

TaDIR13, a Dirigent Protein from Wheat, Promotes Lignan Biosynthesis and Enhances Pathogen Resistance

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Published online: 23 May 2014
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Abstract Dirigent is proposed to be involved in either lignan or lignin biosynthesis. In the present study, sequences for dirigent and dirigent-like proteins from the wheat genome were analyzed. We obtained wheat dirigent (TaDIR13), and dirigent-like (TaDIR4 and TaJA1) recombinant proteins used for biochemical assays. It was shown that TaDIR13 could effectively direct coniferyl alcohol coupling into (+)-pinoresinol. In contrast, TaDIR4 and TaJA1 did not exhibit this activity. In accordance with this role of TaDIR13, overexpression of *TaDIR13* in transgenic tobacco increased total lignan accumulation. Lignan extracts from *TaDIR13* transgenic plants had enhanced antibacterial effects to *Pseudomonas syringae*. Furthermore, these plants did show strong resistance to *P. syringae* and *Phytophthora parasitica*. On the contrary, *TaDIR4* transgenics did not show noticeable changes in either lignan accumulation or pathogen resistance. Overexpression of *TaJA1* did not increase lignan accumulation either. Unlike *TaDIR4* plants though, *TaJA1* plants exhibited high pathogen resistance; yet, this was achieved through its jacalin-related lectins domain and not its dirigent domain. Lignin content and gene expression were not affected in all transgenic plants. Interestingly, expression of the pinoresinol-lariciresinol reductase gene (*NtPLR*) was increased in *TaDIR13* transgenic plant; this is apparently owing to a favored metabolic flux from coniferyl alcohol into the lignan pathway. These data collectively suggest that TaDIR13 is mainly involved in regulating lignan biosynthesis which, in turn, responsible for the observed roles in pathogen resistance. TaDIR4 and TaJA1, both being dirigent-like proteins, have little functional overlap with dirigent proteins.

Keywords Dirigent · Lignan · Lignin · Metabolic flux · Pathogen resistance · *Triticum aestivum*

Introduction

Lignans are phenylpropanoid dimers synthesized via the phenylpropanoid pathway. They occur naturally in a number of plant families and are thought to play the important physiological and ecological roles in the interaction with pathogens and insects, due to their antibacterial and antifeedant activities (Harmatha and Dinan 2003; Schroeder et al. 2006). Coniferyl and sinapyl alcohols are the direct precursors to form lignan dimers. Since coniferyl and sinapyl alcohols, together with other monolignol, *p*-coumaryl alcohol, are also polymerized into macromolecule lignin, lignans are overlapped with lignin in certain biosynthetic pathways (Davin and Lewis 2003). Much is known about lignin biosynthesis, including the action of oxidative enzymes to convert monolignols into corresponding free radicals, but one of the unknowns is how coupling radicals to produce a functional lignin molecule (Boerjan et al. 2003). There are two hypotheses to address this process. The random coupling hypothesis suggests that lignin formation proceeds through coupling of individual monolignols to the growing lignin polymer in a nearly random fashion. Therefore, the amount and species of individual monolignols available will be the main regulators for lignin formation and its final component (Sederoff et al. 1999). In an alternative hypothesis, there is a strict regulation for the formation of individual bonds and hence, the final component of lignin monolignol composition of the resultant lignin molecules (Lewis 1999). The discovery of dirigent proteins provides molecular underpinning for this latter hypothesis (Davin et al. 1997).

A 78-kDa dirigent was first isolated from *Forsythia suspensa* (Davin et al. 1997). An in vitro biochemical analysis

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demonstrated that this protein, in the presence of an oxidase or one electron oxidant, could stereoselectively couple two coniferyl alcohol molecules to give rise to a (+)-pinoresinol (Davin et al. 1997). This dimer, known as lignan, was proposed to couple more monolignols and then form the polymer of lignin (Davin et al. 1997). A dirigent cDNA from *Forsythia intermedia* was found to contain an open-reading frame for 186 amino acids (Gang et al. 1999). Therefore, the native dirigent protein is a trimer molecule, with each subunit being 26 kDa (Gang et al. 1999). Homologs of dirigent genes were recently found in a few other plants, including cotton (Zhu et al. 2007), and *Boea hygrometrica* (Wu et al. 2009). So far, at least 104 more dirigent and dirigent-like protein gene sequences have been discovered from other species (25 from *Arabidopsis*, 54 from rice, and 35 from spruce, Ralph et al. 2006; Ralph et al. 2007). A phylogenetic analysis indicated that these proteins form a super-family consisting of distinct subfamilies (Ralph et al. 2007).

According to gene expression analyses, many dirigent and dirigent-like genes are stress-inducible. Upregulation has been documented for a number of stresses, such as wounding (Ralph et al. 2007), stressed by ABA, dehydration, and low temperature (Wu et al. 2009). Upregulation upon attacks by insects (Ralph et al. 2007) and fungi (Zhu et al. 2007) are of particular interest. The involvement of dirigents in pathogen resistance can be better understood if lignans are brought into the picture. Lignans often exist in high quantities in the heartwood region of trees, potentially preventing degradation by heart-rot fungi (Suzuki and Umezawa 2007). There is a strong implication for dirigents' roles in directing the monolignol-to-pinoresinol (lignan) conversion.

Direct experimental evidence to substantiate dirigent proteins' contributions to lignin biosynthesis is still rare (Davin et al. 2008; Önnnerud et al. 2002). Neither is known whether or not dirigent and dirigent-like proteins of different subfamilies play similar roles. In this report, (1) gene sequences for wheat dirigent and dirigent-like proteins were analyzed, (2) corresponding cDNAs were isolated and recombinant proteins were examined for their coupling properties on monolignols, and (3) functions of dirigents in lignan/lignin biosynthesis and stress responses were studied in transgenic tobacco plants.

Materials and Methods

Plant Materials

Wheat plants (*Triticum aestivum* L. cv. H4564) were grown in a naturally lit glasshouse. Total RNA was isolated using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). Genomic DNA was isolated as described by Edwards et al. (1991).

Phylogenetic Analysis of Dirigent and Dirigent-Like Proteins

Dirigent and dirigent-like protein sequences from *Arabidopsis*, rice, and spruce plants were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>). A similarity search was conducted in a wheat EST database (<http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=wheat>). For each plant species, partial sequences and incorrect or repetitive sequences were discarded. Sequences with more than 97 % identity within any species were treated as one entity. Sequence were compared using the SIM alignment tool (Altschul et al. 1997) with an open gap penalty of 10 and an extend gap penalty of 0.05. Phylogenetic analysis was performed by the maximum parsimony method using the PAUP 4.0 software (Sinauer Associates, Sunderland, MA).

Heterologous Expression and Purification of Dirigent and Dirigent-Like Proteins

TaDIR13 cDNA was amplified using primers 5'-CGgaattcATGCAAGGCCTCGCGGCATC-3' and 5'-CCCaaagctTCAGATGTAGCACTCGTAGAG-3' (with an *EcoRI* and a *HindIII* site, respectively). Similarly, *TaDIR4* cDNA was amplified using primers 5'-CGgaattcATGGCTTGCTTCAAGCTCAAC-3' and 5'-CCCaaagctTCATGGGTGACGTAGAGTC-3'. Each PCR mixture contained 5 ng template, 1 $\mu\text{mol/L}$ each primer, 0.4 mmol/L each dNTP, and 2.5 U Taq DNA polymerase (Gibco, Beijing). Each PCR product was then cloned into vector pET-32a (Novagen, Beijing), and the recombinant plasmids were designated as pET-TaDIR13 and pET-TaDIR4, respectively. Each recombinant plasmid was introduced into competent *E. coli* strain BL21 cells. Bacterial culture and protein extraction-purification were conducted as reported previously (Ma and Tian 2005). TaJA1, another wheat dirigent-like protein of the subclade C, was expressed as previously reported (Ma et al. 2013).

Analysis of Coupling Properties of Dirigent and Dirigent-Like Proteins

Coupling assays were performed by following the method of Davin et al. (1997). The reaction mixture consisted of ammonium peroxydisulfate (2 $\mu\text{mol/mL}$), coniferyl alcohol (10 $\mu\text{mol/mL}$), and recombinant protein (1.5 nmol/mL). After 1-h incubation at 30 °C, the mixture was extracted with ethyl acetate for three times. The extract was evaporated to dryness under vacuum, and the residue was re-dissolved in 50 % methanol. Reversed-phase column chromatography (HPLC) was performed, following the procedure of Zheng et al. (2011). A Dionex HPLC system (Sunnyvale, California) was used, which was equipped with a P680 HPLC pump, an UltiMate 3000 autosampler, a TCC-100 thermostated column

compartment, a Dionex PDA100 photodiode array detector, and a C18 column of ODS 80Ts QA (150 mm×4.6 mm, 5 μm i.d., Tosoh, Tokyo).

Generation of Transgenic Tobacco

The full-length *TaDIR13* cDNA was PCR-amplified using primers 5'-GCCaagcttCTGGTAATTAATAGTTGCG-3' and 5'-ATActcgagGGCACATCACTCCTTATT-3' (with *HindIII* and *XhoI* sites, respectively). The full-length *TaDIR4* cDNA was amplified using primers 5'-TCTaagcttGTCTTCCAATCATCCCG-3' and 5'-TTTctcgagGTAATGCTGCCACAGGT-3'. After digestion with *HindIII* and *XhoI*, the product was ligated into vector pKYLX71 (An et al. 1985), resulting in binary plasmids designated as pKYLX-TaDIR13 and pKYLX-TaDIR4, respectively. The insert was subcloned into *E. coli* DH5α cells and confirmed by enzymatic digestion and sequencing. The vector was transferred into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method, and tobacco (*Nicotiana tabacum* cv. Wisconsin 38) was transformed (Ma et al. 2008). Transgenic tobacco with over-expressed *TaJAI* gene, for subclade C of dirigent from wheat, has been reported previously (Ma et al. 2013). Tobacco plants were also transformed with an empty pKYLX71 vector to generate controls.

The two groups of transgenic plants were identified by PCR with two sets of specific primers (for *TaDIR13*, 5'-CTGGTAATTAATAGTTGCG-3' and 5'-GGCACATCACTCCTTATT-3'; for *TaDIR4*, 5'-GTCTTCCAATCATCCCG-3' and 5'-GTAATGCTGCCACAGGT-3'). The abundance of the *TaDIR13* and *TaDIR4* transcripts in transgenic plants were examined by RT-PCR, using the same primer sets.

Semi-quantitative RT-PCR analysis for lignin and lignan biosynthetic genes was conducted following the procedure of Ma et al. (2011). For lignin genes, primer sequences for cinnamyl alcohol dehydrogenase (*NtCAD*, Genbank accession no. X62343), cinnamoyl-CoA reductase (*NtCCR*, A47101), and caffeic acid *O*-methyltransferase (*NtCOMT*, X74453) can be found in Ma et al. (2011). For lignan biosynthetic genes, pinoreosinol-lariciresinol reductase (*NtPLR*) and piperitol-sesamin synthase (*NtPSS*) primer sequences were as follows: 5'-GCTGTCAAACAGGTGGAT-3' plus 5'-CTTGTCAGTGTCTTGG-3' and 5'-TCTGCAAGAATCAGAACAG-3' plus 5'-TCTCCTTCCCATTCCAAAT-3', respectively. The tobacco actin gene was used as a reference, with primers 5'-CCAATCGAGAGAAGATGACCCA-3' and 5'-CCATCTGGCAGCTCATAGCTCT-3'. The expression intensity of each target gene relative to the actin gene was estimated on the images from electrophoresis.

Quantitative RT-PCR reactions were conducted using a SYBR Green RT-PCR Master Mix (Toyobo, Tokyo) on a Stratagene Mx3000P thermocycler (Stratagene, La Jolla, CA) under conditions of an initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 55 °C for 20 s, and extending at 72 °C for 15 s. Dissociation curves were verified for each reaction. Quantification was performed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) using tobacco actin as a reference gene. The relative value of the gene expression was normalized through the reference gene and calculated as the mean of three biological replicates.

Lignin and Lignan Content Analyses for Transgenic Plants

Samples from air-dried stems were subjected to quantitative analysis for lignin by the Klason method (Kirk and Obst 1988). Lignin content was expressed relative to the cell wall residue (%CWR). Total lignan was extracted from leaf samples using the method of Chen et al. (2010). The extract was evaporated to dryness under vacuum, and the residue was re-dissolved in water. Total lignan was determined using a chromotropic acid spectrophotometric method (Lu et al. 2011) with schisantherin A as a standard.

Determination of Antibacterial Effects of Lignan Extracts

A *Pseudomonas syringae* pv. *tabaci* liquid culture at the exponential phase (OD₆₀₀=0.3) was diluted by 1:1,000, and a 5-μL diluted bacteria sample was spread on a LB plate supplemented with the lignan extract (9:1, v/v). After incubation for 24 h at 28 °C, bacterial colonies were counted.

Pathogen Resistance Assays

T₂ generation plants homologous for the *TaDIR13* and *TaDIR4* transgenes, respectively, were used for disease resistance analyses. Two pathogens, *Phytophthora parasitica* var. *nicotianae* and *P. syringae* pv. *tabaci*, were used. These pathogens, on susceptible plants, cause tobacco black shank and wildfire disease, respectively. The fourth to sixth leaves from the top on 2-month-old plants were inoculated with either pathogen. Methods for pathogen growth, inoculation, and disease scoring were performed according to Guo et al. (2004). For tobacco black shank disease, the lesion diameter was recorded. For tobacco wildfire disease, bacterial population inside leaf disks was determined from cultures on King's B plates. The Student's *t* test for independent samples (Geng and Hills 1989) was applied to

compare transgenic with control lines, with probability at $P_{0.05}$ and $P_{0.01}$.

Results

Dirigent and Dirigent-Like Proteins in Wheat

In the wheat EST database, 12 dirigent and dirigent-like proteins were identified. Along with sequences from *Arabidopsis*, rice, and spruce, a phylogenetic tree was constructed (Fig. 1). These proteins from wheat are members of either the dirigent subfamily (also referred to as the DirA subclade) or the dirigent-like subfamily. In the dirigent-like subfamily, DirB/D subclade proteins are commonly found in all seed plants listed. Other subclades of the dirigent-like subfamily exhibit narrower distributions (DirE in *Arabidopsis* and rice, DirF in spruce, and DirG in rice). DirC is only found in monocot plants. The functions of DirC proteins have been investigated in maize, rice, and wheat (see a review by Ma 2013).

Phylogenetic analysis showed that members of the DirA subclade had higher degrees of sequence conservation than those of DirB/D. Members of DirA found in eudicots (*Arabidopsis*), monocots (rice), and gymnosperms (spruce) were clustered to the different branches (Fig. 1). Members of the DirB/D subclade exhibited higher sequence similarities in inter-taxon than intra-taxon; for example, AtDIR1 and AtDIR2 were more close to OsDIR32 and OsDIR34, while AtDIR4 was more close to TaDIR1. This implies that DirB/D may be a very ancient group of genes, which diverged into different classes with eudicots, monocots, and gymnosperms.

In the current study, we chose TaDIR13 and TaDIR4 as representatives of the DirA and DirB/D subclades, respectively, for further analysis. The full length *TaDIR13* cDNA has 1,002 nucleotides, with a 609-nucleotide open-reading frame (ORF), flanked by 5' and 3' untranslated regions of 58 and 335 nucleotides, respectively. The predicted protein consists of 202 amino acids with a relative molecular mass of 22 kDa and a theoretical isoelectric point of 4.85. The *TaDIR4* cDNA has 782 nucleotides, with a 498-nucleotide ORF, flanked by 5' and 3' untranslated regions of 52 and 232 nucleotides, respectively. The predicted protein consists of 165 amino acids with a relative molecular mass of 17 kDa and a theoretical isoelectric point of 7.34. One DirC isoform from wheat, TaJA1 (Wang and Ma 2005), was also included in this project.

Recombinant TaDIR13 Protein Directs Pinoresinol Formation

Recombinant TaDIR13 protein expressed and purified from *E. coli* (Fig. 2) was tested for activity to couple monolignols. In the presence of ammonium peroxydisulfate as an oxidant,

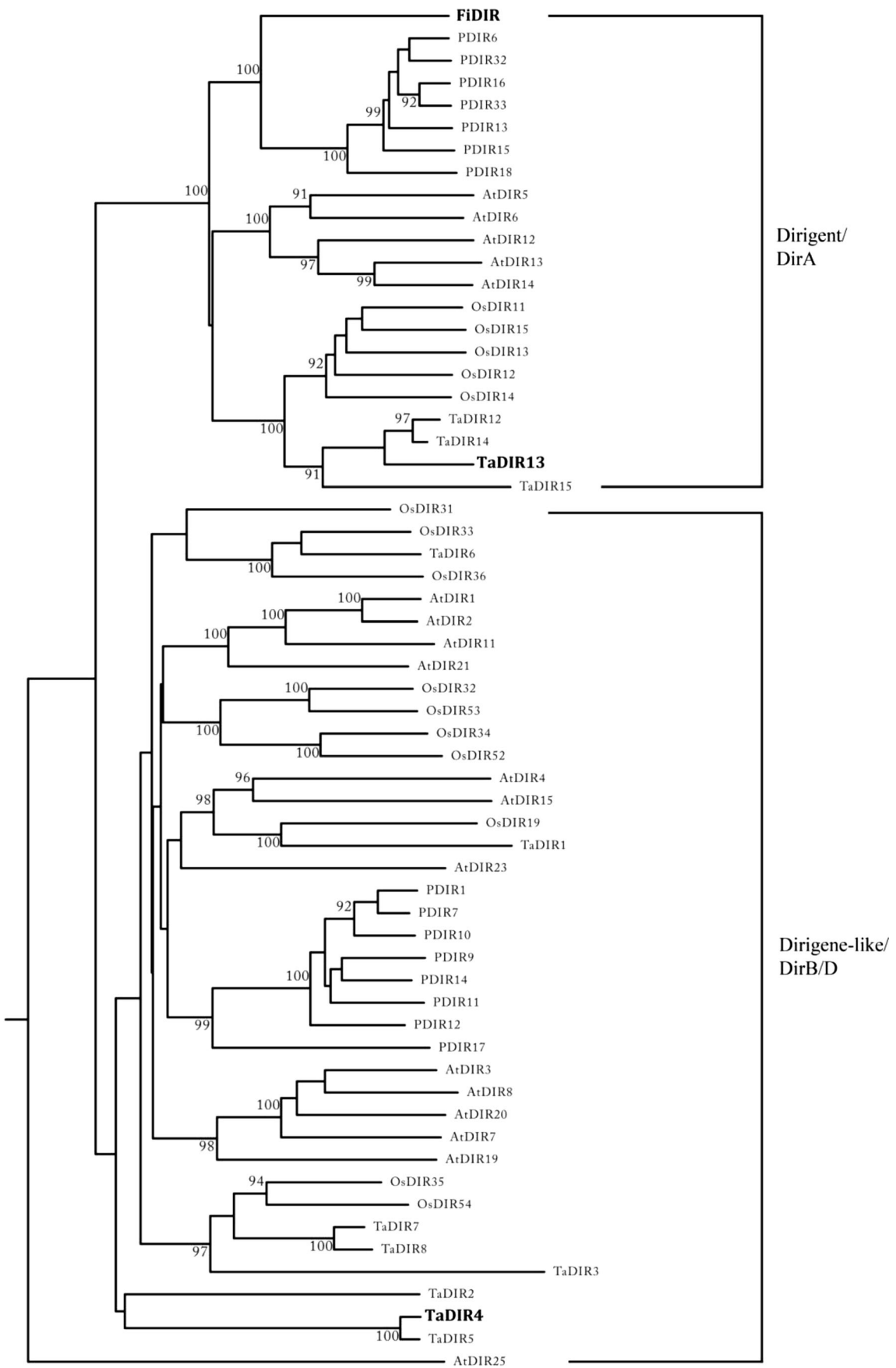
TaDIR13 could direct conversion of coniferyl alcohol into (+)-pinoresinol (Fig. 3). The calculated specific activity for this conversion was about 3.4 nmol (+)-pinoresinol/nmol TaDir13 protein. This result is similar as that reported for *F. suspensa* (Davin et al. 1997). By contrast, recombinant TaDIR4 and TaJA1 proteins did not show this activity in the same reaction condition (data not shown).

TaDIR13 and *TaDIR4* Transgenic Plants Exhibit No Morphological and Developmental Abnormalities

Transgenic plants carrying *TaDIR13* or *TaDIR4* were confirmed by PCR and RT-PCR, and four *TaDIR13* transgenic lines (DIR13-1, DIR13-2, DIR13-3, and DIR13-4) (Fig. 4a) and four *TaDIR4* transgenic lines (DIR4-1, DIR4-3, DIR4-4, and DIR4-9) (Fig. 4b) were examined in detail.

Seeds of T₁ progeny of transgenic plants were screened on a kanamycin-complimented medium. The segregation ratio of drug resistance versus sensitivity was approximately 3:1 for three DIR13 lines (DIR13-2, DIR13-3, and DIR13-4) and three DIR4 lines (DIR4-3, DIR4-4, and DIR4-9). These transgenic lines were essentially identical to wide-type plants in morphology (height and leaf number) and flowering time when grown in greenhouse.

Fig. 1 Molecular phylogenetic tree for dirigent and dirigent-like proteins from *Arabidopsis*, rice, spruce, and wheat. The tree was constructed using Clustal W with PAM 250 residue weight table. The bootstrap values were shown in each branch. AtDIR25 was selected as out-group and FiDIR from *Forsythia* was used for landmark of DirA subclade. FiDIR, TaDIR13, and TaDIR4 were highlighted in the figure. GenBank and EST accession numbers for these sequences are as follows: *Forsythia x intermedia* FiDIR (AAF25357); *Arabidopsis thaliana* AtDIR1 (ABR46205), AtDIR2 (AAM13094), AtDIR3 (NP_199715), AtDIR4 (NP_179707), AtDIR5 (BAD43018), AtDIR6 (AAO00799), AtDIR7 (BAC42662), AtDIR8 (NP_187976), AtDIR11 (BAD44205), AtDIR12 (AAO42052), AtDIR13 (AAP88352), AtDIR14 (NP_192860), AtDIR15 (NP_195582), AtDIR19 (BAC42538), AtDIR20 (AAM91539), AtDIR21 (NP_176762), AtDIR23 (AAO64191), and AtDIR25 (AAP49521); *Picea* spp. PDIR1 (ABD52112), PDIR6 (ABD52117), PDIR7 (ABD52118), PDIR9 (ABD52120), PDIR10 (ABD52121), PDIR11 (ABD52122), PDIR12 (ABD52123), PDIR13 (ABD52124), PDIR14 (ABD52125), PDIR15 (ABD52126), PDIR16 (ABD52127), PDIR17 (ABD52128), PDIR18 (ABD52129), PDIR32 (ABR27728), and PDIR33 (ABR27729); *Oryza sativa* OsDIR11 (NP_001060395), OsDIR12 (NP_001060396), OsDIR13 (NP_001060404), OsDIR14 (BAC19943), OsDIR15 (NP_001060409), OsDIR19 (NP_001051654), OsDIR31 (NP_001066288), OsDIR32 (NP_001065892), OsDIR33 (NP_001065888), OsDIR34 (AAX96293), OsDIR35 (NP_001065890), OsDIR36 (NP_001065889), OsDIR52 (AAX96290), OsDIR53 (ABA93522), and OsDIR54 (AAX96314); and *Triticum aestivum* TaDir1 (TC307806), TaDir2 (TC309798), TaDir3 (TC362616), TaDir4 (TC297337), TaDir5 (TC326720), TaDir6 (TC429903), TaDir7 (TC424593), TaDir8 (CV777987), TaDir12 (TC317574), TaDir13 (TC299725), TaDir14 (BJ284006), and TaDir15 (CK154646)



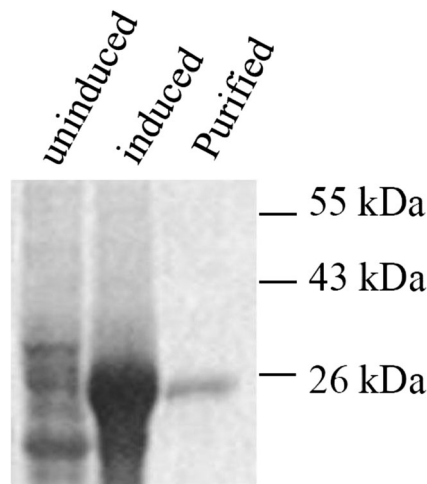


Fig. 2 Gel electrophoresis images showing recombinant TaDIR13 expressed in *E. coli*. Proteins were separated from the total protein fractions of uninduced and IPTG-induced *E. coli* harboring pET-TaDIR13 expression plasmids, and after His-Tag resin purification of the soluble fraction from induced cells. Molecular markers are indicated on the right of the figure

Overexpression of *TaDIR13* Gene Increase Disease Resistance

Lignan extracts from leaves of transgenic plants were tested on *P. syringae* pv. *tabaci*. After incubation for 24 h, extracts from *TaDIR13* transgenic plants significantly inhibited bacterial growth, compared with extracts from control tobacco (Fig. 5). Extracts from either *TaDIR4* or *TaJAI* transgenic plants did not show significant changes in bacterial growth (Fig. 5).

P. syringae and *P. parasitica* var. *nicotianae* were further used in disease-resistant bioassays. Growth of *P. syringae* was significantly inhibited in *TaDIR13* transgenic lines (DIR13-2, DIR13-3, and DIR13-4) compared to the control (Fig. 6a). Pathogen growth in *TaDIR4* transgenic lines (DIR4-3, DIR4-4, and DIR4-9) was not affected (Fig. 6a).

A more careful examination revealed that *TaDIR13* transgenic lines exhibited less severe symptoms of the disease than control after the inoculation of *P. parasitica* (Fig. 6b). Again, *TaDIR4* transgenic lines showed similar disease lesion as the control tobacco (Fig. 6b). Taken together, the data indicate that overexpression of *TaDIR13* in tobacco plants enhances resistance to both bacterial and fungal pathogens, while *TaDIR4* is not involved in these processes.

As shown previously, *TaJAI* gene enhances the disease resistance to both bacterial and fungal pathogens (Ma et al. 2010; Ma et al. 2013), although extracts from

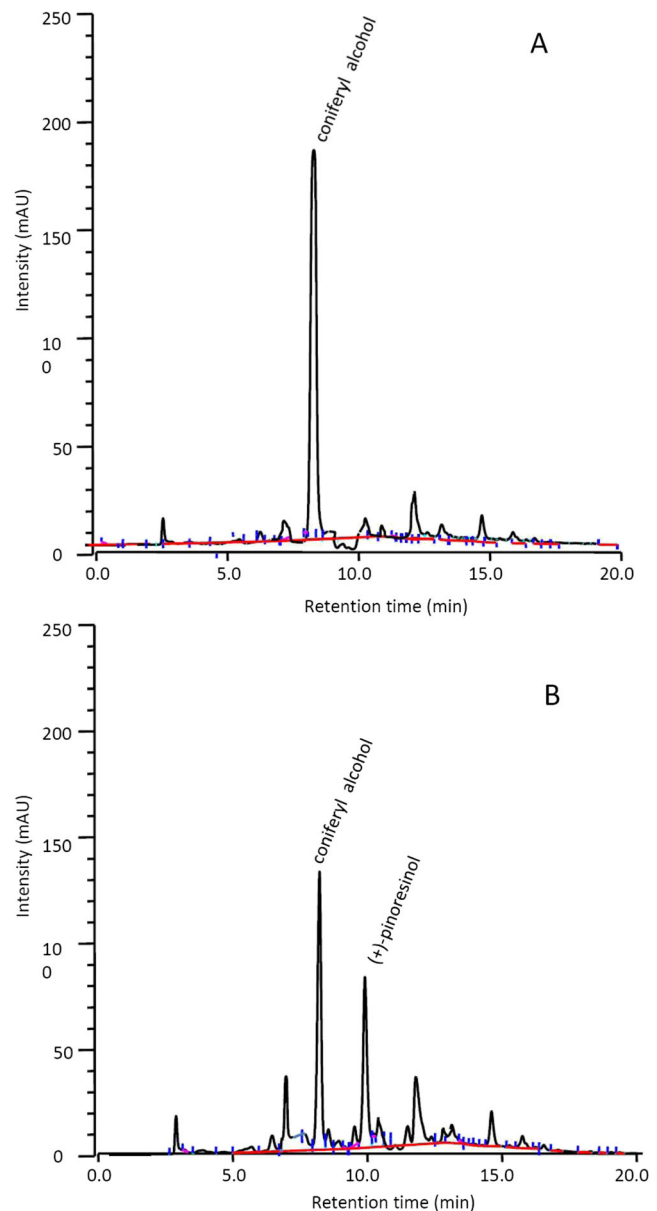


Fig. 3 HPLC chromatograms of the coupling reactions with TaDIR13 recombinant protein. Ultraviolet detection was under 280 nm. **a** Without TaDIR13 protein. **b** With TaDIR13 proteins

TaJAI transgenic plants did not show antibacterial effects (Fig. 5).

Overexpression of *TaDIR13* Gene Increases Lignan Accumulation

Compared to control plants, *TaDIR13* transgenic lines exhibited 13.1 to 15.4 % increases in lignan content. *TaDIR4* and *TaJAI* lines showed little change in lignan content (Fig. 7a).

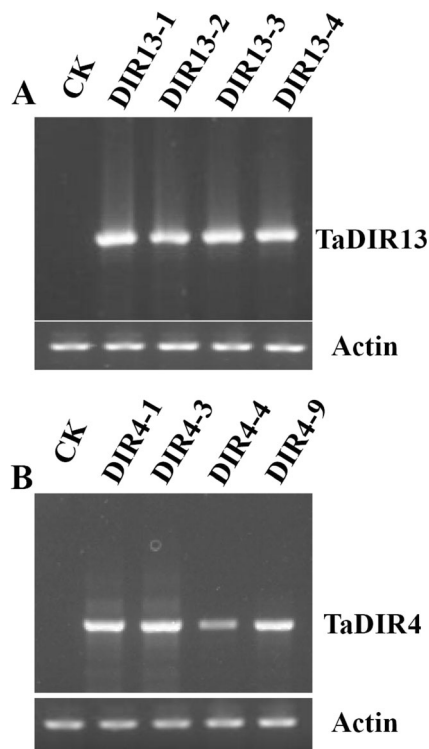


Fig. 4 RT-PCR analyses of *TaDIR13* and *TaDIR4* gene expression in transgenic tobacco plants. Actin was included as an internal control. **a** Four *TaDIR13* lines. **b** Four *TaDIR4* lines. CK control transgenic line with empty pKYLX71 vector

By contrast, acid-insoluble lignin remained nearly unchanged in all transgenic plants relative to controls (Fig. 7b).

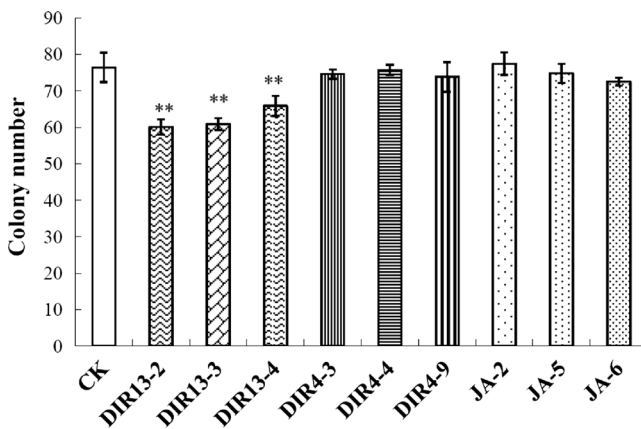


Fig. 5 Antibacterial effects of the lignan extracts from different transgenic tobacco lines. A sample of 5- μ L *Pseudomonas syringae* culture (10^4 CFU/mL) was spread on LB plate supplemented with the lignan extracts. The colonies were counted after 24 h. DIR13-2, DIR13-3, and DIR13-4 lines for *TaDIR13* transgene; DIR4-3, DIR4-4, and DIR4-9 lines for *TaDIR4* transgene; JA-2, JA-5 and JA-6 lines for *TaJAI1* transgene. Values are means of three different experiments, with SE bars. Probability values between the control and transgenic tobacco plants were estimated by the Student's *t* test, with significant difference at $**P_{0.01}$

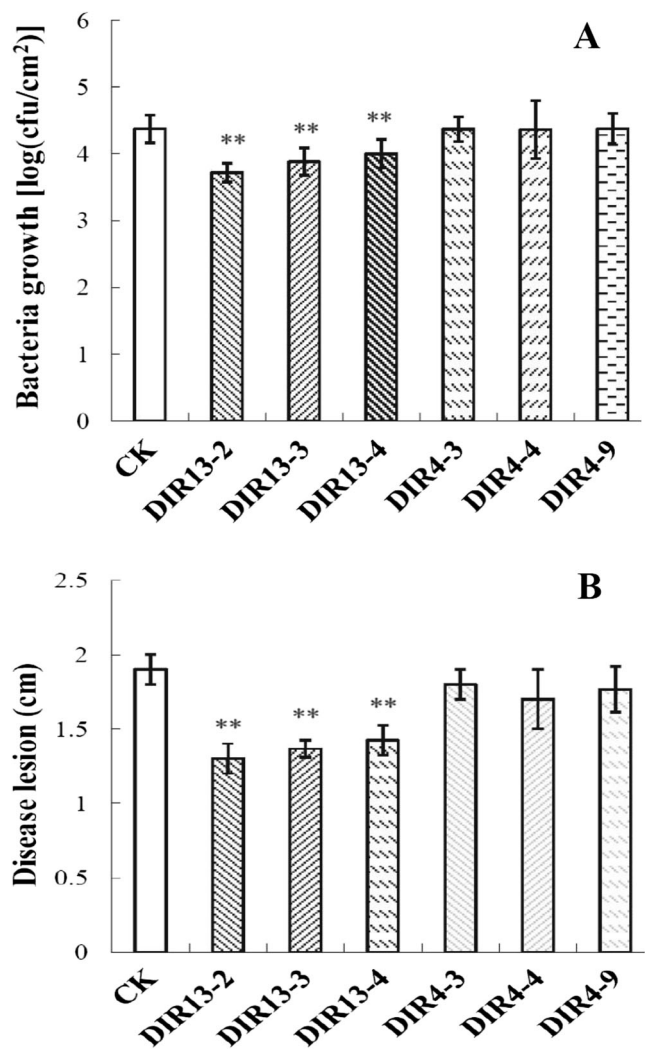


Fig. 6 Analysis of pathogen resistances to *Pseudomonas syringae* pv. *tabaci* and *Phytophthora parasitica* var. *nicotianae* in transgenic plants. **a** Resistance to *Pseudomonas syringae*. **b** Resistance to *Phytophthora parasitica*. Other symbols are the same as to Fig. 5

Overexpression of *TaDIR13* Affects Lignan Biosynthetic Genes

Initiating from coupling two coniferyl alcohol molecules into pinoresinol, lignan biosynthesis branches into two pathways. The branch-point enzymes for which are piperitol-sesamin synthase (PSS) and pinoresinol-lariciresinol reductase (PLR), respectively (Kim et al. 2009). As detected by RT-PCR with RNA from tobacco leaf tissues, *TaDIR13* tobacco lines exhibited strong expression of *NiPLR* but not *NiPSS* (Fig. 8a). The analysis from quantitative real-time PCR confirmed the elevated transcripts of *NiPLR* gene (Fig. 8b). These results were consistent with the observed lignan accumulation pattern (Figs. 3 and 7a). Overexpression of either *TaDIR4* or

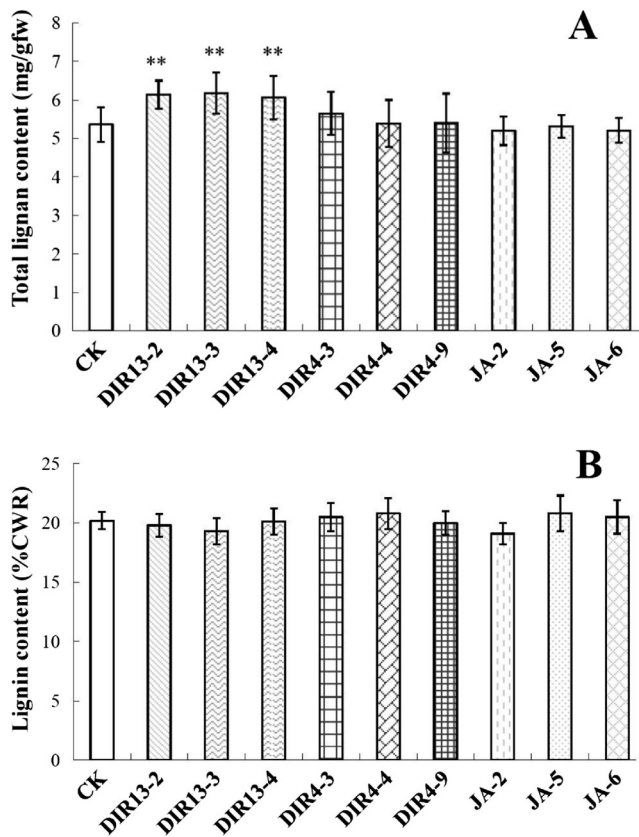


Fig. 7 Lignan and lignin contents in *TaDIR13*, *TaDIR4*, and *TaJAI* lines. **a** Lignan content. **b** Lignin content. Other symbols are the same as to Fig. 5

TaJAI showed no influence on *NtPLR* and *NtPSS* expressions (Fig. 8), consistent with the lignan measurement data for these transgenic plants (Fig. 7a).

Abundances of *NtCAD*, *NtCCR*, and *NtCOMT* transcripts were not affected in all transgenic plants, as detected either by RT-PCR (Fig. 9a) or quantitative real-time PCR (Fig. 9b) with RNA from tobacco stem tissues. This result was parallel to the unchanged lignin content in these plants (Fig. 7b). It is clear that although

lignin and lignan pathways share coniferyl alcohol as the common substrate, our analyses suggest that they are not closely linked to each other.

Discussion

Dirigent Proteins Promote Lignan Accumulation and Enhance Pathogen Resistance

Although dirigent proteins were found for more than a decade (Davin et al. 1997), its concert biological roles are still ambiguous. The data mining showed that homologous genes of *DirA* and *DirB/D* existed in wheat. From the phylogenetic tree of dirigent proteins (Fig. 1), we chose wheat *TaDIR13* and *TaDIR4*, as representatives of *DirA* and *DirB/D*, respectively, to investigate their functions.

We demonstrated that recombinant *TaDIR13* was able to direct the coniferyl alcohol to pinoresinol conversion (Fig. 3b), as reported for the *Forsythia* dirigent (Davin et al. 1997), confirming that *TaDIR13* is indeed a dirigent protein. In accordance with this result, overexpression of the *TaDIR13* gene was found to increase lignan accumulation (Fig. 7a). Further analyses revealed that *TaDIR13* was able to upregulate *PLR* expression, leading to a favored metabolite flux into the lignan pathway (Fig. 8). In other systems, there have been similar observations. During seed development in flax, accumulation of lignan secoisolariciresinol diglucoside was found to be parallel to *PLR* expression, suggesting *PLR*'s regulatory roles in lignan synthesis (Hano et al. 2006). Overexpression of *Forsythia PLR* gene in wheat elevated lignan levels for more than two times (Ayella et al. 2007). Mutation analysis in *Arabidopsis* showed the involvement of both dirigent protein and *PLR* in the enantiomeric control in lignan biosynthesis (Nakatsubo et al. 2008). Taken together, all these studies support the mechanism that dirigent regulates lignan synthesis together with activation of the *PLR* genes.

Fig. 8 Transcript abundance of genes (*NtPLR* and *NtPSS*) involved in the lignan biosynthesis in transgenic tobacco lines. Actin was included as an internal control and reference gene. Data for one line of each gene were shown. **a** Semi-quantitative RT-PCR. **b** Quantitative real-time PCR

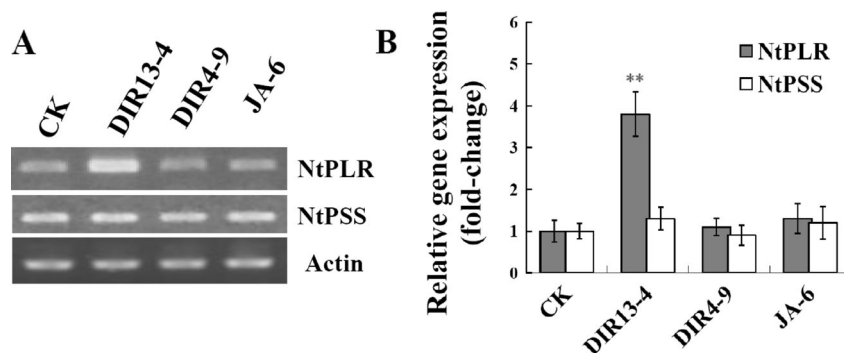
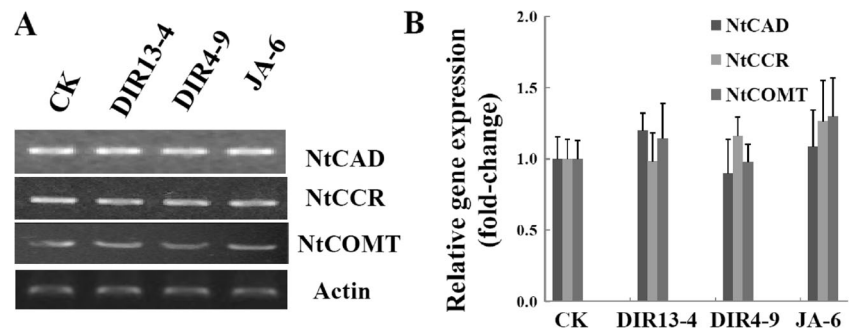


Fig. 9 Transcript abundance of genes (*NtCAD*, *NtCCR*, and *NtCOMT*) involved in the lignin biosynthesis in transgenic tobacco lines. Actin was included as an internal control and reference gene. Data for one line of each gene were shown. **a** Semi-quantitative RT-PCR. **b** Quantitative real-time PCR



On the contrary, lignin biosynthesis was not affected by *TaDIR13* overexpression, neither in lignin content (Fig. 7b) nor lignin biosynthetic genes (*CAD*, *CCR*, and *COMT*) (Fig. 9). This result provides further evidence that dirigent-directed pinosresinol formation is mainly, if not completely, involved in lignan biosynthesis. This is conceivable when spatial separation of lignin and lignan synthesis systems is considered; the former occurs in the cell wall compartment, while the latter in the cytoplasm (Davin et al. 2008; Vanholme et al. 2010; Bonawitz and Chapple 2010).

Lignans are thought to have the important physiological roles in defense process, particularly for their interaction with insects (Harmatha and Dinan 2003; Schroeder et al. 2006). However, their actions on pathogens remain to be elucidated. Constitutive expression of *DRR206* (originally categorized as a disease resistance gene in authors' paper, and we recognized it as a dirigent gene by sequence similarity analysis) increased resistance to blackleg disease (*Leptosphaeria maculans*) in transgenic canola (Wang et al. 1999). Our results demonstrated that lignan accumulation was increased when *TaDIR13* was overexpressed (Fig. 7a). Intensive investigations confirmed that the increased lignan level in transgenic tobacco enhanced its resistance to pathogens, both in in vitro (Fig. 5) and in vivo assays (Fig. 6). The current study on *TaDIR13* strengthens the functional connection of dirigent-regulated lignan synthesis to pathogen resistance.

Dirigent-Like Proteins Have Different Mechanisms for Stress Responses

TaDIR4 and *TaJA1*, both being dirigent-like proteins, did not exhibit to couple coniferyl alcohol into (+)-pinosresinol and promote lignan accumulation (Fig. 7a). Clearly, these proteins are functionally distinct to dirigent protein *TaDIR13*.

Many members of the DirB/D subclade were speculated to play roles in plants' responses to biotic and abiotic stresses. Examples include biotic and abiotic, such as *Gbd1* and *Gbd2* from cotton (*Gossypium hirsutum*) (Zhu et al. 2007), *BhDir1* from *B. hygrometrica* (Wu et al. 2009), and some pDIRs from spruce (see Fig. 1; Ralph et al. 2007). However, all pieces of evidence were from association analysis by gene expression

with relation to stress responses; hence, the roles that DirB/D proteins may play in stress responses are not clear. We demonstrated that *TaDIR4*, a dirigent-like protein of the DirB/D subclade, did not enhance pathogen resistance (Figs. 5; and 6). This is in sharp contrast to the functions of the dirigent protein *TaDIR13* but simply supports the proposed correlation between lignan and pathogen resistance. On the other hand, we observed that the transgenic *TaDIR4* plants exhibited longer roots than controls did on media supplemented with NaCl or mannitol (unpublished data), indicating better resistance to osmotic stress. Detailed studies for biotic and abiotic challenges are needed to understand the underlying mechanisms of DirB/D functions.

TaJA1, another dirigent-like protein, yet from the DirC subclade, was found to be responsible for the increased pathogen resistance in transgenic plants (Ma et al. 2010); but this function is achieved through *TaJA1*'s agglutinating activity by its jacalin domain (Ma et al. 2013), rather than through lignan metabolism (Fig. 7a). Most DirC proteins contain a dirigent-related domain in the N-terminus and a jacalin-related lectin (JRL) domain in the C-terminus (see review by Ma (2013) and references therein). Besides these chimeric DirC, some DirC sequences with only dirigent-related domain were also found in monocot plants, such as *SoDIR1* (Casu et al. 2004) and *ShDIR16* (Damaj et al. 2010) from sugarcane (*Saccharum hybrid*), and *OsDIRs* from rice (Ralph et al. 2007). What the dirigent-related domain does remains unknown.

In conclusion, our results suggest that dirigent and dirigent-like proteins are distinct in their biochemical action, in which dirigent proteins direct the coupling reaction of two molecules of coniferyl alcohol into pinosresinol, and this leads to increase lignan accumulation and pathogen resistance. Dirigent-like proteins have distinct subclades, in which DirB/D may be involved in stress response with an unknown mechanism; DirC increase disease resistance to pathogen by its jacalin-related lectin domain.

Acknowledgments This work was supported by grants from the National Natural Science Foundation of China (No. 31170277, No. 31370336, and No. 31070235), the Natural Science Foundation of Beijing (No. 5122024), and the Innovation Project of Chinese Academy of Sciences.

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