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Research paper

Isolation and characterization of a subgroup IIa WRKY transcription factor PtrWRKY40 from *Populus trichocarpa*

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Salicylic acid (SA) is a defense-related key signaling molecule involved in plant immunity. In this study, a subgroup IIa WRKY gene *PtrWRKY40* was isolated from *Populus trichocarpa*, which displayed amino acid sequence similar to *Arabidopsis AtWRKY40*, *AtWRKY18* and *AtWRKY60*. *PtrWRKY40* transcripts accumulated significantly in response to SA, methyl jasmonate and hemibiotrophic fungus *Dothiorella gregaria* Sacc. Overexpression of *PtrWRKY40* in transgenic poplar conferred higher susceptibility to *D. gregaria* infection. This susceptibility was coupled with reduced expression of SA-associated genes (*PR1.1*, *PR2.1*, *PR5.9*, *CPR5* and *SID2*) and jasmonic acid (JA)-related gene *JAZ8*. Decreased accumulation of endogenous SA was observed in transgenic lines overexpressing *PtrWRKY40* when compared with wild-type plants. However, constitutive expression of *PtrWRKY40* in *Arabidopsis thaliana* displayed resistance to necrotrophic fungus *Botrytis cinerea*, and the expression of JA-defense-related genes such as *PDF1.2*, *VSP2* and *PR3* was remarkably increased in transgenic plants upon infection with fugal pathogens. Together, our findings indicate that *PtrWRKY40* plays a negative role in resistance to hemibiotrophic fungi in poplar but functions as a positive regulator of resistance toward the necrotrophic fungi in *Arabidopsis*.

Keywords: jasmonate, pathogen resistance, *Populus*, salicylic acid.

Introduction

Plant innate immunity consists of two interconnected branches termed pathogen-associated molecular pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). Pattern-triggered immunity is initiated by recognition of common features of microbial pathogens by plant cell-surface receptors, while ETI is acquired by resistant (R) proteins that either directly or indirectly recognize pathogen effector proteins (Chisholm et al. 2006, Jones and Dangl 2006). The activation of PTI and ETI enhances plant disease resistance and restricts pathogen growth. Induction of PTI and ETI also results in activation of diverse plant hormonal signaling pathways, such as ethylene (ET), salicylic acid (SA) and jasmonic acid (JA) pathways (Pieterse et al. 2012). In plants, SA is required for the establishment of systemic-acquired resistance (SAR) against biotrophic and hemibiotrophic pathogens (Durrant and Dong 2004, Vlot et al. 2009, Thaler et al. 2012). Two SA induction-deficient (*sid*) mutants *sid1* and *sid2* did not accumulate SA after pathogen inoculation and displayed sensitivity to both virulent and avirulent forms of *Pseudomonas syringae* and *Peronospora parasitica* (Nawrath and Metraux 1999). Jasmonic acid is another vital plant hormone in defense against pests and wounding. Jasmonic acid derivatives also act as conserved elicitors of secondary metabolite production in plants and affect a variety of processes, including fruit ripening, production of viable pollen, root growth, tendril coiling, response to wounding and abiotic stress (Creelman and Mullet 1997, Wasternack 2014).

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Furthermore, it has been demonstrated that the SA and JA signaling pathways are often mutually antagonistic and provide capability to control resistance against various pathogens with different infection strategies in plants (Kunkel and Brooks 2002, Glazebrook 2005).

Both SA- and JA-related pathways are regulated by defense-related transcription factors (TFs) (Spoel and Loake 2011). WRKY TFs, as one of the largest family in plants, play a major role in transcriptional reprograming during a variety of immune responses (Eulgem 2005). The defining feature of WRKY TFs is the highly conserved 60 amino acids WRKY domain containing a heptapeptide WRKYGQK signature at the N-terminus and a novel zinc finger motif at the C-terminus, both of which are required for the high binding affinity of WRKY proteins to the consensus *cis*-elements termed W box (TTGACT/C) that are present in the promoters of the target genes such as pathogenesis-related (*PR*) genes (Eulgem et al. 2000). WRKY proteins comprise three main groups (I, II and III), and II group is further divided into IIa, IIb, IIc, IId and IIe subgroups depending on the number of WRKY domains and the structure of zinc finger motif (Rushton et al. 2010).

Many WRKY TFs from various species have been described as positive or negative regulators in plant defense against different types of pathogens at multiple levels. First of all, some WRKY proteins are associated with the synthesis of defense-related phytohormones. In *Arabidopsis*, *wrky54*/*wrky70* double mutant shows an impressively enhanced level of SA and SA-glucoside (Li et al. 2013). WRKY28 and WRKY46 are transcriptional activators of isochorismate synthase gene (*ICS1*) and AVR_{PPHB}*SUSCEPTIBLE 3* (*PBS3*), whose mutants accumulate drastically reduced levels of SA and SA-glucoside, respectively (van Verk et al. 2011). WRKY11 and WRKY17 are demonstrated to act upstream of JA by positively regulating the *P. syringae*-stimulated accumulation of JAs via increasing the expression of *LOX2* and *AOS*, encoding key enzymes in the JA biosynthesis pathway (Bell et al. 1995, Laudert et al. 1996, Journot-Catalino et al. 2006). Salicylic acid-induced nuclear translocation of the TF NONEXPRESSOR OF PR1 (NPR1), which is a key central regulator of the SA signaling pathway and activates many genes required for disease resistance, can be regulated by WRKY proteins (Kinkema et al. 2000, Yu et al. 2001). In addition, WRKY TFs integrate signals from some mutually antagonistic pathways. For instance, AtWRKY70 acts as an activator of SA-induced genes and a repressor of JA-responsive genes (Li et al. 2004). AtWRKY33 regulates the antagonistic interaction between defense pathways mediating responses to necrotrophic pathogens and *P. syringae* (Zheng et al. 2006). Most of the WRKY proteins can bind to the conserved W box (C/T)TGAC(T/C) in the promoters of target genes. WRKY proteins may directly modulate the expression of defense genes, such as *PR* genes, because W boxes are found on their promoters (Eulgem et al. 2000). For example, *PcPR1-1* was shown in vivo to be indeed a target of PcWRKY1 in parsley cells (Ülker and Somssich 2004). Interestingly, plenty of W boxes are also predicted in the promoters of some *WRKY* genes (Jiang et al. 2014). In *Arabidopsis*, *AtWRKY18* was proved to be regulated by itself (Chen and Chen 2002), indicating that it is either autoregulated by itself or cross regulated by other WRKY proteins. Finally, the interactions between WRKY proteins and between WRKY and other TF proteins indicate the existence of complicated transcriptional networks in the regulation of various biological processes. In *Arabidopsis*, AtWRKY18, AtWRKY40 and AtWRKY60 formed homologous or heterologous dimers to change the resistance to pathogens (Xu et al. 2006). To date, it has been demonstrated that some WRKY TFs directly and indirectly regulate plant defense pathways; however, little is known about potential roles of WRKY TFs in regulating host defense in plants.

Poplar (*Populus* spp.) is an important economic and ecologic species, but many hybrid poplars are susceptible to many bacterial and fungal pathogens, which severely affect wood productivity (Huang and Su 2003). The *Populus* genome contains at least 100 *WRKY* genes (Jiang et al. 2014). Increasing evidence has confirmed important roles of WRKY TFs in poplar defense processes, and several poplar *WRKY* genes have been identified to be involved in defense response. Overexpression and underexpression of *PtWRKY23* in poplar (*Populus tremula* × *Populus alba*) were shown to make trees susceptible to *Melampsora* infection and disrupted the redox homeostasis (Levée et al. 2009). The constitutive expression of the poplar WRKY TFs PtoWRKY60 and PtrWRKY89 has been shown to enhance resistance to *Dothiorella gregaria* in transgenic poplar plants (Jiang et al. 2014, Ye et al. 2014). More recently, overexpression of PtrWRKY73, a SA-inducible poplar WRKY TF, increased resistance to a virulent strain of the bacterial pathogen *P. syringae* (*Pst*DC3000) and enhanced sensitivity to the necrotrophic fungal pathogen *Botrytis cinerea* in *Arabidopsis thaliana* (Duan et al. 2015). The diverse roles of poplar WRKY proteins may reflect the complex signaling and transcriptional networks of plant defense that require tight regulation and fine-tuning. Therefore, functional characterization of more *WRKY* genes in *Populus*, especially their roles in plant immune responses, needs to be investigated.

In this study, we isolated and characterized a subgroup IIa *WRKY* gene, *PtrWRKY40*, from *Populus trichocarpa*. Phylogenetic analysis showed that PtrWRKY40 is similar to *Arabidopsis* AtWRKY40, AtWRKY18 and AtWRKY60. *PtrWRKY40* was induced in response to treatments of SA, methyl jasmonate (MeJA) and infection of *D. gregaria* in poplar. Constitutive expression of *PtrWRKY40* in poplar enhanced sensitivity to the hemibiotrophic fungus *D. gregaria*, coupled with downregulated transcript levels of SA-associated genes (*PR1.1*, *PR2.1*, *PR5.9*, *CPR5* and *SID2*) and the JA-related gene (*JAZ8*) in transgenic lines when compared with wild-type (WT) plants. However, transgenic *Arabidopsis* plants overexpressing *PtrWRKY40* displayed increased

resistance to necrotrophic fungus *B. cinerea*, and expression of JA-related genes such as *PDF1.2*, *VSP2* and *PR3* was activated. Thus, we provide evidence that PtrWRKY40 functions as a negative regulator of resistance to hemibiotrophic fungi by suppressing expression of *PR* genes but enhances the resistance to the necrotrophic fungi in plants.

Materials and methods

Plant materials

Populus tomentosa (clone 741) and *P. trichocarpa* were grown in the greenhouse at 25 °C under a 14-h/10-h light/dark cycle with supplemental light (4500 lux). *Arabidopsis thaliana* (ecotype Columbia-0) was grown in an illumination incubator at 22 and 23 °C with a 16-h/8-h light/dark cycle and 80% relative humidity.

Cloning of PtrWRKY40 gene

PtrWRKY40 gene was amplified from the total RNA extracted from the leaves of *P. trichocarpa* by reverse transcription polymerase chain reaction (RT-PCR) using gene-specific primers (PtrWRKY40-F: 5′-TAGCTAGCTGCAGCTGCAGC-3′; PtrWRKY40-R: 5′-TCACCACTTAGTGTGATTTTGC-3′). The primers were designed according to the annotated sequence (POPTR_0003s18060.1) from the *P. trichocarpa* genome. The PCR was carried out with PrimeSTAR HS polymerase (Takara, Dalian, China) in a total volume of 25 μl at 94 °C for 3 min; 36 cycles of 98 °C for 2 min, 56 °C for 30 s and 72 °C for 2 min, followed by a final extension of 72 °C for 10 min. The PCR products were cloned into the plant binary vector pCXSN (Chen et al. 2009) and sequenced for verification. The resulting vector, p*35S:PtrWRKY40*, with the *PtrWRKY40* open reading frame (ORF) driven by the cauliflower mosaic virus (CaMV) 35S promoter and the hygromycin phosphotransferase (*Hpt*) gene, which conferred resistance to hygromycin, was transformed into *Escherichia coli* strain DH5α and then introduced into *Agrobacterium tumefaciens* strain EHA105 by the freeze–thaw method.

Sequence comparisons and phylogenetic analysis

Amino acid sequence alignments of four WRKY proteins were performed with DNAMAN version 7. A total of 13 WRKY proteins were used to construct a phylogenetic tree by neighbor-joining (NJ) method using program MEGA5.10 (Tamura et al. 2011).

Transformation of P. tomentosa plants

Agrobacterium tumefaciens strain EHA105 containing p*35S:PtrWRKY40* were incubated in liquid yeast extract peptone (YEP) medium supplemented with 40 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin at 28 °C with constant shaking (200 r.p.m.). *Populus tomentosa* was transformed using the leaf disc method as described by Jia et al. (2010). Transgenic plants were selected on woody plant medium supplemented with 9 mg l^{-1} hygromycin. Rooted plantlets were acclimatized in a humid chamber (16 h photoperiod, 25 °C and 70% relative humidity) for 2 weeks and finally transferred into the greenhouse.

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 RT-PCR, 1.0 μg 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. For *PtrWRKY40* expression analysis, PCR amplification was performed for 36 cycles, with each cycle consisting of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and finally 10 min at 72 °C. The PCR conditions of temperature and number of PCR cycles may vary for different genes. The constitutively expressed *18S* gene was used as control to confirm equal amount of cDNA in each reaction. All the primers used for RT-PCR are listed in Table S1 available as Supplementary Data at *Tree Physiology* Online.

Quantitative real-time PCR

Quantitative real-time PCR was performed on a TaKaRa TP800 real-time PCR detection system using specific primers for *PtrWRKY40* and *18S* as mentioned in Table S1 available as Supplementary Data at *Tree Physiology* Online. 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 ExTaqTM (Takara). Each measurement was carried out in triplicate, and the error bars represent standard error (SE) of the mean of fold changes for the three biological replicates.

Subcellular localization

The coding sequence of *PtrWRKY40* without a termination codon was ligated into pCX-DG vector (Chen et al. 2009) to generate the *GFP:PtrWRKY40* construct. *Agrobacterium tumefaciens* strain EHA105 containing *GFP:PtrWRKY40* was cultured in YEP medium (optical density (OD) 1.0 at 600 nm) and cells were diluted with Murashige and Skoog (MS) liquid medium (OD 0.8 at 600 nm). The empty *CaMV 35S:GFP* vector was used as a control. The fusion construct and the control were transformed into onion (*Allium cepa*) epidermal cells by particle bombardment and placed upside down on the MS plates containing MS with agar for 24 h in dark at 28 °C. Samples were stained with 4′,6-diamidino-2-phenylindole (DAPI), transferred to glass slides and images under green fluorescent protein (GFP), UV and brightfield were taken by light microscope (Olympus BX53, Olympus, Tokyo, Japan).

Transactivation activity assay

The coding region of *PtrWRKY40* without a termination codon was amplified using forward primer 5′-CCGGAATTCTAGCTAGCTGCAGCCACAAAG-3′ and reverse primer 5′-CGCGGATCCTCACCACTTAGTGTGATTTTGC-3′ (EcoRI and BamHI sites underlined in primer sequences). The amplified fragment was inserted into

pGBKT7 yeast expression vector containing GAL4-BD domain (Clontech Laboratories, Inc., Dalian, China). An empty vector pGBKT7 was used as a negative control and PtoMYB216 (Tian et al. 2013) was used as a positive control. The constructs above were transformed into yeast strain *Saccharomyces cerevisiae* Gold2 (using the manufacturer's instructions). The transformants yeast cells were grown on synthetic dropout (SD)-Trp-Ade-His selective medium at 30 °C for 3 days. The α-galactosidase activity assay was performed with the transformed cell lines grown in liquid SD-Trp medium using X-α-Gal as a substrate according to the manufacturer's manual. The experiments were repeated three times.

Transformation of P. tomentosa

Poplar transformation methods were described previously by Jia et al. (2010). In brief, leaves of *P. tomentosa* were excised from in vitro plantlets, cut into disks and dipped in the diluted *Agrobacterium* culture for 10 min. Regenerated plants were screened on selection medium with 9 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.

Transformation of A. thaliana

Transformation of *A. thaliana* (Columbia-0) plants with *Agrobacterium* strain EHA105 carrying the recombinant constructs was performed using the floral dip method (Clough and Bent 1998). Transgenic seedlings were selected for hygromycin resistance and confirmed by PCR.

Pathogen challenge of transgenic plants

For disease resistance to fungal pathogens, mature leaves of 3-month-old poplar plants were incubated with *D. gregaria*, which was grown on potato dextrose agar medium for 3 days at 28 °C. For disease resistance to *B. cinerea*, the fungal spores (5×10^5 spores ml^{-1}) were sprayed on to 4-week-old *Arabidopsis* plants as described previously (Zheng et al. 2006). The plants were covered with a transparent plastic dome to maintain high humidity, and disease development was evaluated 5 days later.

SA and MeJA treatments

Salicylic acid (5 mM in water) and MeJA [1 mM in 0.1% (v/v) ethanol] solutions were applied to whole plants. At least three plants were selected for one time interval for each treatment. The treated plants were immediately covered with a transparent film sheet. The leaves were collected and frozen in liquid nitrogen until RNA extraction.

SA extraction and quantification by high-performance liquid chromatography

Salicylic acid was extracted from 1.25 g of fresh poplar leaves as described previously (Ogawa et al. 2005). Each sample was extracted three times with methanol and 5 μl of 1 mg ml^{-1} *m*-hydroxybenzoic acid as an internal standard. The mixture was centrifuged at 12,000 r.p.m. for 5 min and the supernatant was collected. The solution was evaporated to dryness. The residue was dissolved in 200 μl of methanol, and then 1 ml of 1 mM KOH was added. Lipophilic substances were removed by extraction twice with chloroform. The aqueous phase was transferred to a new tube and then 10 μl of phosphoric acid and 700 μl of ethyl acetate were added. All supernatants were dried by vacuum evaporation. The dried material was dissolved in a solution of 50% (v/v) methanol and analyzed by high-performance liquid chromatography (HPLC; Shimadzu Co., Kyoto, Japan). The diode array detector was used to record absorption at 309 nm. The mobile phase was 20 mM sodium acetate (pH 2.5) containing 20% methanol. The highly purified SA was used to create standard absorption curves.

Accession numbers of WRKYs from different species

The accession numbers of the *WRKY* genes are *PtrWRKY40* (XP_002332076), *PtrWRKY77* (EEE78831.2), *PtoWRKY60* (AIA66985.1), *PtrWRKY89* (XP_002309186), *AtWRKY18* (At4g31800), *AtWRKY40* (At1g80840), *AtWRKY60* (At2g25000), *OsWRKY76* (ABC02813.1), *OsWRKY71* (BAF80893.1), *GhWRKY15* (AIE43890.1), *GaWRKY1* (AAR98818.1), *VpWRKY3* (AEN71143.1) and *NtWIZZ* (AB028022).

Results

Isolation and characterization of PtrWRKY40 from P. trichocarpa

Based on the sequence (POPTR_0003s18060.1) from the *Populus* genome database, *PtrWRKY40* was isolated by RT-PCR from the total RNA from the leaves of *P. trichocarpa*. The nucleotide sequence of *PtrWRKY40* cDNA is 957 bp and contains an ORF encoding a putative Group IIa protein of the WRKY family of 318 amino acids. Multiple sequence alignment of the deduced amino acid sequence of PtrWRKY40 showed high similarity with *A. thaliana* AtWRKY40, AtWRKY18 and AtWRKY60 (Figure 1a). Further sequence analysis showed that all four proteins possessed the same type of potential zinc ligands ($CX_5CX_{23}HXH$) and contained potential leucine zippers at the C-terminus, which is known to allow protein dimerization (Eulgem et al. 2000).

A phylogenetic tree was constructed to analyze the phylogenetic relationship based on the amino acids of PtrWRKY40 and other known WRKYs from different species, such as *Nicotiana attenuata*, *A. thaliana*, *P. trichocarpa*, *P. tomentosa*, *Glycine max*, *Oryza sativa*, *Gossypium arboretum*, *Gossypium hirsutum* and *Vitis pseudoreticulata*. As shown in Figure 1b, PtrWRKY40 was much closer to OsWRKY76, AtWRKY18 and NtWIZZ, which are involved in SA- and MeJA- or wounding-mediated plant defense responses (Hara et al. 2000, Chen and Chen 2002, Xu et al. 2006, Wenke et al. 2012).

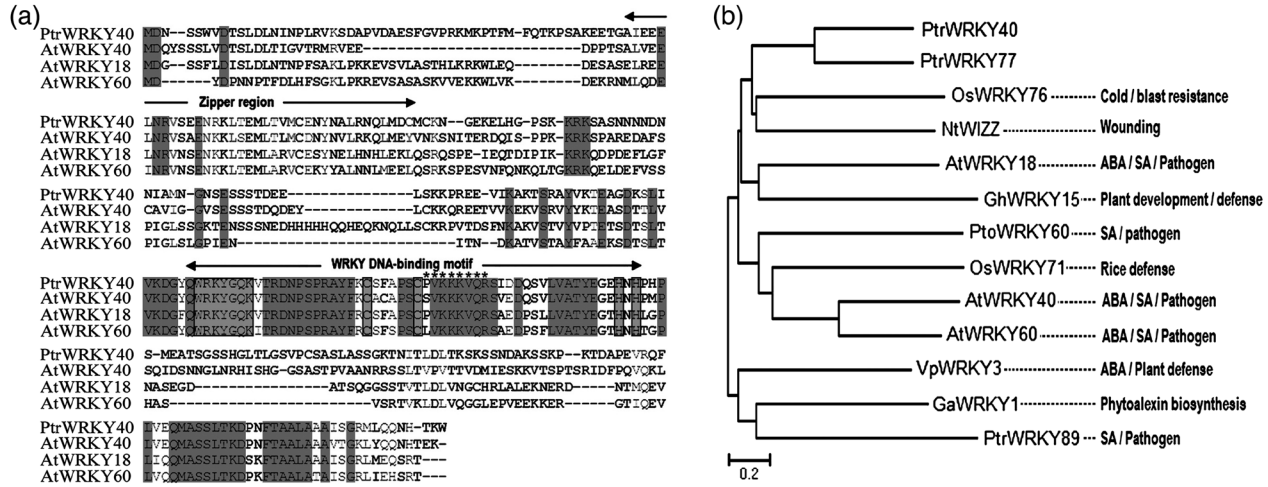

Figure 1. Relationship of *PtrWRKY40* and its homologs. (a) Sequence comparison of amino acids of PtrWRKY40, AtWRKY18, AtWRKY40 and AtWRKY60. Amino acids identical in the four proteins are black in gray background. The WRKY domains at the C-terminus and the Leu zipper motifs with six modules at the N-terminus of the four sequences are indicated. The highly conserved WRKYGQK residues and the residues forming the C_2H_2 zinc fingers are highlighted in box. Nuclear location signal 'PVKKKVQR' is indicated by asterisks. (b) Phylogenetic relationships of *PtrWRKY40* and proteins from selected species. Sequences are from *P. trichocarpa* (PtrWRKY40, PtrWRKY77 and PtrWRKY89), *P. tomentosa* (PtoWRKY60), *A. thaliana* (AtWRKY18, AtWRKY40 and AtWRKY60), *N. attenuata* (NtWIZZ), *O. sativa* (OsWRKY71 and OsWRKY76), *G. arboretum* (GaWRKY1), *G. hirsutum* (GhWRKY15) and *V. pseudoreticulata* (*VpWRKY3*). Sequence alignment was made by DNAMAN version 7, and phylogenetic tree was constructed by the NJ method using MEGA version 5.10. Bar represents 0.2 substitutions per site.

Expression patterns of PtrWRKY40 in poplar and in response to various stresses

Expression level of *PtrWRKY40* in various tissues of *P. trichocarpa* was analyzed by RT-PCR. *PtrWRKY40* was expressed in all adult plant tissues except young leaves and phloem (Figure 2a). The highest accumulation of *PtrWRKY40* transcripts was observed in the roots but a relatively low expression was seen in the petiole of poplar.

To investigate the role of PtrWRKY40 in poplar defense, we analyzed its expression over a time course of 24 h after treatments of SA, MeJA and *D. gregaria* inoculation by quantitative RT-PCR (qRT-PCR). As shown in Figure 2b, *PtrWRKY40* transcripts were elevated in response to SA at 2, 5 and 8 h post infiltration (hpi) relative to that at 0 hpi. The levels of *PtrWRKY40* transcripts also reached the maximum at 8 hpi in plants incubated with MeJA. Interestingly, in plants inoculated with *D. gregaria*, the mRNA level of *PtrWRKY40* was slightly enhanced but decreased suddenly at 8 hpi. These results implied that PtrWRKY40 was involved in defense responses in poplar.

Subcellular localization and transcriptional activity of PtrWRKY40

To determine whether PtrWRKY40 functioned as a TF, we first analyzed its subcellular localization. A nuclear location signal 'PVKKKVQR' was found in the amino acid sequence of PtrWRKY40 (Figure 1a), suggesting that it might be a nucleus-localized protein. Green fluorescent protein was fused at the 5′-terminal of the WRKY protein under the control of the constitutive CaMV 35S promoter. The fusion construct was directly delivered into onion epidermal cells. The green fluorescence was exclusively observed in the nucleus of the onion epidermal cells with *35S:GFP::PtrWRKY40* vector construct (Figure 3a). In contrast, green fluorescence was detected in both the nucleus and cytoplasm when the *35S:GFP* vector construct as a control was used. This finding indicated that PtrWRKY40 specifically localizes in the nucleus.

To test transcriptional activation of PtrWRKY40, the ORF of *PtrWRKY40* was fused with the GAL4 DNA-binding domain (BD) and transformed into Y2HGold yeast cells. All transformants grew well on SD medium lacking Trp. In contrast, only transformants containing BD-PtoMYB216, which was demonstrated to be a transcriptional activator (Tian et al. 2013), could grow on SD medium lacking Trp/His/Ade, but those cells containing BD (empty vector) or BD-PtrWRKY40 could not (Figure 3b). Therefore, PtrWRKY40 protein was not a transcriptional activator.

Constitutive expression of PtrWRKY40 enhanced susceptibility to D. gregaria in poplar

To investigate the role of *PtrWRKY40* in plant defense, 13 transgenic poplar plants overexpressing *PtrWRKY40* were regenerated. The transgenic lines displayed no phenotypic difference compared with WT plants (see Figure S1a available as Supplementary Data at *Tree Physiology* Online). The transcript levels of *PtrWRKY40* in transgenic plants were measured by RT-PCR (see Figure S1b and c available as Supplementary Data at *Tree Physiology* Online). Two transgenic lines (L11 and L12) with high transcript levels were selected for further evaluation

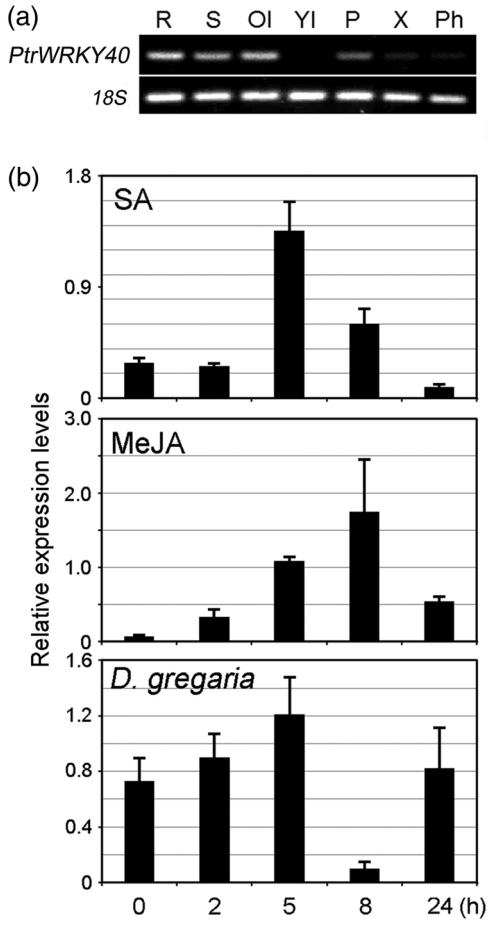

Figure 2. Expression patterns of *PtrWRKY40* in various organs and treatments with SA, MeJA and fungal pathogen. (a) RT-PCR analysis of PtrWRKY40 transcript levels in various organs and tissues of *P. trichocarpa*. Poplar *18S* expression was used as a control. Total RNA was isolated from root (R), stem (S), petiole (P), old leaf [the sixth leaf from the apex (OI)], young leaf [the first leaf (YI)], xylem (X) and phloem (Ph). (b) qRT-PCR analysis of PtrWRKY40 expression in the leaves of WT *P. trichocarpa* in response to SA (5 mM), MeJA (1 mM) and *D. gregaria*. The RNA was extracted from the leaves at 0, 2, 5, 8 and 24 h post treatment. Leaves from second and third node with same age and size were selected for treatments. Transcript levels of *PtrWRKY40* relative to 18S are presented for each treatment at each time interval. Each value represents the mean ± SE of three independent measurements.

of disease resistance. The leaves of 3-month-old transgenic and control plants were inoculated with agar plugs containing *D. gregaria* mycelium. The transgenic lines displayed remarkably greater disease symptoms with larger necrotic lesions on inoculated leaves when compared with WT plants (Figure 4a). The lesion areas on inoculated leaves were measured relative to the total leaf area using digitalized images. Lesion areas of transgenic lines (L11, 12.88%; L12, 15.09%) were significantly ($P < 0.05$) higher than that (4%) of WT plant (Figure 4b). These results indicated that overexpression of *PtrWRKY40* resulted in an increase of susceptibility to *D. gregaria* in the transgenic poplar.

PtrWRKY40 downregulated the expression of SA-pathway genes

Increased susceptibility to hemibiotrophic and biotrophic pathogens in plants is usually caused by reduced expression of *PR* genes associated with the SA-mediated defense pathway (Durrant and Dong 2004, Xu et al. 2006). We further determined whether *PtrWRKY40* was involved in regulating the expression of *Populus PR* genes, which were shown to be highly expressed in disease defense to *D. gregaria* in poplar (Jiang et al. 2014, Ye et al. 2014). Semi-quantitative RT-PCR analysis showed that expression of *PtoPR1.1*, *PtoPR2.1*, *PtoPR5.9* and *PtoCPR5* was significantly downregulated in transgenic *35S:PtrWRKY40* lines when compared with WT plants (Figure 5). Due to the antagonistic relationship between SA and JA pathways, we further tested the mRNA levels of jasmonate ZIM-domain (JAZ) proteins, which act as negative regulators of JA signaling (Chini et al. 2007, Chung et al. 2009). No obvious difference in transcript levels of *PtoJAZs* proteins except for *PtoJAZ8* was observed in transgenic lines when compared with the WT (Figure 5). These results suggested that *PtrWRKY40* may play an important role in disease resistance through regulating *PR* genes and SA/JA signaling pathways in poplar.

Overexpression of PtrWRKY40 affected SA accumulation in transgenic poplar

In *Arabidopsis*, *SID2* (also called *ICS1*) was found to be a key enzyme involved in SA biosynthesis and defects in *SID2* led to more susceptibility to pathogens with low SA accumulation (Nawrath and Metraux 1999, Wildermuth et al. 2001). Moreover, *AtWRKY40* showed binding activity to the promoter of *JAZ8* in vivo (Pandey et al. 2010). In this study, *PtoJAZ8* expression was downregulated in transgenic *35S:PtrWRKY40* lines (Figure 5), suggesting that *PtrWRKY40* might cause a change in *SID2* expression. Semi-quantitative RT-PCR analysis revealed that the expression of *PtoSID2* in transgenic lines was significantly lower than the WT (Figure 6a). To further test endogenous SA levels, SA contents were quantified by HPLC analysis and lower accumulation of SA levels was detected in transgenic lines in comparison with the WT plant (Figure 6b). Together, these results suggested that constitutive expression of *PtrWRKY40* in poplar resulted in a decreased SA accumulation, which was correlated to the enhanced susceptibility against hemibiotrophic pathogens.

PtrWRKY40 increased resistance to B. cinerea in transgenic Arabidopsis

The SA signaling pathway mediates plant defense against biotrophic and hemibiotrophic pathogens and interacts antagonistically with the JA/ET pathway, which is involved in defense against necrotrophic pathogens like *B. cinerea* (Kunkel and Brooks 2002). To examine the role of *PtrWRKY40* against

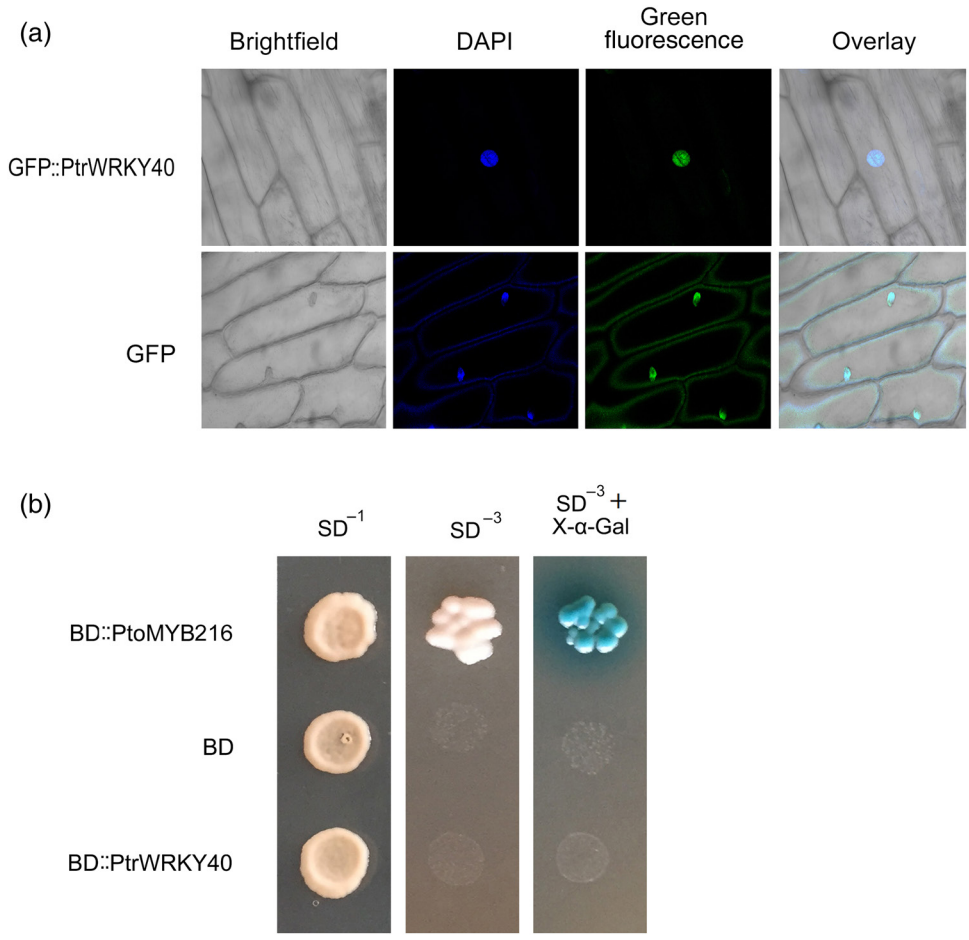

Figure 3. Subcellular localization and transactivation assay of PtrWRKY40. (a) To determine its subcellular localization, *PtrWRKY40* was ligated into pCX-DG vector to yield *GFP-PtrWRKY40* construct. The resultant vector was transformed into onion (*A. cepa*) epidermal cells and stained with DAPI. The fusion protein exhibited its localization to the nucleus of epidermal cells as manifested by GFP (upper column). Green fluorescent protein alone was localized to both the nucleus and the cytoplasm because of its small size (lower column). Brightfield and overlay images of the onion epidermal cells are also shown. (b) *PtrWRKY40* was cloned into pGBKT7 vector with BD domain and transformed into Gold2 yeast cells. The transformants were grown on SD^{-1} medium lacking tryptophan for positive clone selection and then on SD^{-3} medium lacking tryptophan, histidine and adenine for the transactivation assay according to the manufacturer's instructions. Transformants grown on SD^{-3} with X-α-Gal are also shown. BD (empty vector) is a negative control. BD-PtoMYB216 represents a positive control.

necrotrophic pathogens, *PtrWRKY40* was introduced into *Arabidopsis* plants by floral dip method. The transgenic *Arabidopsis* plants showed no phenotypic difference under normal conditions when compared with WT plants (see Figure S1d available as Supplementary Data at *Tree Physiology* Online). Four-week-old transgenic and WT seedlings were sprayed with spore suspension of *B. cinerea*. Inoculated leaves of transgenic lines displayed mild symptoms of disease when compared with WT plants (Figure 7a). To measure the growth of fungal pathogen, total RNAs were extracted from the leaves of *Arabidopsis* after 3 days of incubation. The *actin* gene of *B. cinerea* (*BcActin*) was amplified to quantify the biomass of the fungus. The transgenic lines displayed significantly low expression of *BcActin* transcripts when compared with the WT control (Figure 7b).

We further investigated the role of *PtrWRKY40* in JA metabolism and signaling. Transcript levels of JA-responsive marker genes, including plant defensin gene (*PDF1.2*) (Penninckx et al. 1996, 1998), vegetative storage protein 2 gene (*VSP2*) (Leon-Reyes et al. 2010) and *PR3* (Thomma et al. 1998), were compared with WT and transgenic *35S:PtrWRKY40* plants by semi-quantitative RT-PCR. As shown in Figure 7c, JA-regulated marker genes showed elevated expressions in transgenic lines when compared with the control. Thus, the expression levels of JA-regulated genes could be positively correlated with the resistance to *B. cinerea* in transgenic *Arabidopsis*. These results suggested a positive role of *PtrWRKY40* in JA-mediated signaling against *B. cinerea* in *Arabidopsis*.

Discussion

Plants are constantly challenged by various pathogens such as fungi, viruses and bacteria in nature. To fight microbial attacks,

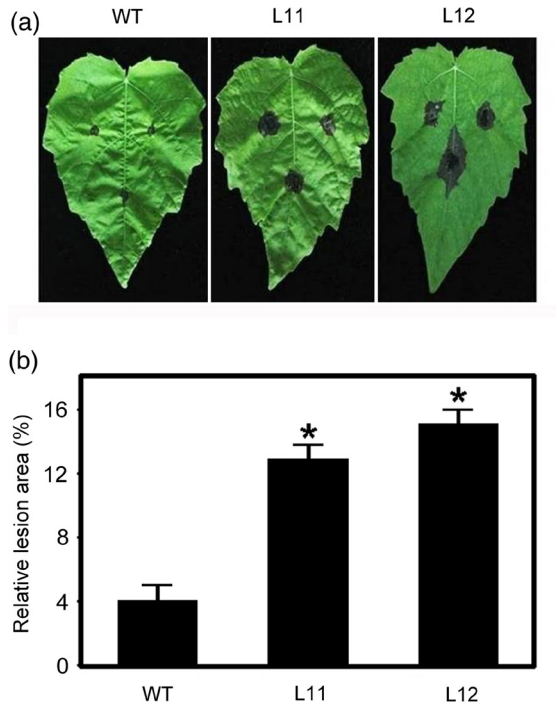

Figure 4. Susceptibility of transgenic poplar plants inoculated with *D. gregaria*. (a) The leaves from WT and transgenic plants inoculated with fungus *D. gregaria*. Leaves of transgenic lines show larger lesion areas caused by fungus when compared with control. (b) Measure of susceptibility of disease by percentage of lesion area to total area of leaf. L11 and L12 were transgenic plants with high expression levels of *PtrWRKY40*. Asterisks indicate a statistically significant difference between WT and transgenic plants. Values are means of three replications. Error bars indicate standard deviations ($P < 0.05$ by Student's *t*-test).

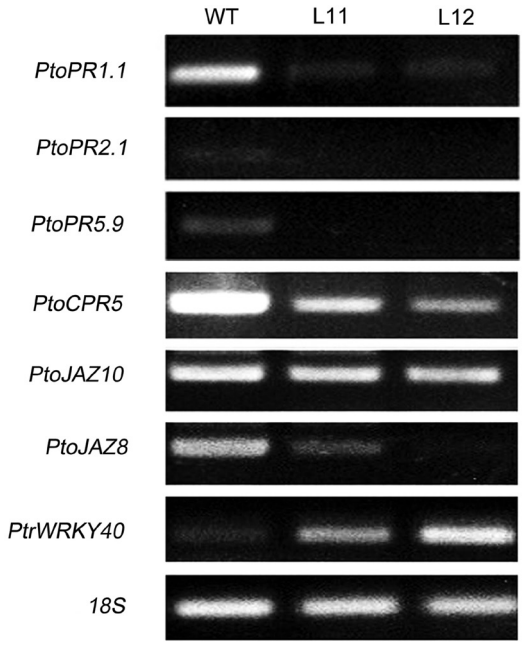

Figure 5. Analysis of expression patterns of defense-related genes in poplar. Transcript levels of *PtoPR1.1*, *PtoPR2.1*, *PtoPR5.9*, *PtoCPR5*, *PtoJAZ10*, *PtoJAZ8* and *PtrWRKY40* in WT and transgenic plants were tested by semi-quantitative RT-PCR. Poplar *18S* was used as a control.

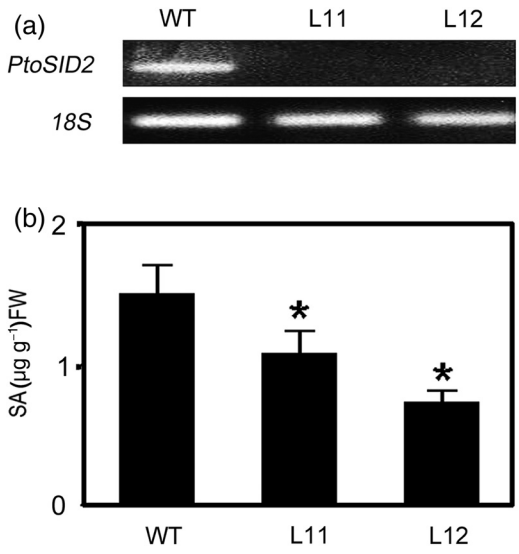

Figure 6. Salicylic acid accumulation in transgenic and WT poplar. (a) Transcript levels of *PtoSID2* in WT and overexpression lines. *18S* was used as a control. (b) Susceptibility to *D. gregaria* in transgenic plants was correlated to low SA accumulation in leaf tissues when compared with WT poplar. Salicylic acid was extracted and quantified by HPLC analysis from fresh leaves of plants. Error bars represent standard deviation. Asterisks indicate statistically significant difference. The experiment was repeated in three replicates with similar results ($P < 0.05$ by Student's *t*-test).

plants have evolved multiple and complex defense strategies. Plant hormones play an important role in various defense signaling. Salicylic acid and JA are the key plant endogenous secondary signaling molecules in response to pathogens (Glazebrook 2005). Previous studies demonstrated that WRKY TFs were induced by SA and pathogens and participated in many biotic and abiotic stress responses including pathogen defense (Pandey and Somssich 2009, Rushton et al. 2010, Bakshi and Oelmüller 2014, Jiang et al. 2014). In this study, a poplar *WRKY* gene, designated *PtrWRKY40*, was identified and its expression was induced to maximum by SA and JA at 5 and 8 hpi, respectively (Figure 2b), indicating that PtrWRKY40 was involved in both SA- and JA-mediated defense pathways. Interestingly, transcript level of *PtrWRKY40* was slightly increased after *D. gregaria* infection but reduced suddenly at 8 hpi (Figure 2b). Furthermore, transgenic poplar plants overexpressing *PtrWRKY40* showed more susceptibility to *D. gregaria* when compared with WT plants (Figure 4).

As shown by phylogenetic analysis in Figure 1, PtrWRKY40 belongs to the subgroup IIa of WRKY TFs (Eulgem et al. 2000). AtWRKY40, AtWRKY18 and AtWRKY60 have been well elaborated in their role in plant immunity and have been shown to play a partially redundant role in regulation of the basal defense response to different pathogens (Xu et al. 2006). Similarly, overexpression of *PtrWRKY40* enhanced sensitivity to hemibiotrophic *D. gregaria* in poplar and increased resistance to necrotrophic *B. cinerea* in *Arabidopsis*. However, our results indicated that PtrWRKY40 in poplar might have a different role in pathogen

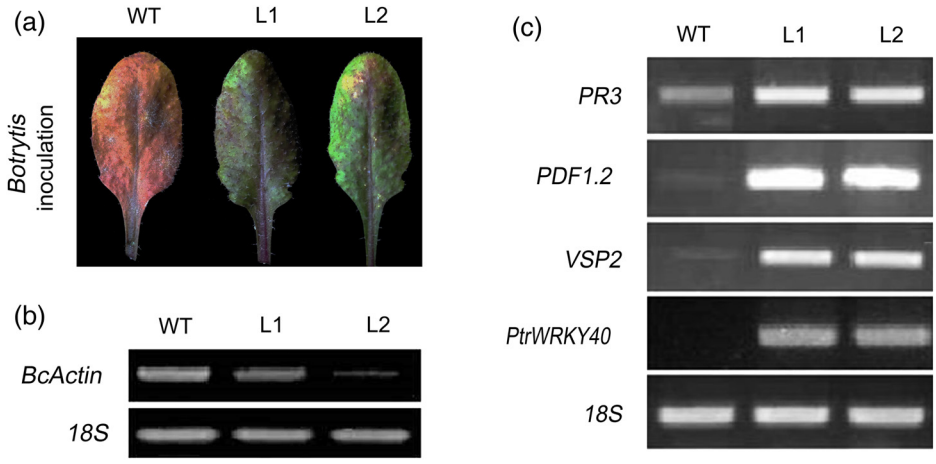

Figure 7. PtrWRKY40 shows resistance to *B. cinerea* in transgenic *Arabidopsis*. (a) Four-week-old WT and transgenic lines were inoculated by spraying spore suspension of *B. cinerea* and kept at high humidity. (b) Measurement of the biomass of the fungal pathogen on infected plants. Total RNA was isolated from inoculated plants three days after infection and RT-PCR with *actin* gene of *Botrytis* (*BcActin*) was carried out to quantify the biomass of the *B. cinerea* in transgenic lines and WT. *18S* was used as a control. (c) Expression of JA-pathway genes *PDF1.2*, *VSP2* and *PR3* in response to *B. cinerea* inoculation in transgenic lines and WT *Arabidopsis*. Total RNA was isolated from leaves harvested 24 h after spraying fungi. *18S* was used as a control.

response in comparison with AtWRKY40. The single mutant and overexpression lines of *AtWRKY40* did not show significant differences in susceptibility or resistance to pathogens *P. syringae* and *B. cinerea* when compared with WT plants (Xu et al. 2006). In addition, amino acid sequence alignment showed that the identity of PtrWRKY40 and AtWRKY40 has been only 54% (Figure 1a), indicating a partial difference in function by PtrWRKY40 from AtWRKY40.

Salicylic acid plays a key role in plant defense by establishing the SAR and activating defense-related genes (Durrant and Dong 2004). Plants defective in SA accumulation were shown to be susceptible to pathogens and exhibited suppression in PR genes involved in the SA pathway. In this study, overexpression of *PtrWRKY40* in poplar exhibited more susceptibility to *D. gregaria*, which is a hemibiotrophic fungus and downregulated *PR*s accumulation (Figures 4 and 5), suggesting a suppression in the SA signaling pathway. As shown in Figure 6, the expression of *PtoSID2*, a gene encoding an isochorismate synthase required for SA biosynthesis, was decreased in transgenic poplar plants compared with the WT control. Salicylic acid accumulation in transgenic *35S:PtrWRKY40* poplar plants was significantly decreased, which is in agreement with *PtoSID2* transcript levels. On the other hand, JA is generally antagonistic with the SA-dependent pathway (Dempsey et al. 1997, Kunkel and Brooks 2002, Robert-Seilaniantz et al. 2011, Pieterse et al. 2012). The transgenic *Arabidopsis* overexpressing *PtrWRKY40* exhibited enhanced resistance to *B. cinerea*, and that is consistent with the increased *PDF1.2* accumulation (Figure 7), indicating a boost in JA signaling. These results showed that PtrWRKY40 acted as a repressor for SA signaling pathway and an activator for JA-dependent pathway.

Recently, JAZ repressor proteins have been shown to play a role in JA–SA antagonistic crosstalk (Van der Does et al. 2013). The increase in JAZ proteins is proposed to repress EIN3 and EIL1, which relieve the suppression on *SID2* exerted by EIN3 and EIL1 and hence SA biosynthesis increases (Kazan and Manners 2012). As shown in Figure 5, transgenic *35S:PtrWRKY40* poplars exhibited lower accumulation of *PtoJAZ8* when compared with WT plants, which is in agreement with the reduced SA contents (Figure 6b). WRKY proteins were described as TFs capable of binding W-box-containing sequences that are present in the promoter regions of downstream target genes (Eulgem et al. 2000). In this study, due to the occurrence of W boxes in the promoter of *PtoJAZ8* (see Figure S2 available as Supplementary Data at *Tree Physiology* Online), we speculate that PtrWRKY40 might regulate the *PtoJAZ8* directly to contribute to the antagonistic relationship of SA/JA signaling pathways via repressing *PtoSID2*.

The data presented in the present study suggest the involvement of PtrWRKY40 in both SA and JA signaling pathways. In *Arabidopsis*, AtWRKY70 has also been demonstrated to be involved in defense against biotrophic and necrotrophic pathogens (AbuQamar et al. 2006, Li et al. 2006). However, the difference in susceptibility observed between transgenic lines overexpressing *PtrWRKY40* in poplar and *Arabidopsis* toward the selected fungi is obvious and may be due to the use of a heterologous plant system. Further works using a homologous expression system in which PtrWRKY40 will determine its endogenous target genes and/or partners will provide new insights into the biological functions of *WRKY* genes in poplar and their role in enhancing protection against various fungi.

Supplementary data

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

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Conflict of interest

None declared.

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