to fungal pathogens by increasing defense-regulated gene expression. However, the result of yeast one-hybrid assays showed that PtoWRKY60 was not a transcriptional activator in vivo (Figure 5). Moreover, no LxLxL motif existed in the PtoWRKY60 sequence (Figure 1). This limited evidence indicated that PtoWRKY60 might be a transcriptional repressor. Therefore, it is implicated that expression of *PR5s* is indirectly up-regulated by *PtoWRKY60* in poplar.

Salicylic acid-induced defense responses are mediated by an ankyrin repeat protein NPR1 (Cao et al. 1997, Spoel et al. 2003). But there also exists an NPR1-independent pathway in plants (Bowling et al. 1997, Shah et al. 1999, Clarke et al. 2000, Li et al. 2004). For example, constitutive expression of *PR* genes in *cpr6* and *ssi2* was not found to be compromised by the *npr1-1* mutation (Clarke et al. 1998, Shah et al. 2001). In transgenic *35S:PtoWRKY60* plants, the expression level of *NPR1* was not changed compared with the WT control (Figure 7a). Together, these results indicate that PtoWRKY60 may be partially involved in the *NPR1*-independent SA signaling pathway.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

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