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A *Nicotiana benthamiana* AP2/ERF transcription factor confers resistance to *Phytophthora parasitica*

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Abstract

Diseases caused by *Phytophthora* species seriously affect global crop production and food security. Identification of key factors involved in plant resistance is valuable for disease management. Previously, we characterized the transcriptome of *Nicotiana benthamiana* which was infected with *Phytophthora parasitica*. Here, we selected *NbERF173,* one of the most strongly up-regulated genes of *N. benthamiana* in response to *P. parasitica* infection, for further investigation. First, *NbERF173* encodes a conserved transcription factor in our tested plant species. Second, overexpression of *NbERF173* in *N. benthamiana* enhanced its resistance to *P. parasitica*, and silencing of *NbERF173* significantly promoted the infection of both *P. parasitica* and *Botrytis cinerea*. Additionally, we demonstrated that NbERF173 can participate in reprograming of gene expression during *P. parasitica* infection and manipulate expression patterns of many defense-related genes, including two proteinase inhibitors encoding genes (*PI1-B* and *KTI1*). Furthermore, overexpression of *PI1-B* and *KTI1* strengthened plant resistance to *P. parasitica* and partially restored the deficiency in resistance of *NbERF173*-silenced *N. benthamiana* plants. Finally, we found that NbERF173 could not bind to the promoters of *PI1-B* and *KTI1* using yeast one-hybrid assay. Together, our results suggest that NbERF173 positively regulate the disease resistance, probably by reprograming of defense-related genes.

Keywords: *Nicotiana benthamiana*, *Phytophthora parasitica*, AP2/ERF, Proteinase inhibitor

Background

Phytophthora species belong to oomycetes and cause devastating diseases in numerous important agricultural and ornamental crops (Kroon et al. 2012; Kamoun et al. 2015). Increasing attention has been focused on *Phytophthora parasitica* (syn. *Phytophthora nicotianae* Breda de Haan), which has a broad host range and is responsible for serious damage to agricultural production and natural ecosystems (Meng et al. 2014). For disease management, the most effective method is to breed disease-resistant cultivars. However, the available resistance resource for *P. parasitica* is still limited and the resistance-related genes are rarely reported.

Nicotiana benthamiana, a highly tractable experimental model organism for research of plant pathology, appears to adopt a distinct strategy that is avoidance rather than resistance in response to pathogens. A mutation that occurred one million years ago gave rise to a dominant resistance-related gene (Rdr1) in *N. benthamiana,* enabling the plant to grow faster by shortening its growth cycle, although it possesses low resistance to most pathogenic organisms (Bally et al. 2015). *N. benthamiana* is also a frequently-used model plant for studying the interactions between hosts and *Phytophthora* species because it is highly sensitive to *Phytophthora* pathogens, and it is easy to implement virus-induced gene silencing and transient expression assays in this plant (Goodin et al. 2008). This phytopathology system will help us to understand not only the pathogenesis of *Phytophthora* pathogens but also the resistance mechanism of plants.

AP2/ERF (APETALA2/ethylene-responsive element binding factor) transcription factors belong to one of the largest plant transcription factor families, which are characterized by the conserved AP2/ERF DNA binding domain of 57–66 amino acid residues in length

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(Okamuro et al. 1997). Originally, the AP2/ERF family was reported to only exist in the plant kingdom, but recent studies have indicated that AP2/ERF genes are also present in protists and ciliates (Rashid et al. 2012; Licausi et al. 2013). The AP2/ERF family consists of four subfamilies (AP2, CBF/DREB, ERF, and RAV) based on their sequence similarities and numbers of AP2/ERF domain (Sakuma et al. 2002). Most proteins in the ERF family have a single AP2 domain (Nakano et al. 2006). Nevertheless, the AP2 family consists of members characterized by tandem repeats of one or two AP2 domains (Shigyo and Ito 2004). The RAV family proteins possess an ERF domain associated with a B3 DNA-binding domain (Swaminathan et al. 2008). A wide variety of *ERF* genes have been characterized, including *Arabidopsis thaliana AtERF1–5* (Fujimoto et al. 2000), *Lycopersicum esculentum LeERF1–4* (Tournier et al. 2003), *Glycine max GmERF3* (Zhang et al. 2008) and *Petunia hybrid PhERF2/3* (Liu et al. 2011). But their functions in *N. benthamiana* disease resistance are largely unknown.

Plant defense proteins, including proteinase inhibitors, play an important role in plant defense response, which have been well documented (Haq et al. 2004; Kim et al. 2009; Valdes-Rodriguez et al. 2010). For example, Proteinase inhibitors possess potent broad-spectrum growth-inhibitory activity against various plant pathogens (Ryan 1990; Kim et al. 2009). Transgenic rice plants expressing a potato proteinase inhibitor gene displayed enhanced resistance to two pathogens, *Magnaporthe oryzae* and *Fusarium verticillioides* by inhibiting fungal carboxypeptidases (Quilis et al. 2007). A pair of genes is highly induced by pathogen infection in both tobacco and tomato, and the corresponding proteins cause lysis of sporangia and growth inhibition of *P. infestans* (Woloshuk et al. 1991). *P. infestans* also cause an accumulation of serine protease inhibitors in potato tubers (Valueva et al. 1998; Valueva et al. 2003), but their roles in pathogen inhibition are not determined yet. Stacking two protease-inhibitor genes in transgenic tobacco plants showed resistance to both insects and pathogens, including *Pythium aphanidermatum* infection (Senthilkumar et al. 2010).

Previously, we found that many *N. benthamiana* ERF transcription factors were up-regulated during *P. parasitica* infection using RNA-Seq analysis (Shen et al. 2016), suggesting their potential role in resistance to *Phytophthora* pathogens. Here, we selected an ERF family member (*NbERF173*) that was strongly induced by *P. parasitica* infection for further characterization. Results revealed that overexpression or down-regulation of *NbERF173* altered the resistance levels to *P. parasitica* in transgenic *N. benthamiana* plants. Furthermore, we demonstrated that down-regulation of *NbERF173* could lead to constitutive expression impairment of two proteinase inhibitor encoding genes. Overall, these results indicate that *NbERF173* positively regulate disease resistance in *N. benthamiana* by affecting expression levels of defense-related genes.

Results

Characterization of *NbERF173* in *N. benthamiana* during *P. parasitica* infection

To precisely analyze the functions of ERFs, we designated all 266 ERF transcription factors in *N. benthamiana* as NbERF1 to NbERF266 (Additional file 1: Table S1). Based on the RNA-Seq data (Shen et al. 2016), we selected 64 genes that were up-regulated with > 2–folds changes upon *P. parasitica* infection for further analysis (Additional file 2: Table S2). Total RNA was isolated from *N. benthamiana* seedlings infected with *P. parasitica* zoospores at 0, 3, 6, and 9 h post inoculation (hpi), and used for further confirmation of the up-regulated *ERF* genes through RT-PCR with gene-specific primers. We successively validated their expression patterns by using qRT-PCR, and finally 9 *ERF* genes were successfully confirmed as up-regulated genes, including *NbERF173* (Additional file 2: Table S2). Furthermore, qRT-PCR results showed that expression of *NbERF173* was also significantly induced in response to *P. parasitica* infection and reached a peak at 6 hpi (Fig. 1a). Since *NbERF173* was up-regulated by more than 250 folds (Fig. 1a), we selected *NbERF173* for further functional characterization.

To isolate the full-length cDNA of *NbERF173*, we searched the corresponding protein in *N. benthamiana* genome using the gene ID (Niben101Scf01430g00007.1), and then confirmed by RT-PCR. Analysis of the obtained sequence (https://web.expasy.org/compute_pi/) revealed that *NbERF173* contained a 780-bp open reading frame encoding a 259-aa protein with a predicted molecular mass of 29.27 kDa (pI = 5.64). NbERF173 contains multiply nuclear localization signals (NLSs) predicted by software (http://nls-mapper.iab.keio.ac.jp/) and a nuclear export signal (NES) at residues from 203 to 212 using NetNES1.1 Server (http://www.cbs.dtu.dk/services/NetNES-1.1). Sequence blast and alignment showed that NbERF175, NbERF236, NbERF244, and NbERF246 are homologs of NbERF173 in *N. benthamiana*, but none of them exhibited induced expression during infection. Furthermore, NbERF173 shared varying degrees of similarity with other plant ERFs. We constructed a phylogenetic tree of these ERF proteins using the alignment results with the MEGA7 NJ method (Tamura et al. 2011). As shown in Fig. 1b, NbERF173 shared similarity of 86% with NtERF071 from *N. tabacum,* similarity of 73% with CaERF071 (Lee et al. 2004), 59% with SlERF2 (Tournier et al. 2003; Pirrello et al. 2006), and 50% with AtERF (NP_188299.1) from *Arabidopsis* (Buttner and Singh 1997) (Fig. 1b). As shown in Fig. 1c, NbERF173 was predicted to contain a conserved

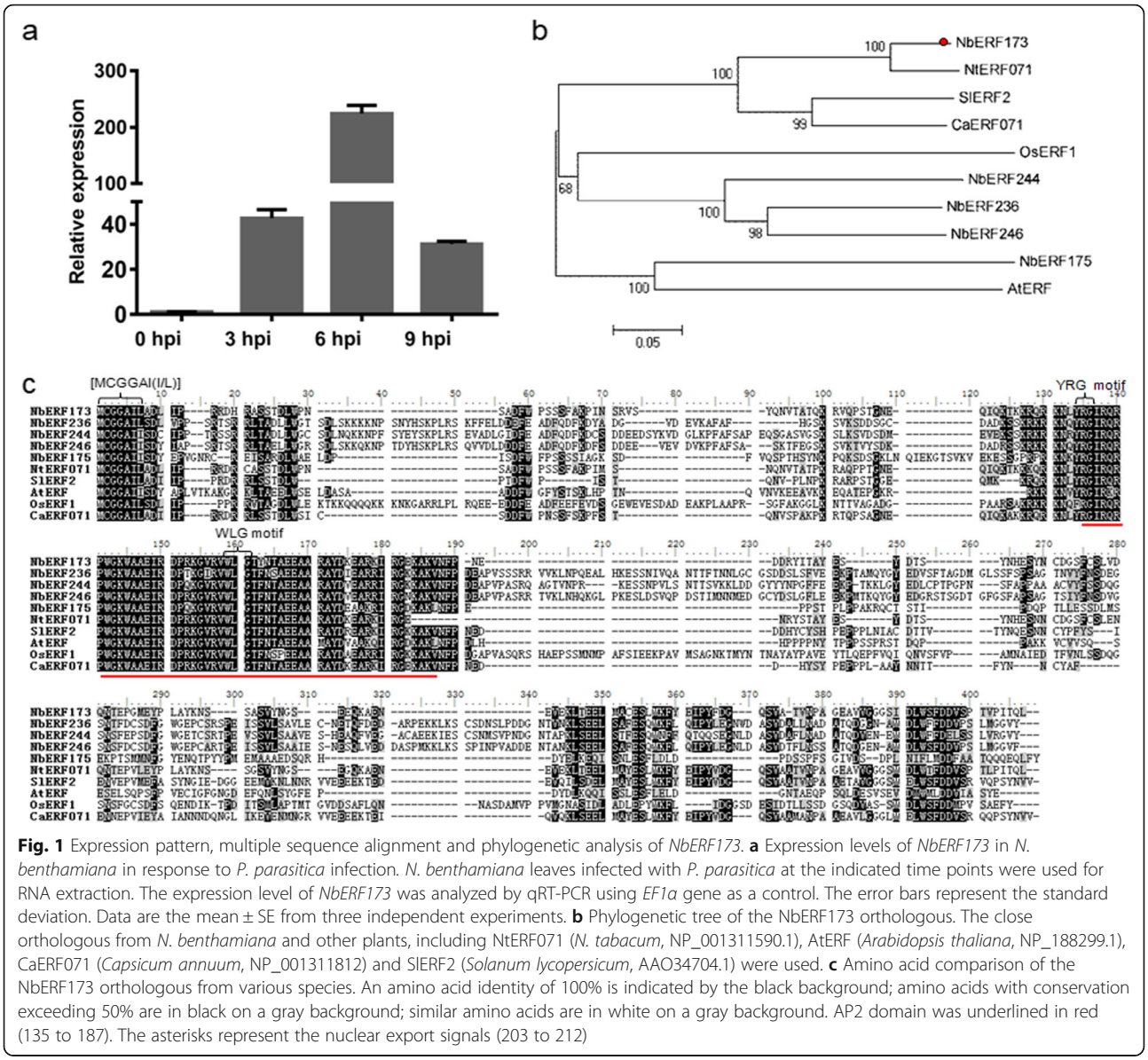

Fig. 1 Expression pattern, multiple sequence alignment and phylogenetic analysis of *NbERF173*. **a** Expression levels of *NbERF173* in *N. benthamiana* in response to *P. parasitica* infection. *N. benthamiana* leaves infected with *P. parasitica* at the indicated time points were used for RNA extraction. The expression level of *NbERF173* was analyzed by qRT-PCR using *EF1a* gene as a control. The error bars represent the standard deviation. Data are the mean ± SE from three independent experiments. **b** Phylogenetic tree of the NbERF173 orthologous. The close orthologous from *N. benthamiana* and other plants, including NtERF071 (*N. tabacum*, NP_001311590.1), AtERF (*Arabidopsis thaliana*, NP_188299.1), CaERF071 (*Capsicum annuum*, NP_001311812) and SlERF2 (*Solanum lycopersicum*, AAO34704.1) were used. **c** Amino acid comparison of the NbERF173 orthologous from various species. An amino acid identity of 100% is indicated by the black background; amino acids with conservation exceeding 50% are in black on a gray background; similar amino acids are in white on a gray background. AP2 domain was underlined in red (135 to 187). The asterisks represent the nuclear export signals (203 to 212)

AP2/ERF DNA binding domain with 52-aa residues, suggesting that NbERF173 belongs to the AP2/ERF family (Hao et al. 1998). It also contains a conserved [MCGGAI(I/L)] motif in the N-terminal region, suggesting that it belongs to the class IV of ERF family (Tournier et al. 2003). Interestingly, it has a conserved WLG motif, which exists only in a few ERFs (Li et al. 2015). The expression of this gene was induced by hormones, such as salicylic acid, jasmonic acid and ethylene (Additional file 3: Figure S1).

Overexpression of *NbERF173* in *N. benthamiana* enhanced its disease resistance

To investigate the function of *NbERF173* in plant disease resistance, we fused its full-length cDNA with a GFP-tag sequence at the N-terminus and generated a construct, pBINPLUS:GFP:NbERF173. *Agrobacterium tumefaciens*-mediated transient expression assay was implemented to express the GFP:NbERF173 fusion protein in *N. benthamiana* leaves. GFP protein alone, as a negative control, localized in both cytoplasm and nucleus, while the GFP:NbERF173 fusion protein localized only in the nucleus, which was confirmed by DAPI staining (Fig. 2a). After transient expression, the corresponding *N. benthamiana* leaves were inoculated with zoospore suspensions of *P. parasitica*. The lesion diameters were measured at 36 and 48 hpi. In contrast to the *GFP* expression control on one side of the leaves, expression of *NbERF173* on the other side developed significantly smaller lesions (Fig. 2b, c). These results indicated that

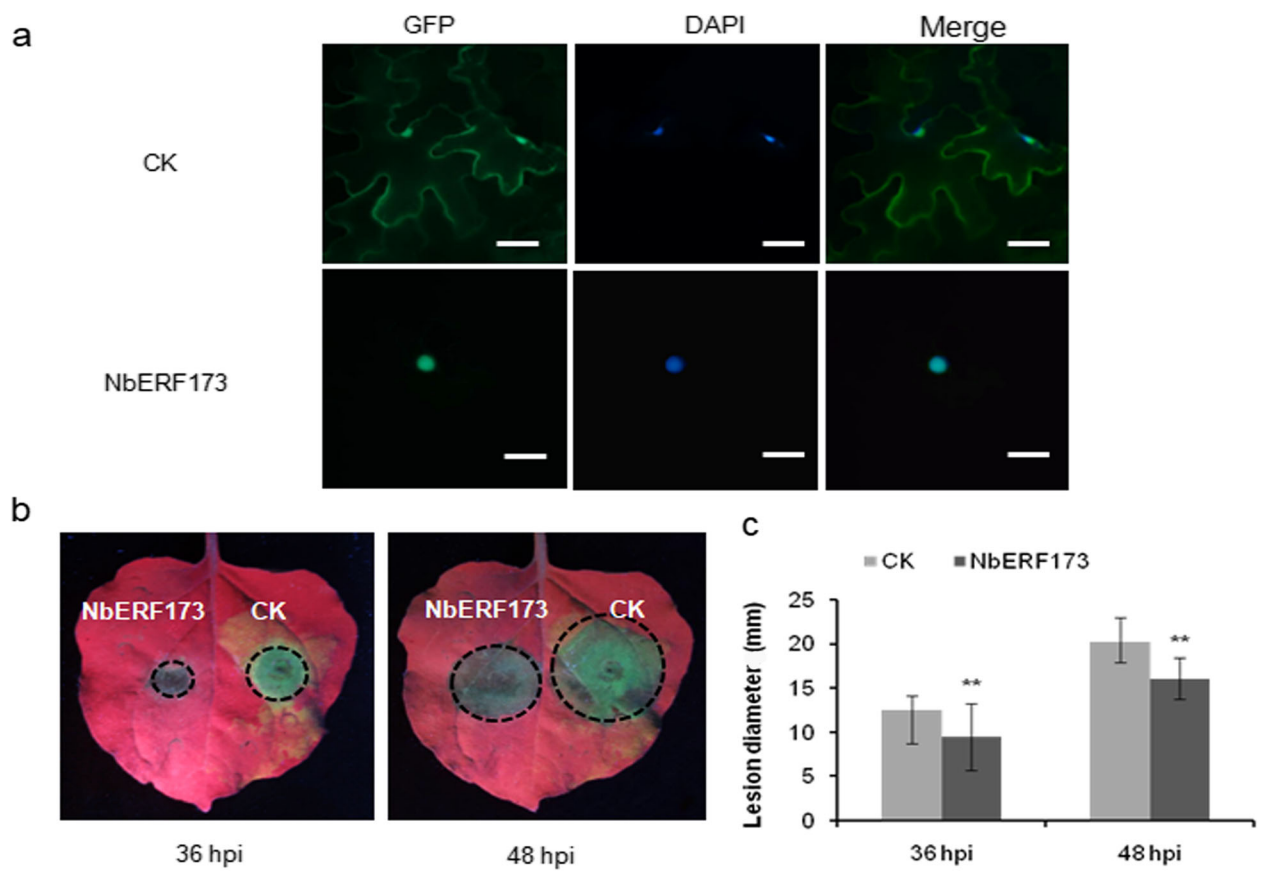

Fig. 2 Overexpression of *NbERF173* boosts resistance of *N. benthamiana* to *P. parasitica*. **a** Subcellular localization of NbERF173. The constructs, pBINPLUS:NbERF173 and pBINPLUS:GFP, were transiently expressed in *N. benthamiana* leaves. The photos were taken using a confocal microscope. The excitation wavelength used was 488 nm for GFP and 405 nm for DAPI. The scale bar indicates 50 μm. **b** Representative photographs of *N. benthamiana* leaves under infection. *A. tumefaciens*-mediated transient expression of CK (right) and NbERF173 (left) fusion proteins in *N. benthamiana* leaves. The infiltrated leaves (after 48 h) were inoculated with *P. parasitica* zoospores, and photographed at 36 hpi and 48 hpi, respectively. **c** Lesion diameters (mm) of *N. benthamiana* leaves. The data was calculated from three independent biological replicates using at least eight leaves each (** $P < 0.01$ compared with GFP; Dunnett's test)

overexpression of *NbERF173* enhanced disease resistance to *P. parasitica* in *N. benthamiana*.

Silencing of *NbERF173* impaired the disease resistance of *N. benthamiana*

Next, we silenced *NbERF173* by using virus-induced gene silencing (VIGS) assay in *N. benthamiana*. qRT-PCR analysis showed that the expression level of *NbERF173* was reduced by more than 96% in the *TRV:NbERF173* plants (Fig. 3a). In contrast, other homologous genes including *NbERF175*, *NbERF236*, *NbERF244* and *NbERF246* were not affected in the *NbERF173*-silenced plants, indicating the high specificity of gene silencing of *NbERF173* (Additional file 4: Figure S2). It is worth mentioning that *NbERF173*-silenced *N. benthamiana* seedlings displayed normal growth phenotype (Additional file 4: Figure S2). Afterwards, *NbERF173*-silenced *N. benthamiana* leaves were inoculated with *P. parasitica* zoospore suspensions. As shown in Fig. 3b, lesion diameters of the silenced leaves (*TRV:NbERF173*) were significantly larger than those of the controls (*TRV:GFP*) at 36 and 48 hpi (Fig. 3b). To dissect pathogen development, we stained the inoculated tissue by trypan blue to visualize hyphal extension in host cells. Consist with the above results, abundant *P. parasitica* hyphae were observed in *TRV:NbERF173* leaves while few hyphae were present in the control leaves (*TRV:GFP*) (Fig. 3c). Taken together, these results suggested that *NbERF173* is required for the resistance of *N. benthamiana* to *P. parasitica.* To further analyze the resistance conferred by *NbERF173*, we inoculated *NbERF173*-silenced *N. benthamiana* leaves with *Botrytis cinerea*, a typical necrotrophic fungal pathogen, and found that *NbERF173*-silenced plants also showed more sensitivity than the control leaves to *B. cinerea* infection (Fig. 3d). Collectively, NbERF173 positively regulates the resistance of *N. benthamiana* to plant pathogens.

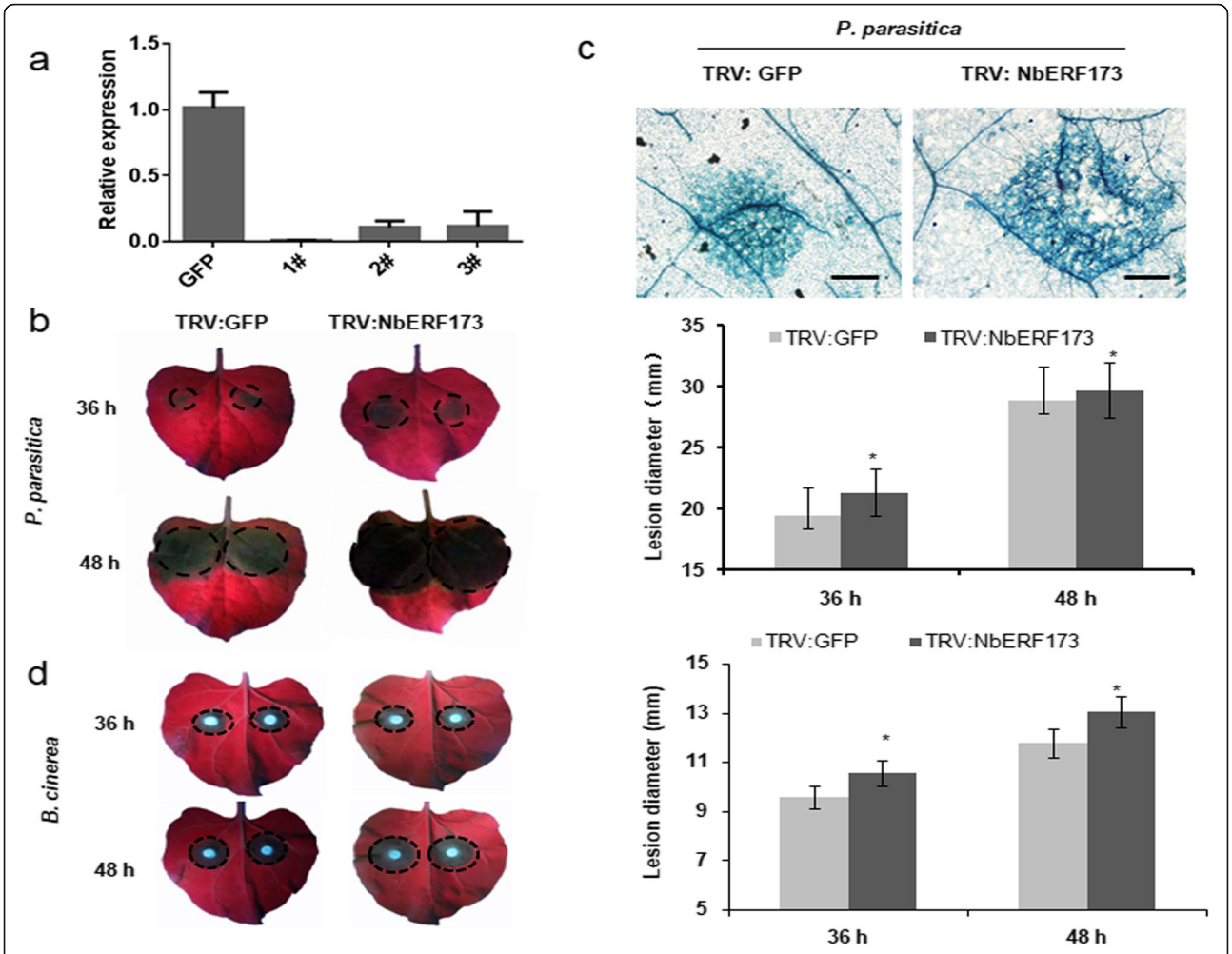

Fig. 3 The enhanced susceptibility of the *NbERF173*-silenced lines. **a** RT-PCR analysis of *NbERF173* expression in three independent silenced lines. To obtain the *NbERF173*-silenced lines, *Agrobacterium* strains harboring the TRV1 vector and TRV:GFP, TRV:PDS (phytoenedesaturase) or TRV:NbERF173 were mixed in a 1:1 ratio to achieve a final OD600 of 0.5 for each strain. The co-cultures were then infiltrated into the lower three leaves of 2-week-old plants. **b** Lesion diameters of the *NbERF173*-silenced leaves inoculated with *P. parasitica*. Lesion diameters of the *NbERF173*-silenced leaves inoculated with *P. parasitica* were measured at 36 and 48 hpi, and calculated from three independent biological replicates using at least eight leaves each (* $P < 0.05$ compared with GFP; Dunnett's test). **c** Trypan blue staining of the hyphal extension. The staining was performed at 24 hpi. **d** Infection of the *NbERF173*-silenced leaves with *B. cinerea*. Lesion diameters of the *NbERF173*-silenced leaves inoculated with *B. cinerea* were measured at 36 and 48 hpi, and calculated from three independent biological replicates using at least eight leaves each (* $P < 0.05$ compared with GFP; Dunnett's test)

Analysis of genes regulated by *NbERF173* during *P. parasitica* infection

To investigate the mechanism underlying the plant defense conferred by NbERF173, we compared the gene expression profiling between *NbERF173*-silenced and non-silenced plants at 0 and 6 hpi upon *P. parasitica* infection by using Illumina-based RNA-Seq assay. In the T6 treatment (*P. parasitica* infected *N. benthamiana* wild-type leaves for 6 h), 6205 genes (5364, 770, 60 and 11) were up-regulated and 8154 (6736, 895, 486 and 37) genes were down-regulated compared with T0 (the non-infected control leaves) (2-fold changes, $P < 0.05$) (Fig. 4a). Among the up-regulated *N. benthamiana* genes upon infection, we found that expression of only 71 genes (60 and 11) were repressed in the E0-T0 (*NbERF173*-silenced lines compared with the wild type without pathogen infection), and 781 genes (770 and 11) were down-regulated in the E6-T6 (*NbERF173*-silenced lines compared with the wild type upon pathogen infection for 6 h). Finally, we obtained 11 candidate genes (Additional file 5: Table S3) that are induced by pathogen, but suppressed in the *NbERF173*-silenced lines in both 0 and 6 hpi (Fig. 4a), including two proteinase inhibitors and two heat shock proteins. These genes were designated as defense-related genes that are positively regulated by NbERF173. Among the 8154 *N.*

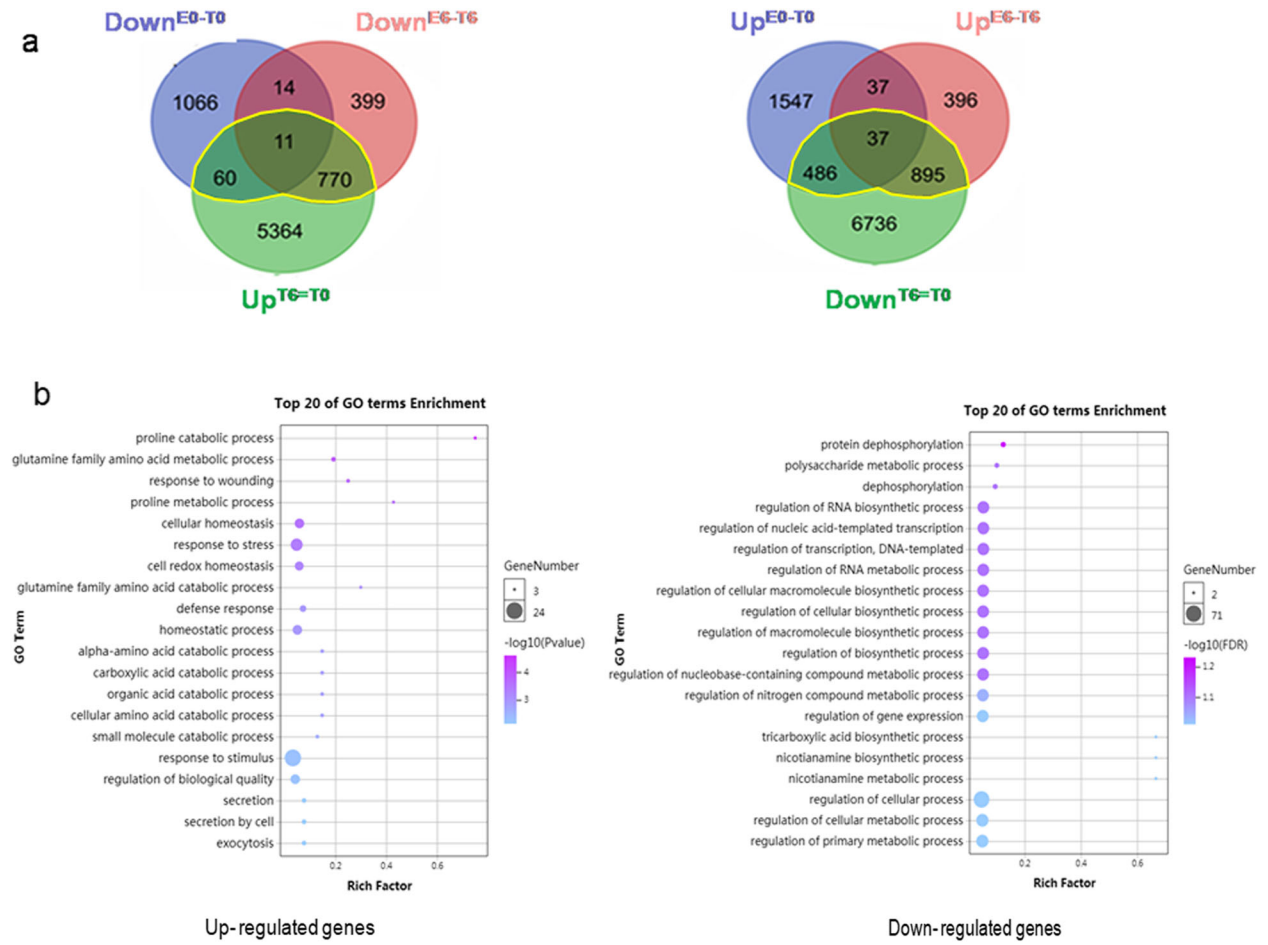

Fig. 4 Analysis of genes regulated by NbERF173 during *P. parasitica* infection. **a** Venn diagram representing the overlap among differentially expressed genes identified by RNA-Seq analysis. $Up^{T6=T0}$ or $Down^{T6=T0}$ represent gene numbers are up-regulated or down-regulated upon infection in the control plants (pTRV), respectively. $Down^{E0\text{-}T0}$, $Down^{E6\text{-}T6}$, $Up^{E0\text{-}T0}$ and $Up^{E6\text{-}T6}$ mean the comparisons of the different genes between the *NbERF173*-silenced (E0, E6) and control (T0, T6) plants at 0 hpi (E0, T0) or 6 hpi (E6, T6), respectively. The gene numbers are the genes that are the 2-fold changes between each pair of comparison. **b** GO analyses of DEGs in response to silencing of *NbERF173* and *P. parasitica* infection in yellow box. The top 20 enriched GO terms of up-regulated and down-regulated genes are shown on the graph. The top *X*-axis indicates DEGs numbers, and the bottom *X*-axis means −log10P-value. The *Y*-axis indicates GO category

benthamiana genes down-regulated by pathogen, 523 genes (486 and 37) were up-regulated in the E0-T0 (*NbERF173*-silenced lines compared with the wild type without infection) and 932 genes (895 and 37) were up-regulated in the E6-T6 (*NbERF173*-silenced lines compared with the wild type upon infection). In summary, 37 candidate genes (Additional file 6: Table S4) were negatively expressed upon infection, which could be reversed in the *NbERF173*-silenced plants. These genes encoded 2-succinylbenzoate-CoA ligase, MYC2 and NAC36 transcription factors. Collectively, a large number of genes are involved in the infection process, and we suggest that NbERF173 plays an important role in regulation of gene expression.

Next, GO enrichment analysis was performed to identify differentially expressed gene sets in response to silencing of *NbERF173* and *P. parasitica* infection based on GO category. The up-regulated genes on the left pattern of Fig. 4b showed genes that were both up-regulated in *P. parasitica* infected *N. benthamiana* and down-regulated in *NbERF173*-silenced lines (770, 60 and 11). The down-regulated genes on the right pattern of Fig. 4b showed genes that were both down-regulated in *P. parasitica* infected *N. benthamiana* and up-regulated in *NbERF173*-silenced lines (895, 486 and 37). The top 20 enriched GO terms were determined (Fig. 4b), among which "response to stress and stimulus" was the most abundant term in the up-regulated GO categories, while "regulation of biosynthetic process" and "regulation of cellular process" were the dominant terms in the down-regulated genes.

Expression of two proteinase inhibitor genes was repressed in *NbERF173*-silenced lines

Based on RNA-Seq results, we selected 7 up-regulated and 5 down-regulated genes involved in defense responses for qRT-PCR validation (Additional file 7: Table S5). The results manifested that these genes exhibited similar expression patterns compared with those derived from RNA-Seq approach (Fig. 5), highlighting the accuracy and reproducibility of the RNA-Seq analysis in this study. As shown in Fig. 5, expression levels of 4 genes were significantly ($P < 0.01$) reduced in the *NbERF173*-silenced lines compared with the non-silenced lines at 0 and 6 hpi, which encode proteinase inhibitor I-B (PI1-B), myb-related protein 305 (MYB305), Kunitz trypsin inhibitor 1 (KTI1) and chaperone protein HtpG, respectively. Notably, the expression levels of two protease inhibitor genes in *NbERF173*-silenced *N. benthamiana* were less than one-fifth of those in control plants (pTRV:GFP). In the previous RNA-Seq data (Shen et al. 2016), *PI1-B* and *KTI1* were also up-regulated upon *P. parasitica* infection, suggesting their potential roles in plant defense response. Furthermore, we demonstrated that the expression of *PI1-B* and *KTI1* in *NbERF173*-overexpressed leaves was up-regulated by performing qRT-PCR assay (Additional file 8: Figure S3). Taken together, these results suggested that NbERF173 tightly regulates the expression of *PI1-B* and *KTI1*.

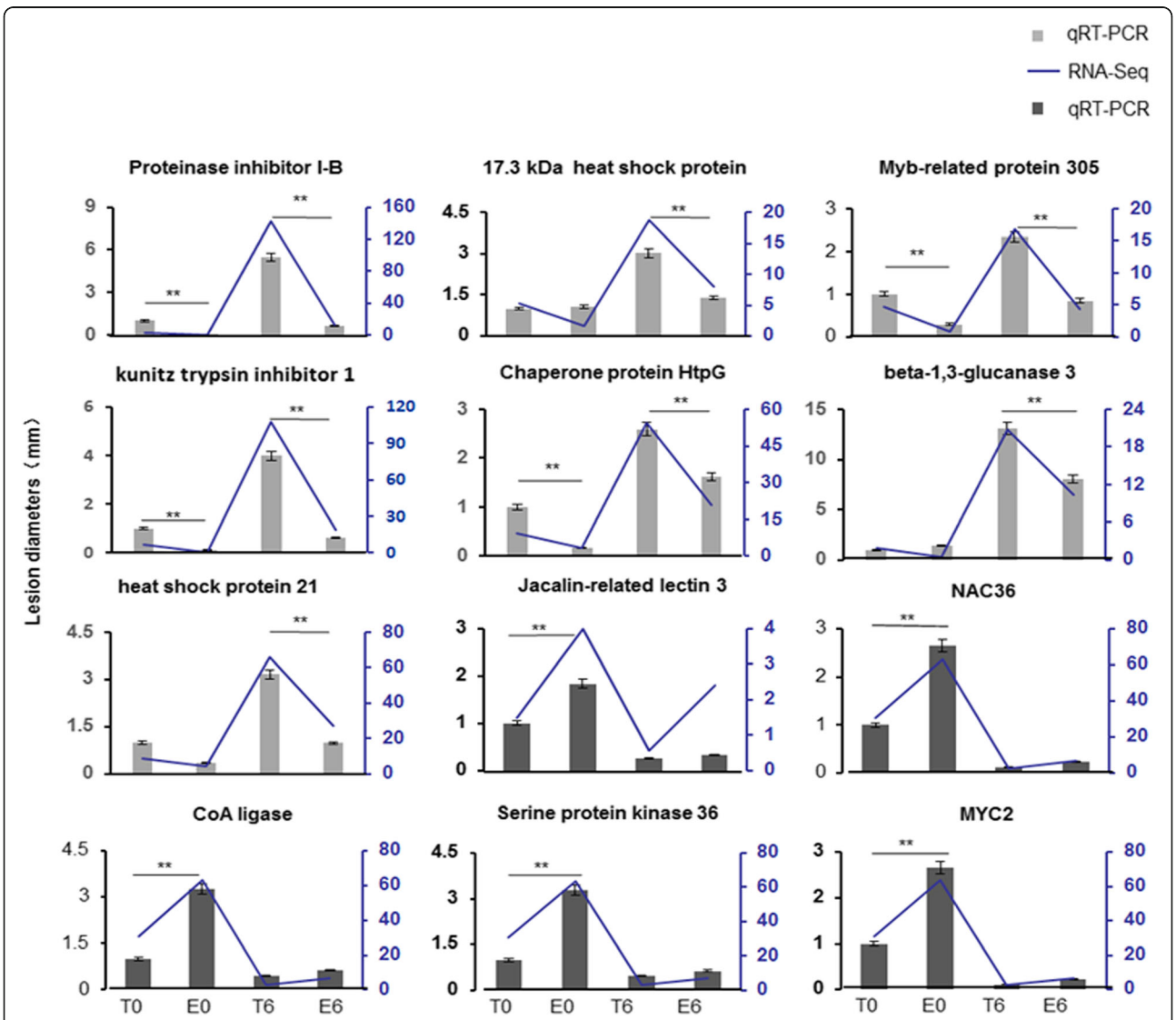

Fig. 5 Validation of the RNA-Seq data by qRT-PCR. The relative expression levels of the indicated genes were verified by qRT-PCR with *EF1a* gene as a reference. T0 and T6 are the control plants (pTRV:GFP) without (T0) or with pathogen at 6 hpi (T6). E0 and E6 are the *NbERF173*-silenced plants without (E0) or with pathogen at 6 hpi (E6). Error bars represent the SD for three independent experiments (**$P < 0.01$ compared with GFP; Dunnett's test). The left *Y*-axis represents the relative expression levels measured by qRT-PCR and the right means data of RNA-Seq

Since proteinase inhibitors were reported to play an important role in defense response against pathogens (Carrillo et al. 2011; Majeed et al. 2011), we speculated that NbERF173 possibly confers the resistance to *P. parasitica* through positively regulating the expression of *PI1-B* and *KTI1* in *N. benthamiana*. To verify our hypothesis, we firstly performed *A. tumefaciens*-mediated transient expression of these two genes in *N. benthamiana*. Afterwards, we found that overexpression of these two PIs (PI1-B and KTI1) in *N. benthamiana* enhanced plant resistance to *P. parasitica* (Fig. 6). Moreover, the deficiency of *NbERF173* gene expression significantly reduced the resistance of *N. benthamiana* to *P. parasitica* (Fig. 3b), but overexpression of thses two PIs in *NbERF173*-silenced lines could partially restore the resistance to *P. parasitica* (Fig. 6). Therefore, these results indicated that NbERF173 confers resistance to *P. parasitica* probably by regulating two *PI* genes expression.

NbERF173 has no binding activities to the promoters of two proteinase inhibitors

To determine whether there are direct physical interactions between NbERF173 and the promoters of two *PI* genes (Additional file 9: Table S6), we conducted the yeast one-hybridization (Y1H) experiment. In the Y1H assay, yeast transformant harboring pGADT53m × pHIS53 serves as a positive control, and pGADT53m × pHIS2 serves as a negative control. The recombinant bait plasmids by pGAD–NbERF173 × pHIS2, pGAD empty×pHIS2–PPI1-B (promoter region of the *PI1-B* gene) and pGAD empty×pHIS2–KTI-B (promoter region of the *KTI* gene) did not grow well in medium without histidine, suggesting they didn't have self-activating activities. Unfortunately, yeast transformants harboring pGADT7–NbERF173 × pHIS2–PPI1-B or pGADT7–NbERF173 × pHIS2–KTI-B also did not grow well in medium without histidine (Additional file 10: Figure S4), indicating that NbERF173 could not bind the promoters of the two *PI* genes. In other words, NbERF173 may indirectly regulate the two *PI* genes expression or need other components as partners to accomplish its function.

Discussion

Previous studies on *ERF* genes are mainly focused on their involvement in plant growth and development. For example, overexpression of rice *OsERF1* affects growth and development in *Arabidopsis* (Hu et al. 2008), SlERF2, a tomato ethylene response factor, is involved in seed germination (Pirrello et al. 2006). ERFs also function in transcriptional regulation of ethylene biosynthesis and hormone responses. For example, SlERF2 is involved in ethylene response (Pirrello et al. 2006). In addition, TERF2/LeERF2 enhances tomato tolerance to freezing

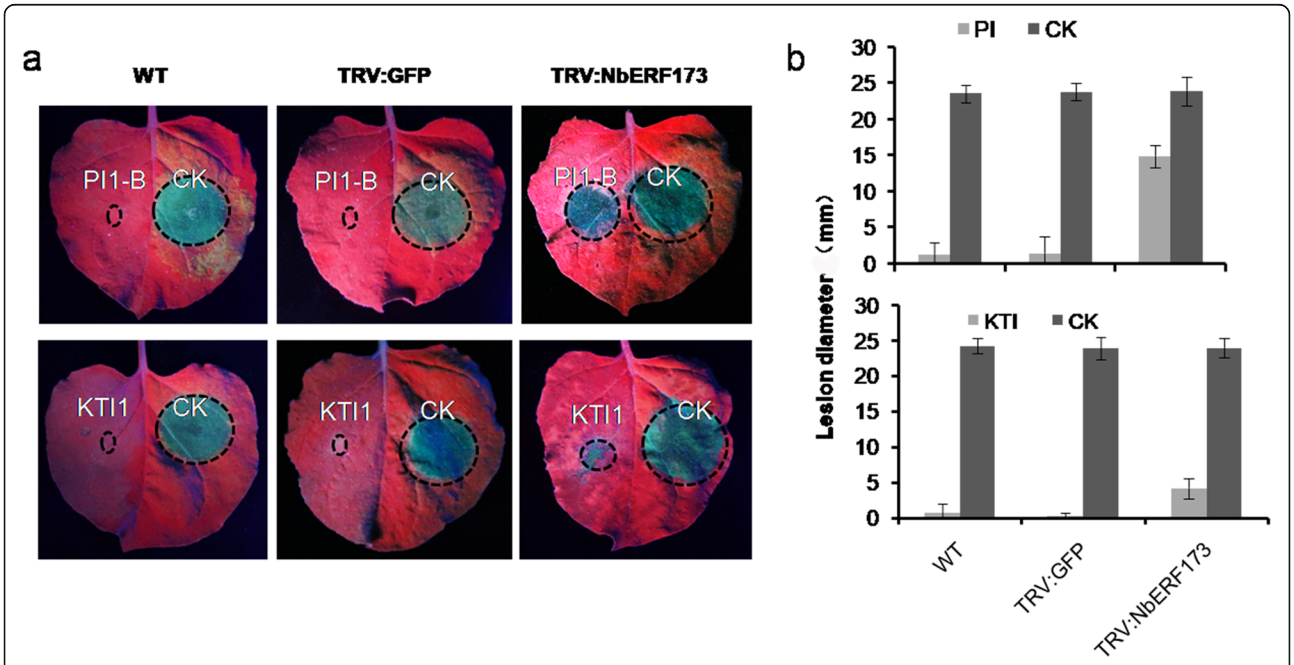

Fig. 6 Overexpression of two *PIs*-encoding genes can partially rescue the resistance reduction caused by *NbERFI73* silencing in *N. benthamiana*. **a** Phenotypes of the infected *N. benthamiana* leaves. *A. tumefaciens*-mediated transient expression of CK (right) or PIs proteins (left) were performed in the *N. benthamiana* leaves. After 48 h of infiltration, *P. parasitica* zoospores were inoculated, and the photos were taken at 48 hpi. **b** Lesion diameters at 48 hpi. The corresponding data was measured and calculated from three independent biological replicates using at least eight leaves each (** $P < 0.01$, Dunnett's test)

(Zhang and Huang 2010). However, ERFs that function in disease resistance are not fully studied, especially in *N. benthamiana*. In this study, we systematically identified the *N. benthamiana* ERFs, and found NbERF173 conferred *N. benthamiana* resistance to *P. parasitica* with induced expression in the early stages during infection. Overexpression of *NbERF173* in *N. benthamiana* enhanced its resistance to *P. parasitica*. Furthermore, suppressed expression of *NbERF173* by VIGS led to a dramatic reduction in *N. benthamiana* resistance to *P. parasitica* and *B. cinerea*. These results suggested that NbERF173 participates in *N. benthamiana* resistance to pathogens.

Some ERFs are involved in plant defense responses against pathogen infection by regulating different gene sets, including pathogenesis-related genes (Shin et al. 2002; Gutterson and Reuber 2004). For example, *CaPTI1* from *Capsicum annuum* is involved in the regulation of defense response to *P. capsici* (Jin et al. 2015). The wheat pathogen-induced ERF transcription factor TaPIE1 positively mediates host responses to a necrotrophic pathogen *Rhizoctonia cerealis* (Zhu et al. 2014). Interestingly, it also regulates resistance of freezing stress by activating a subset of defense- and stress-related genes downstream of the ethylene signaling pathway. Our results revealed that NbERF173 acted as a positive regulator for defense against both semibiotrophic and necrotrophic pathogens. GmERF5 is a soybean EAR motif-containing ERF and binds to the GCC-box element to act as a repressor of gene transcription. Its overexpression in soybean confers high resistance to *P. sojae* (Dong et al. 2015). Unfortunately, we failed to obtain the target *cis*-elements of NbERF173 using RNA-seq data, which is perhaps caused by the fact that many genes are indirectly regulated. We found that a majority of genes was negatively regulated by NbERF173, which are enriched in GO terms of "regulation of biosynthetic process" and "regulation of cellular process". It will be interesting to illustrate whether NbERF173 acts as a repressor or activator of gene transcription. Another soybean ERF, GmERF113, also positively regulates *P. sojae* resistance. It interacts with a basic helix-loop-helix transcription factor to control expression of the pathogenesis-related genes (Zhao et al. 2017). Many defense-related genes were identified to be manipulated by NbERF173 in this study. Additional studies of mechanisms are needed to clarify its direct integration with the *cis*-elements or combination with other essential regulators.

It has been well documented that PIs possess potent and broad-spectrum growth-inhibitory activity against plant pathogens or insects (Ryan 1990; Haq et al. 2004; Kim et al. 2009; Valdes-Rodriguez et al. 2010). Transgenic plants have been generated and their resistance to pathogens has also been tested (Schlüter et al. 2010). Numerous reports have underlined the potential of plant PIs for the utilization in genetically modified plants for disease-resistance (Benchabane et al. 2010; Schlüter et al. 2010; Senthilkumar et al. 2010). However, whether these genes are regulated by other components is largely unknown. In our study, NbERF173 affected the expression of defense-related genes. To analyze the mechanism of NbERF173 in conferring the resistance of *N. benthamiana* to *P. parasitica*, we used Illumina-based RNA-Seq assay to analyze gene expression profiling between *NbERF173*-silenced and non-silenced *N. benthamiana* leaves. Finally, we identified two *PI genes* in *N. benthamiana* which were positively regulated by NbERF173. We found overexpression of two *PIs* (*PI* and *KTI*) in wild-type *N. benthamiana* enhanced its resistance to *Phytophthora* infection, which is consistent with previous studies. Furthermore, overexpression of *PI* and *KTI* in *NbERF173*-silenced leaves partially restored the resistance, suggesting they might act at the downstream of NbERF173. Unfortunately, we found that NbERF173 failed to directly bind to the promoters of *PI* and *KTI*. We speculate that NbERF173 may indirectly regulate the two *PI* genes expression or the regulation needs other component as partners, which need to be addressed in the future.

Conclusions

In this study, we identified a *N. benthamiana ERF* gene *NbERF173* that plays a positive role in plant resistance to *Phytophthora* pathogens. NbERF173 can regulate expression of many defense-related genes, including proteinase inhibitors. Our results revealed a potential mechanism for *NbERF173* regulating the disease resistance of *N. benthamiana*.

Methods

Microbial strains, plants, and culture conditions

P. parasitica (isolate Pp025) was cultured for 3 days on 2.5% vegetable (V8) juice medium at 25 °C in the dark (Dou et al. 2008). To prepare *P. parasitica* zoospores, the mycelia were cultured in 2.5% liquid V8 juice medium for 3 days and washed five times with sterilized water at room temperature and incubated at 25 °C until sporangia formed. To initiate zoospore release, fresh cold sterilized water (4 °C) was added and the plates were incubated at 4 °C. The zoospore concentration was estimated with a hemocytometer. *N. benthamiana* was grown at 25 °C under a photoperiod of 16 h light/8 h dark in an environmentally controlled growth room.

Bioinformatics analysis and primer design

The produced clean reads were mapped to the *N. benthamiana* V1.0.1 reference genome (https://solgenomics.net/organism/Nicotiana_benthamiana/genome)

using TopHat (Trapnell et al. 2009) software with default parameters. Only the reads that could be uniquely mapped to the *N. benthamiana* genome were used for subsequent processing. The retained reads were quantified using Cufflinksv1.0.3 program (Trapnell et al. 2009), and the expression level of each gene was calculated by normalizing to the fragment perkilobase of exon per million mapped reads (FPKM) value. Weakly expressed genes were removed, and only genes with FPKM larger than 1 were included in the analysis. Differentially expressed genes were identified using the GFOLD algorithm (Feng et al. 2012), which was biologically meaningful for single replicate experiments. Genes with four fold change (GFOLD [1or-1]) were considered differentially expressed between two samples. MapMan ontology tool was used to obtain an overview of *N. benthamiana* genes involved in metabolic pathway in which a plant-specific ontology classifies genes into hierarchical categories and was denominated BIN (Thimm et al. 2004). *N. benthamiana* genes were assigned to BINs using the Mercator automated annotation pipeline. Then riched Mapman BINs were identified using Fisher's exact test.

The multiple sequence alignment was constructed by CLUSTALW, and the phylogenetic tree was constructed using the MEGA 5.0 software. Protein domain and motif analyses were conducted using the NCBI conserved domain database and Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). All primers used for RT-PCR, qRT-PCR, and plasmid construction were designed by Primer Premier 5 software (Additional file 11: Table S7). The primer specificity was evaluated by sequence similarity comparison. The reaction conditions were optimized by determining the optimal annealing temperature and primer concentration.

The whole genome and protein sequences of *N. benthamiana* were downloaded from the National Center for Biotechnology Information (NCBI). The protein sequences of putative NbERFs in *N. benthamiana* were aligned with the Clustal X program (version 1.83) using the default parameters. Phylogenetic trees were constructed using the aligned result with the Neighbor-Joining (NJ) method in MEGA7 software (version 7.0.2.6, Tamura et al. 2011). Subcellular localization signal analysis was conducted using NetNES 1.1 Server. (http://www.cbs.dtu.dk/services/NetNES-1.1).

RNA extraction and RT-PCR analysis

Six-week-old *N. benthamiana* leaves were detached and immersed in suspensions of *P. parasitica* containing 1×10^5 zoospores/mL. Leaves were collected at 0, 3, 6, and 9 hpi and frozen in liquid nitrogen. The RNA was extracted with Trizol reagent (TIANGEN), following the manufacturer's instructions, and then treated with DNase I at 37 °C for 30 min. RNA (1 μg) was then reverse-transcribed using a PrimeScript First-strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. A 20 μL aliquot of cDNA was diluted to 60 μL with water, and then 2 μL of the diluted cDNA was used for the analyses. For real-time quantitative RT-PCR (qRT-PCR) analysis, gene-specific primers for *NbERF* genes were designed and synthesized by GenScript (Nanjing) (product size 110–130 bp; Tm 59 °C–61 °C, *EF1α* was used as an internal control. All reactions were performed on an Icycleri Q5 system (Bio-Rad), using the SYBR Green SuperMix Kit (Vazyme Biotech) according to the manufacturer's instructions. The expression levels of these genes were calculated as $2^{-\Delta CT}$ values. The relative temporal expression levels of each gene were also calculated as $2^{-\Delta CT}$ values. At least three biological replicates were used for the fluorescence-quantitative PCR reactions, with each biological repeat having at least three technical replicates.

RNA sequence (RNA-seq) profiling

The *NbERF173*-silenced *N. benthamiana* leaves at 6 hpi and the mock-treated leaves were selected for RNA-Seq analysis. Total RNA was isolated using the Trizol reagent (TIANGEN) according to the manufacturer's instructions and then treated with DNase I (RNase free, TaKaRa) to remove genomic DNA contaminations. The RNA samples from three biological replicates for each sample were pooled and sequenced with 100 bp paired-end reads on Illumina HiSeq 2000 platform.

Agrobacterium-mediated transient expression in *N. benthamiana* leaves

The cDNA fragments of *NbERF173* and proteinase inhibitors (PIs) were cloned into pBINPLUS with a GFP-tag (Song et al. 2015). Transformation of *Agrobacterium* GV3101 by electroporation and infiltration of *Agrobacterium* suspensions were done as described by Asai et al. (2008).

Subcellular localization of NbERF173

To observe the subcellular localization of green fluorescent protein pBINPLUS:NbERF173 fusion protein, *A. tumefaciens*-mediated transient expression assays were used to express GFP (CK) and GFP:NbERF173 fusion proteins in *N. benthamiana*. After treatment for 48 h, *N. benthamiana* leaves treated with *A. tumefaciens* were infiltrated with 5 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) solution. The infiltrated plant leaves were cut into small squares and mounted in water under a cover slip. Fluorescence and DAPI staining were visualized by epifluorescence microscopy using ultraviolet light. The excitation wavelength was 488 nm for GFP and 405 nm for DAPI (Song et al. 2015). The GFP transgenic *N. benthamiana* leaves were used as controls.

Virus-induced gene silencing

VIGS of *N. benthamiana* was performed as described previously (Ratcliff et al. 2001). Briefly, an *NbERF173* fragment exhibiting high sequence similarity to other members of this family was cloned in an antisense manner into the tobacco rattle virus (TRV) vector to create a silencing construct (pTRV:NbERF173). *Agrobacterium* strains harboring the TRV1 vector and TRV:GFP, TRV:PDS (phytoenedesaturase) or TRV:NbERF173 were mixed in a 1:1 ratio to achieve a final OD600 of 0.5 for each strain. The co-cultures were then infiltrated into the lower three leaves of 2-week-old plants. VIGS was done as described by Ratcliff et al. (2001). Additional file 10: Table S7 lists the primers used to amplify cDNA fragments from the *N. benthamiana* cDNA library (Yoshioka et al. 2003). Restriction sites were added to the 5′-ends of the forward and reverse primers for cloning into the TRV vector pTRV2.

Phytophthora infection assays

To assay the *P. parasitica* infection of gene-silenced *N. benthamiana* leaves 2 weeks after infiltrating *Agrobacterium* strains harboring vectors for VIGS, the upper leaves corresponding to the photo-bleached leaves in plants separately infected with TRV:PDS were detached and used for qRT-PCR analysis and *P. parasitica* inoculation assays. The lesion diameters were measured at 36 and 48 hpi. Significant differences were identified using *t*-tests. For the *B. cinerea* inoculation assays, the lesion diameters were measured at 36 and 48 hpi and significant differences were identified using *t*-tests. To analyze pathogen development, the hyphal extension in the host cells was visualized by trypan blue staining (Yoshioka et al. 2003). At 24 hpi, abundant *P. parasitica* hyphae were observed in tissues of *TRV:NbERF173* leaves.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s42483-020-0045-3.

Additional file 1: Table S1. The ERF family of *N. benthamiana*.

Additional file 2: Table S2. The RT-PCR results of some *N. benthamiana* ERF factors.

Additional file 3: Figure S1. Expression profiles of *NbERF173* in leaves in response to salicylic acid (SA), jasmonic acid (JA) and ethylene (ETH) treatment. The expression levels at each time point were normalized by the expression levels in the samples treated with fresh water and harvested at the corresponding time point (control). Three biological replications were conducted. The error bars represent the standard deviation (*, $P < 0.05$; ** $P < 0.01$, Dunnett's test).

Additional file 4: Figure S2. Validation of gene silencing specificity. **a** Relative expressions of *NbERF173* homologous. RNA-Seq data of *NbERF236*, *NbERF244*, *NbERF246* and *NbERF175* were compared in the *NbERF173*-silenced and control leaves. **b** VIGS-treated *N. benthamiana* seedlings. The *N. benthamiana* seedlings were treated with pTRV:PDS (a positive control to silence the phytoene desaturase gene line), pTRV:NbERF173 and pTRV:GFP (the negative control).

Additional file 5: Table S3. Eleven candidate genes were up-regulated by NbERF173.

Additional file 6: Table S4. Thirty-seven candidate genes were down-regulated by NbERF173.

Additional file 7: Table S5. The downstream genes regulated by NbERF173.

Additional file 8: Figure S3. The relative expression levels of proteinase inhibitor I-B (*PI1-B*) and Kunitz trypsin inhibitor 1 (*KTI1*) were verified in the *NbERF173*-overexpressed and control leaves by qRT-PCR with *EF1a* gene as a reference.

Additional file 9: Table S6. The promoter sequences of the two proteinase inhibitors.

Additional file 10: Figure S4. Yeast one-hybrid assay of NbERF173 binding to promoters of two *PI* genes. PPI1-B and PKTI indicate the promoters of the two *PI* genes, respectively. The left yeast colonies showed that yeasts were grown on SD medium (−Leu-Trp). The right colonies indicated that yeasts were grown on SD medium (−His-Leu-Trp), suggesting of positive binding activities. The combination of pGAD53m/pHIS2 was used as negative controls while the combination of pGAD53m/pHiS53 was used as positive controls.

Additional file 11: Table S7. The primers involved in this study.

Abbreviations

ERF: Ethylene response factor; KTI1: Kunitz trypsin inhibitor 1; PDS: Phytoene desaturase; PI1-B: Proteinase inhibitor I-B; TF: Transcription factor; TRV: Tobacco rattle virus; VIGS: Virus induced gene silencing; Y1H: Yeast one-hybridization

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Not applicable.

Authors' contributions

JY and CC conceived and designed the experiments. YJ and WL conducted the plant inoculation and RNA extraction. TB conducted the qRT-PCR validation and statistics analysis on experimental data. GA and XZ performed all the bioinformatics analysis. YC and DD provided technical support and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

Asai S, Ohta K, Yoshioka H. MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in Nicotiana benthamiana. Plant Cell. 2008;20:1390–406.

Bally J, Nakasugi K, Jia F, Jung H, Ho SYW, Wong M, et al. The extremophile *Nicotiana benthamiana* has traded viral defence for early vigour. Nat Plants. 2015;1:15165.

Benchabane M, Schlüter U, Vorster J, Goulet MC, Michaud D. Plant cystatins. Biochimie. 2010;92:1657–66.

Büttner M, Singh KB. *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. Proc Natl Acad Sci U S A. 1997;94:5961–6.

Carrillo L, Martinez M, Álvarez-Alfageme F, Castañera P, Smagghe G, Diaz I, et al. A barley cysteine-proteinase inhibitor reduces the performance of two aphid species in artificial diets and transgenic *Arabidopsis* plants. Transgenic Res. 2011;20:305–19.

Dong L, Cheng Y, Wu J, Cheng Q, Li W, Fan S, et al. Overexpression of *GmERF5*, a new member of the soybean EAR motif-containing ERF transcription factor, enhances resistance to *Phytophthora sojae* in soybean. J Exp Bot. 2015;66:2635–47.

Dou D, Kale SD, Wang X, Jiang RHY, Bruce NA, Arredondo FD, et al. RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. Plant Cell. 2008;20:1930–47.

Feng J, Meyer CA, Wang Q, Liu JS, Liu SX, Zhang Y. GFOLD: a generalized fold change for ranking differentially expressed genes from RNA-seq data. Bioinformatics. 2012;28:2782–8.

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell. 2000;12:393–404.

Goodin MM, Zaitlin D, Naidu RA, Lommel SA. *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. Mol Plant-Microbe Interac. 2008;21:1015–26.

Gutterson N, Reuber TL. Regulation of disease resistance pathways by AP2/ERF transcription factors. Curr Opin Plant Biol. 2004;7:465–71.

Hao D, Ohme-Takagi M, Sarai A. Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. J Biol Chem. 1998;273:26857–61.

Haq SK, Atif SM, Khan RH. Protein proteinase inhibitor genes in combat against insects, pests, and pathogens: natural and engineered phytoprotection. Arch Biochem Biophys. 2004;431:145–59.

Hu Y, Zhao L, Chong K, Wang T. Overexpression of *OsERF1*, a novel rice ERF gene, up-regulates ethylene-responsive genes expression besides affects growth and development in *Arabidopsis*. J Plant Physiol. 2008;165:1717–25.

Jin JH, Zhang HX, Tan JY, Yan MJ, Li DW, Khan A, et al. A new ethylene-responsive factor *CaPTI1* gene of pepper (*Capsicum annuum* L.) involved in the regulation of defense response to *Phytophthora capsici*. Front Plant Sci. 2015;6:1217.

Kamoun S, Furzer O, Jones JDG, Judelson HS, Ali GS, Dalio RJD, et al. The top 10 oomycete pathogens in molecular plant pathology. Mol Plant Pathol. 2015;16:413–34.

Kim JY, Park SC, Hwang I, Cheong H, Nah JW, Hahm KS, et al. Protease inhibitors from plants with antimicrobial activity. Int J Mol Sci. 2009;10:2860–72.

Kroon LPNM, Brouwer H, de Cock AWAM, Govers F. The genus *Phytophthora* anno 2012. Phytopathology. 2012;102:348–64.

Lee JH, Hong JP, Oh SK, Lee S, Choi D, Kim W. The ethylene-responsive factor like protein 1 (CaERFLP1) of hot pepper (*Capsicum annuum* L.) interacts *in vitro* with both GCC and DRE/CRT sequences with different binding affinities: possible biological roles of CaERFLP1 in response to pathogen infection and high salinity conditions in transgenic tobacco plants. Plant Mol Biol. 2004;55:61–81.

Li MY, Xu ZS, Huang Y, Tian C, Wang F, Xiong AS. Genome-wide analysis of AP2/ERF transcription factors in carrot (*Daucus carota* L.) reveals evolution and expression profiles under abiotic stress. Mol Gen Genomics. 2015;290:2049–61.

Licausi F, Ohme-Takagi M, Perata P. APETALA2/ethylene responsive factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. New Phytol. 2013;199:639–49.

Liu J, Li J, Wang H, Fu Z, Liu J, Yu Y. Identification and expression analysis of *ERF* transcription factor genes in petunia during flower senescence and in response to hormone treatments. J Exp Bot. 2011;62:825–40.

Majeed A, Makhdoom R, Husnain T, Riazuddin S. Assessment of potato proteinase inhibitor-II gene as an antifungal and insecticidal agent. Acta Agric Scand Sect B. 2011;61:92–6.

Meng Y, Zhang Q, Ding W, Shan W. *Phytophthora parasitica*: a model oomycete plant pathogen. Mycology. 2014;5(2):43–51.

Nakano T, Suzuki K, Fujimura T, Shinshi H. Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. Plant Physiol. 2006;140:411–32.

Okamuro JK, Caster B, Villarroel R, Montagu MV, Jofuku KD. The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in *Arabidopsis*. Proc Natl Acad Sci U S A. 1997;94:7076–81.

Pirrello J, Jaimes-Miranda F, Sanchez-Ballesta MT, Tournier B, Khalil-Ahmad Q, Regad F, et al. Sl-ERF2, a tomato ethylene response factor involved in ethylene response and seed germination. Plant Cell Physiol. 2006;47:1195–205.

Quilis J, Meynard D, Vila L, Avilés FX, Guiderdoni E, San SB. A potato carboxypeptidase inhibitor gene provides pathogen resistance in transgenic rice. Plant Biotechnol J. 2007;5:537–53.

Rashid M, He G, Yang G, Hussain J, Xu Y. AP2/ERF transcription factor in rice: genome-wide canvas and syntenic relationships between monocots and eudicots. Evol Bioinforma. 2012;8:321–55.

Ratcliff F, Martin-Hernandez AM, Baulcombe DC. Technical advance: tobacco rattle virus as a vector for analysis of gene function by silencing. Plant J. 2001;25:237–45.

Ryan CA. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu Rev Phytopathol. 1990;28:425–49.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K. DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem Biophys Res Commun. 2002;290:998–1009.

Schluter U, Benchabane M, Munger A, Kiggundu A, Vorster J, Goulet MC, et al. Recombinant protease inhibitors for herbivore pest control: a multitrophic perspective. J Exp Bot. 2010;61:4169–83.

Senthilkumar R, Cheng CP, Yeh KW. Genetically pyramiding protease-inhibitor genes for dual broad-spectrum resistance against insect and phytopathogens in transgenic tobacco. Plant Biotechnol J. 2010;8:65–75.

Shen D, Chai C, Ma L, Zhang M, Dou D. Comparative RNA-Seq analysis of *Nicotiana benthamiana* in response to *Phytophthora parasitica* infection. Plant Growth Regul. 2016;80:59–67.

Shigyo M, Ito M. Analysis of gymnosperm two-AP2-domain-containing genes. Dev Genes Evol. 2004;214:105–14.

Shin R, Park JM, An JM, Paek KH. Ectopic expression of *Tsi1* in transgenic hot pepper plants enhances host resistance to viral, bacterial, and oomycete pathogens. Mol Plant-Microbe Interac. 2002;15:983–9.

Song T, Ma Z, Shen D, Li Q, Li W, Su L, et al. An oomycete CRN effector reprograms expression of plant HSP genes by targeting their promoters. PLoS Pathog. 2015;11:e1005348.

Swaminathan K, Peterson K, Jack T. The plant B3 superfamily. Trends Plant Sci. 2008;13:647–55.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–9.

Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, et al. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J. 2004;37:914–39.

Tournier B, Sanchez-Ballesta MT, Jones B, Pesquet E, Regad F, Latché A, et al. New members of the tomato ERF family show specific expression pattern and diverse DNA-binding capacity to the GCC box element. FEBS Lett. 2003;550:149–54.

Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 2009;25:1105–11.

Valdes-Rodriguez S, Cedro-Tanda A, Aguilar-Hernandez V, Cortes-Onofre E, Blanco-Labra A, Guerrero-Rangel A. Recombinant amaranth cystatin (AhCPI) inhibits the growth of phytopathogenic fungi. Plant Physiol Biochem. 2010;48:469–75.

Valueva TA, Revina TA, Gvozdeva EL, Gerasimova NG, Ozeretskovskaia OL. Role of proteinase inhibitors in potato protection. Russ J Bioorg Chem. 2003;29:454–8.

Valueva TA, Revina TA, Kladnitskaya GV, Mosolov VV. Kunitz-type proteinase inhibitors from intact and Phytophthora-infected potato tubers. FEBS Lett. 1998;426:131–4.

Woloshuk CP, Meulenhoff JS, Sela-Buurlage M, van den Elzen PJ, Cornelissen BJ. Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell. 1991;3:619–28.

Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, et al. *Nicotiana benthamiana* gp91^{phox} homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant Cell. 2003;15:706–18.

Zhang G, Chen M, Chen X, Xu Z, Guan S, Li LC, et al. Phylogeny, gene structures, and expression patterns of the ERF gene family in soybean (*Glycine max* L.). J Exp Bot. 2008;59:4095–107.

Zhang Z, Huang R. Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor *TERF2/LeERF2* is modulated by ethylene biosynthesis. Plant Mol Biol. 2010;73:241–9.

Zhao Y, Chang X, Qi D, Dong L, Wang G, Fan S, et al. A novel soybean ERF transcription factor, *GmERF113*, increases resistance to *Phytophthora sojae* infection in soybean. Front Plant Sci. 2017;8:299.

Zhu X, Qi L, Liu X, Cai S, Xu H, Huang R, et al. The wheat ethylene response factor transcription factor pathogen-induced ERF1 mediates host responses to both the necrotrophic pathogen *Rhizoctonia cerealis* and freezing stresses. Plant Physiol. 2014;164:1499–514.