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Research article

Overexpression of a Chinese cabbage *BrERF11* transcription factor enhances disease resistance to *Ralstonia solanacearum* in tobacco

Yan Lai [a,b,1], Fengfeng Dang [a,b,1], Jing Lin [a], Lu Yu [a], Youliang Shi [a], Yuhua Xiao [a], Mukun Huang [a], Jinhui Lin [a], Chengcong Chen [a], Aihua Qi [a], Zhiqin Liu [a], Deyi Guan [b], Shaoliang Mou [a], Ailian Qiu [a], Shuilin He [a,b,*]

[a] College of Life Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China
[b] National Education Minster Key laboratory of Plant Genetic Improvement and Comprehensive Utilization, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China

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ABSTRACT

Ethylene-responsive factors (ERFs) play diverse roles in plant growth, developmental processes and stress responses. However, the roles and underlying mechanism of ERFs remain poorly understood, especially in non-model plants. In this study, a full length cDNA of ERF gene was isolated from the cDNA library of Chinese cabbage. According to sequence alignment, we found a highly conservative AP2/ERF domain, two nuclear localization signals, and an ERF-associated Amphiphilic Repression (EAR) motif in its C-terminal region. It belonged to VIIIa group ERFs sharing the highest sequence identity with *At*ERF11 in all of the ERFs in Arabidopsis and designated *Br*ERF11. *BrERF11*-green fluorescence protein (GFP) transient expressed in onion epidermis cells localized to the nucleus. The transcript levels of *BrERF11* were induced by exogenous salicylic acid (SA), methyl jasmonate (MeJA), ethephon (ETH), and hydrogen peroxide (H_2O_2). Constitutive expression of *BrERF11* enhanced tolerance to *Ralstonia solanacearum* infection in transgenic tobacco plants, which was coupled with hypersensitive response (HR), burst of H_2O_2 and upregulation of defense-related genes including HR marker genes, SA-, JA-dependent pathogen-related genes and ET biosynthesis associated genes and downregulation of *CAT1*, suggesting *BrERF11* may participate in pathogen-associated molecular pattern (PAMP)- and effector-triggered immunity (PTI and ETI) mediated by SA-, JA- and ET-dependent signaling mechanisms.

1. Introduction

As sessile organisms, plants inevitably encounter challenges in their natural habitats from various pathogens, which usually impair growth and development of plants and cause heavy crop yield loss in agriculture production. In addition to a range of preformed barriers, plants have developed a complicated inducible immunity mechanism to defend themselves against microbial invasion over the course of evolution. Inducible plant defenses are based on two modes of immunity termed pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) [1]. PTI is triggered by recognition of Microbe-Associated Molecular Patterns (MAMPs), resulting in activation of basal (or non-host) defense. To overcome PTI, pathogens secrete effectors and inject them into host cells to suppress PTI by targeting the component of PTI and promote parasitism. In the presence of cognate resistance (R) genes, it has been proposed that plants detect the virulence activity of pathogen effectors and give rise to ETI in a gene-for-gene model. This R-mediated ETI is faster and quantitatively stronger than PTI, and is typically accompanied by a hypersensitive response (HR) form of programmed cell death [2]. Plant defense reaction is regulated by complicated signaling networks, which generally include ion fluxes across the plasma membrane [3], oxidative burst, change of phytohormones [4], MAP kinase cascades [5] and defense associated genes regulation by transcription factors [6]. It has been reported that many signaling components are shared by PTI and ETI, including hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) [7]. Consistently, the sets of genes induced during

Abbreviations: ABA, abscisic acid; DAB, diaminobenzidine; DAI, days after inoculation; EAR, ERF-associated amphiphilic repression motif; ERE, ethylene-responsive element; ERF, ethylene-responsive factor; ETH, ethephon; ETI, effector-triggered immunity; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GUS, β-glucuronidase; H_2O_2, hydrogen peroxide; HR, hypersensitive response; MeJA, methyl jasmonate; ORF, open reading frame; PTI, pathogen-associated molecular pattern (PAMP)-triggered immunity; SA, salicylic acid; WT, wild-type.

* Corresponding author. College of Life Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China. Tel./fax: +86 591 83704487.
E-mail address: shlhe2007@yahoo.com.cn (S. He).

[1] These authors contributed equally to this work.

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PTI and ETI overlap and the differences observed between compatible and incompatible interactions are usually temporal and quantitative rather than qualitative [8,9]. Thus plants may share the same signaling machinery in response to different stimuli. As defense responses against pathogens in PTI and ETI include vast transcriptional reprogramming, transcription factors are expected to play important roles in PTI and ETI as well as in the integrating (coordinating) regulation of PTI and ETI, but few reports in this regards are mentioned.

The APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily is characterized by the presence of a highly conserved AP2/ERF DNA-binding domain. On the basis of the similarity of the AP2/ERF domain, this large gene family can be divided into five subfamilies including the AP2, ERF, DREB (dehydration-responsive element binding protein), RAV (for related to ABI3/VP1) and soloist [10]. Among these, the ERF subfamily has a single AP2 domain and well-defined DNA-binding activity. It has been shown that some ERF proteins can bind specifically to the GCC box (AGCCGCC), the core sequence of the ethylene-responsive element (ERE), usually functioning in the regulation of plant responses to biotic stresses such as pathogens [11]. Besides GCC-box, some ERF proteins can bind to DRE/CRT (dehydration-responsive element/C-repeat element) cis-element and involve in abiotic stress responses [12]. The ERF proteins binding to these cis-elements regulate ethylene-responsive gene expression and participate in signaling pathway mediated by other hormones such as SA, JA and abscisic acid (ABA), indicating that they play important roles in plant biotic and abiotic stress responses as positive regulator or repressor [13]. A large body of evidence suggests that ERFs function in plant response to pathogens infection. *At*ERF5 [14] and Pti4 (an ethylene-responsive factor of tomato) [15,16] have been reported to involve in PTI, ETI and HR, respectively. However, the studies on the roles of ERF proteins in plant defense are largely from model plants such as *Arabodipsis*, rice, tomato or tobacco, and their roles in PTI or ETI and the underlying mechanism remain poorly understood, especially in non-model plants such as Chinese cabbage.

Chinese cabbage (*Brassica rapa* L. ssp. pekinensis) is one of the most important Cruciferae vegetables in Asia. However, its growth and productivity are frequently impaired by various diseases, which always cause serious yield loss. Unraveling the intricacies of disease resistance signaling cascade in Chinese cabbage will help to create novel strategies for enhancement of stress tolerance. ERFs have been proven to play important roles in plant defense signaling pathway. Previous studies have already discovered 62 putative AP2/ERF family members by analyzing 142 947 ESTs in *B. rapa* database [17], but the information of specific ERF proteins in disease resistance in Chinese cabbage remains poorly understood. Here, we reported the isolation and characterization of a full length cDNA encoding a new Chinese cabbage ERF protein, designated *BrERF11*, which contained an EAR motif but might acting as a transcriptional activator. The transcripts of *BrERF11* were accumulated after treatments with exogenous SA, MeJA, ethephon (ETH) and hydrogen peroxide. The overexpression of *BrERF11* conferred the resistance to *Ralstonia solanacearum* infection in transgenic tobacco.

2. Results

2.1. Cloning and sequence analysis of the BrERF11 gene

The full-length cDNA sequence of a Chinese cabbage ERF homolog, designated *BrERF11* (GenBank accession number: DQ887755), was isolated from SMART™ cDNA library prepared from Chinese cabbage seedlings. The *BrERF11* cDNA comprised 812 bp, containing an open reading frame (ORF) encoding a polypeptide of 172 residues with a predicted molecular mass of 18.86 kDa and an isoelectric point of 8.86. The deduced protein *Br*ERF11 had a conserved 58 amino acid DNA-binding domain (AP2/ERF domain) with two highly conserved amino acid residues in ERF proteins, 14th alanine (A) and 19th aspartic acid (D) [10]. In addition, *Br*ERF11 protein possessed two putative basic nuclear localization signal (NLS) sequences in the N-terminal region and the C-terminal region (R28KRP and K154RPR). A conserved CMVIII-1 (EAR) motif which might function in transcriptional regulation and a conserved CMVIII-2 motif were also found in the C-terminal region (Fig. 1a). Multiple sequence alignment and phylogenetic analyses showed that *BrERF11* shared high similarity with VIIIa group ERFs containing EAR motif (Fig. 1b). *Br*ERF11 has 67.61% identity with *At*ERF11 from Arabidopsis, 45.05% with *Gm*ERF4 from soybean, 44.84% with *Sl*ERF3 from tomato, and 42.79% with *At*ERF4 at the amino acid level. *Gm*ERF4, *Sl*ERF3 and other members of VIIIa group ERFs were known as function proteins involved in plant stress responses [18–20]. The above results indicate that *Br*ERF11 is a novel member of the VIIIa group family and may involve in Chinese cabbage defense responses.

2.2. BrERF11 transcripts are upregulated by defense-inducing chemicals

To clarify the potential roles of *BrERF11* in response to defense-related signal molecules, the relative transcript levels of *BrERF11* were examined in Chinese cabbage seedlings under SA, MeJA, ETH and H_2O_2 treatments using quantitative real-time PCR analysis. The transcript levels of *BrERF11* were significantly increased at 1 h after treatment with all defense signaling molecules and almost maintained at a high level over the whole experimental period. The strongest response to SA (nearly 9-fold over the control) was observed at 3 h after treatment (Fig. 2a). Compared with SA treatment, the ETH and MeJA treatments caused *BrERF11* transcripts accumulated more rapidly and abundantly. The maximum transcript levels were detected at 1 h with 38- and 21-fold, respectively (Fig. 2b and c). H_2O_2 not only triggers the HR in plant–pathogen interactions, but also functions as signaling molecules to activate plant defense responses [21]. As shown in Fig. 2d, H_2O_2 treatment resulted in a rapid accumulation of the *BrERF11* transcript at 1 h with 6-fold over the control. Endogenous phytohormones play pivotal roles in plant defense signaling network and involve in activation of specific defense-related genes [4]. These results further indicate that *BrERF11* may participate in Chinese cabbage defense responses.

2.3. Nuclear localization of BrERF11 protein

The prediction of two putative NLS (R28KRP and K154RPR) of *Br*ERF11 implies that the protein may localize to the nucleus (Fig. 1a). To verify the subcellular localization of *Br*ERF11, binary vectors containing the *35S*::*BrERF11*-green fluorescence protein (GFP) gene or the control *p35S*::*GFP* gene were used for transformation of onion epidermal cells by particle bombardment. As shown in Fig. 3, *Br*ERF11-GFP fusion protein localized exclusively to the nuclei of onion epidermal cells, while the GFP protein alone was distributed throughout the entire cell.

2.4. BrERF11 overexpression in tobacco confers tolerance to R. solanacearum

Due to the elevation of *BrERF11* transcripts after treatment with defense signaling molecules, transgenic T_2 tobacco plants were generated to further examine the role of *BrERF11* in plant stress response (Fig. 4B). No apparent phenotypic differences between

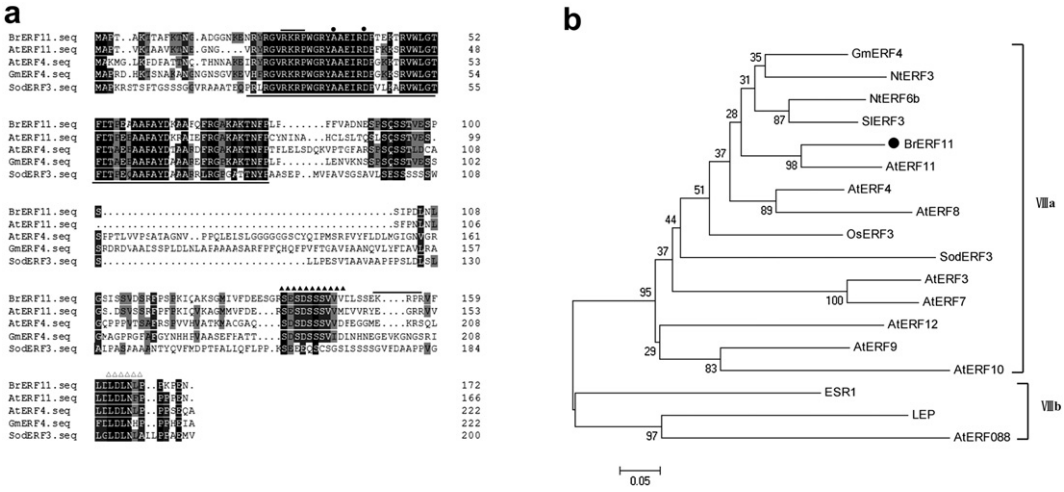

Fig. 1. Amino acid sequence alignment and phylogenetic relationship between *Br*ERF11 and other representative members of the VIII cluster. (a) Comparison of the derived amino acid sequences of *Br*ERF11 with EAR motif-containing ERFs from Arabidopsis *At*ERF11/4, soybean *Gm*ERF4 and sugarcane *Sod*ERF3. The AP2/ERF domain is underlined and two putative nuclear localization signals predicted by WoLF PSORT (http://wolfpsort.org/) are overlined. Two conserved amino acid residues in AP2/ERF domain (the 14th Ala and 19th Asp) are marked by spots (•). The conserved CMVIII-2 motif is shown by solid triangles (▲) and the CMVIII-1 (EAR) motif in C-terminal is shown by open triangles (△). Amino acid residues identical in all five proteins are shown in black; those conserved in at least three sequences are shaded. (b) Phylogenetic comparison of *Br*ERF11 and other VIII cluster proteins. Alignments were made in Clustal X and phylogenetic tree was constructed by neighbor-joining algorithms of MEGA 5.01 software. Bootstrap values (1000 replicates) are shown in percentages at the branch nodes. The Genbank accession numbers for the other VIII cluster proteins are as follows: *Gm*ERF4 (EU747723), *Nt*ERF3 (D38124), *Nt*ERF6b (AB573719), *Sl*ERF3 (AY192369), *At*ERF11 (AB055882), *At*ERF4 (AY140030), *At*ERF8 (AB036884), *Os*ERF3 (AB036883), *Sod*ERF3 (AM493723), *At*ERF3 (CP002684), *At*ERF7 (AB032201), *At*ERF12 (AB055883), *At*ERF9 (AB047648), *At*ERF10 (AB047649), ESR1 (AF353577), LEP (AF216581), *At*ERF088 (CP002684).

wild type and transgenic plants were observed. We first tested the resistance of the *BrERF11* transgenic lines against *R. solanacearum*. Six-week-old tobacco plants were inoculated with *R. solanacearum* by root invasion. All of the three tested transgenic lines exhibited enhanced disease resistance in response to *R. solanacearum* inoculation (not shown). One transgenic line which showed the highest relative transcript levels (not shown) of *BrERF11* of all tested lines was chosen for detailed disease resistance assays. At 7 days after inoculation (DAI), *35S*::*BrERF11* transgenic plants showed approximately 8% wilting symptoms, while the wild-type plants exhibited almost 46% wilting. The disease index at 13 DAI in the transgenic plants remained at 35% compared to wild-type plants (approximately 86%) (Fig. 4D). As shown in Fig. 4A, extremely severe wilting symptoms were observed in wild-type plants at 14 DAI but not in *35S*::*BrERF11* transgenic plants. Disease symptoms on detach leaves of wild-type and transgenic plants were also monitored for 7 DAI. As shown in Fig. 4C, disease symptoms developed on the wild-type plants leaves were more obvious than

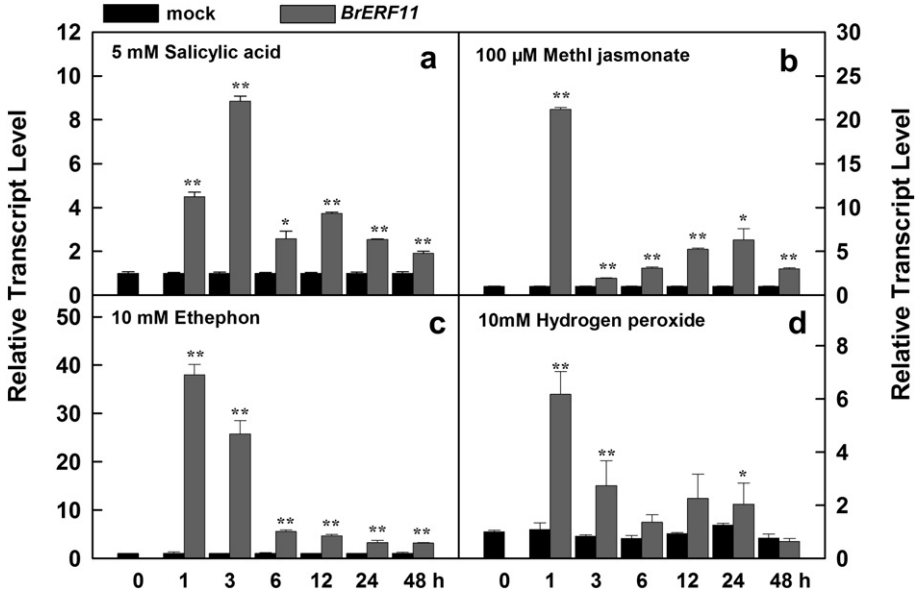

Fig. 2. Analysis of *BrERF11* transcripts after hydrogen peroxide or different hormone treatments. The transcript levels of *BrERF11* were determined by quantitative real-time PCR. Total RNA was prepared from leave tissues of two-week-old seedling sprayed with 5 mM SA (a), 100 MeJA (b), ETH (c) and hydrogen peroxide (d) at the time points indicated. The non-treatment transcript levels at each time points were used as the control and assigned value of 1. Relative transcript levels were normalized to *GAPDH* level. Data represents the average of three independent biological replicates ± SE. Statistically significant differences of transcript levels between treatment and control were determined by SNK test ($^{*}P < 0.05$, $^{**}P < 0.01$).

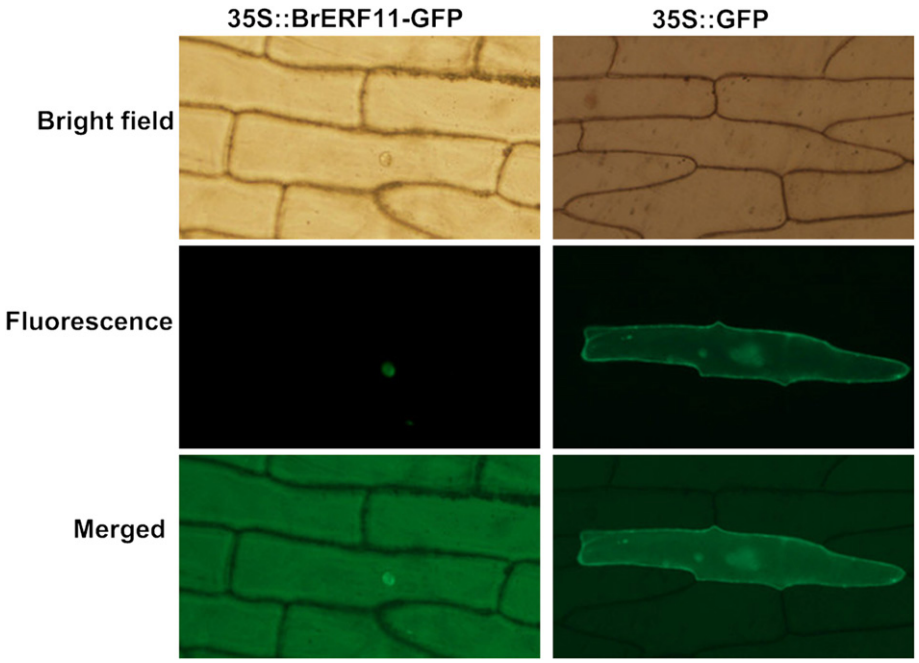

Fig. 3. Subcellular localization of *Br*ERF11-GFP fusion proteins. The constructs of *35S*::*BrERF11-GFP* (left) and the *p35S*::*GFP* (right) were introduced into the onion epidermis cells by the particle bombardment transformation method. The bright field (top), fluorescence (middle), and merged images (bottom) were taken 18 h after bombardment.

those of transgenic plants. Taken together, the results suggest that the ectopic expression of *BrERF11* in tobacco confers enhanced disease resistance against *R. solanacearum*.

To dissect the effects of *BrERF11* overexpression in transgenic tobacco on HR and H_2O_2 generation during the defense response to *R. solanacearum*, leaves of wild-type and *35S*::*BrERF11* transgenic plants were stained with trypan blue and diaminobenzidine (DAB). After 48 h inoculation with *R. solanacearum*, the leaves of *35S*::*BrERF11* transgenic plants exhibited significantly increased hypersensitive cell death phenotypes compared with wild-type plants. The strong induction of oxidative bursts was also observed in inoculated leaves of transgenic plants as compared with wild-type plants (Fig. 4E). This result suggests that the ectopic expression of *BrERF11* in tobacco induces HR and H_2O_2 generation in response to *R. solanacearum* infection.

Quantitative real-time PCR was used to evaluate the role of ectopic expression of *BrERF11* in transcriptional responses of defense-related genes in transgenic tobacco. Since overexpression of *BrERF11* in tobacco activated HR and H_2O_2 generation during the defense response to *R. solanacearum*, two tobacco HR related genes have been assayed: *NtHSR201* and *NtHSR515* [22]. As shown in Fig. 5, the transcript levels of *NtHSR201*, *NtHSR515* in transgenic plants leaves were significantly higher than in the wild-type plants leaves. Likewise, *BrERF11* overexpression also caused an increase in transcript levels of ET-responsive *NtACS6* and ACC oxidase gene *NtEFE26* [23], JA-responsive *NtPR-1b* [24] and SA-responsive *NtPR-1a*/*c*, *NtPR3* and *NtPRQ* [25] genes. Earlier studies showed that *NPR1* (NON-EXPRESSOR OF PR1) functioned as an important regulator in cross-talk between the SA- and JA-dependent signaling pathways [26]. In our study, no statistically significant transcript difference of *NtNPR1* was observed between wild-type and transgenic plants. Furthermore, the transcript levels of *NtCAT1* (for catalase) which involved in removing H_2O_2 production were decreased in *35S*::*BrERF11* transgenic plants.

The transcriptional responses of defense-related genes during *R. solanacearum* infection were also examined using quantitative real-time PCR analysis. As shown in Fig. 6, in comparison with wild-type plants, a set of defense-related genes including *NtHSR201*, *NtHSR515*, *NtEFE26*, *NtPR-1b*, *NtPR-1a*/*c*, *NtPR3* and *NtPRQ* in transgenic plants were significantly upregulated at both 24 h and 48 h after infection with *R. solanacearum*. The transcript levels of *NtACS6* in transgenic plants were significantly increased at 48 h after infection. In wild-type plants, the transcripts of *NtCAT1* presented significantly high level at 48 h after *R. solanacearum* infection. These genes also exhibited constitutive upregulated or down-regulated transcript levels in *35S*::*BrERF11* transgenic plants. However, *NtNPR1* showed different transcript pattern in response to *R. solanacearum* infection. The transcripts of *NtNPR1* were significantly increased at 24 h after infection, and no significantly transcript difference at 48 h was observed between wild-type and transgenic plant.

3. Discussion

The ERF proteins belong to the AP2/ERF superfamily, which are characterized by a single ERF DNA-binding domain. In *Arabidopsis* genome, 122 *ERF* genes have been identified and further divided into 12 groups [27]. Among these groups, some members of VIII group including AtERF3/4 and AtERF7-12 harbor a conserved ERF-associated amphiphilic repression (EAR) motif or CMVIII-1 motif in their C-terminal region. ERF4 [28] and *At*ERF11 [29] have been described as nuclear-localized proteins and bind to the GCC box or dehydration-responsive element, acting as transcriptional repressors. Previous studies have showed that the conserved EAR motif (contain (L/F) DLN (L/F) xP sequence) is essential for repression [30]. Recently, a few EAR-motif-containing ERFs have been reported acting as transcriptional activator. The EAR-motif-containing protein *Sod*ERF3 may function as a transcriptional activator in tobacco drought and osmotic resistance [18]. In another case, Tiwari et al. [31] found that an EAR-motif-containing ERF *At*ERF98 was a transcriptional activator due to the unique EDLL activation motif.

In this study, the full length cDNA of *BrERF11* was isolated from the cDNA library of Chinese cabbage. *BrERF11* exhibited a high

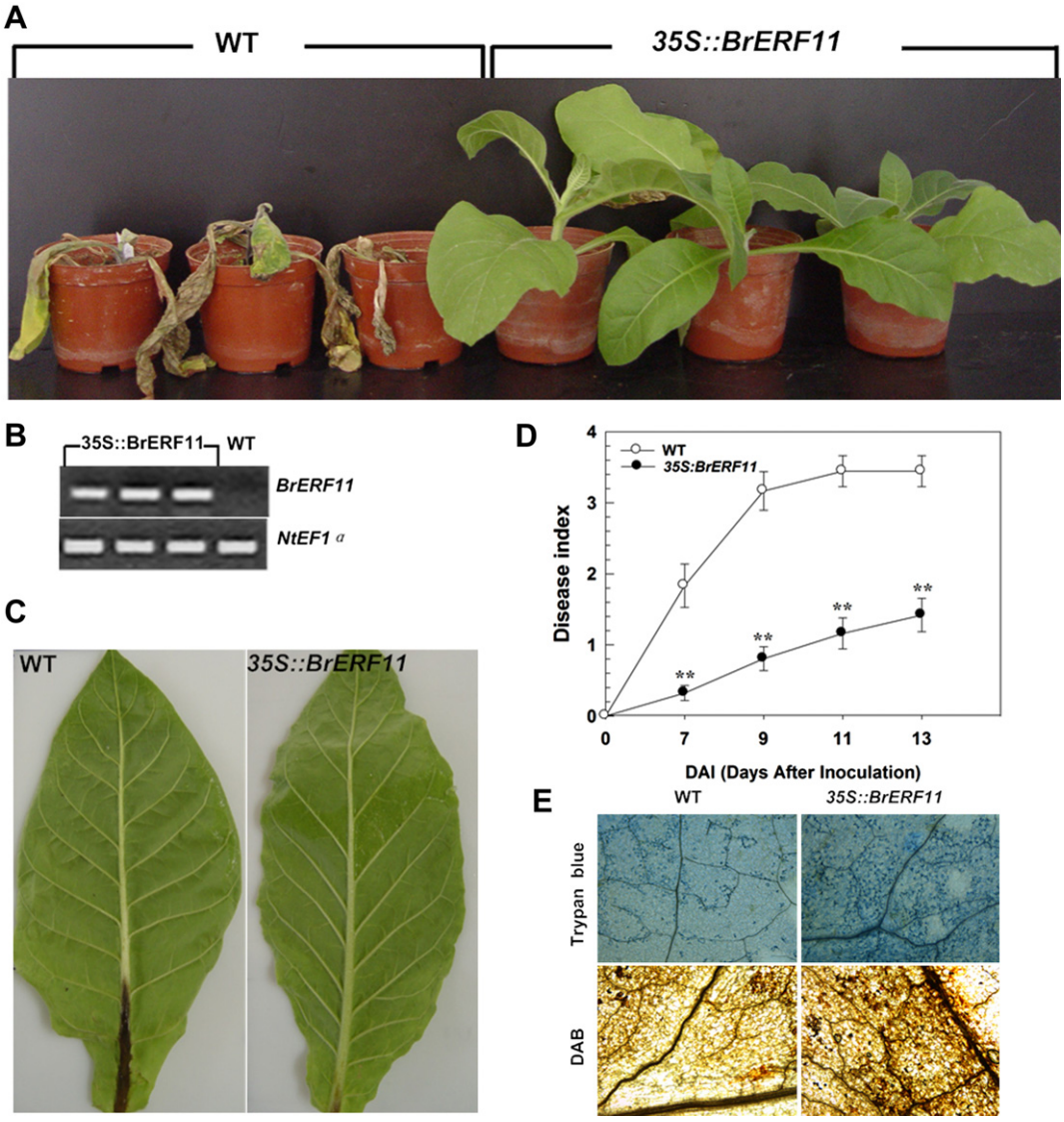

Fig. 4. Overexpression of *BrERF11* enhanced resistance to *R. solanacearum* in transgenic tobacco. (A) Phenotype of 6-week-old wild-type CB1 and 35*S*::*BrERF11* transgenic tobacco plants inoculated with 10^8 cfu/mL *R. solanacearum* by root invasion for 14 days. (B) RT-PCR analysis of *BrERF11* in wild-type and transgenic 35*S*::*BrERF11* plants. The *NtEF1α* gene was used as a constitutive control. (C) Disease symptoms of detached leaves of wild-type and transgenic 35*S*::*BrERF11* plants. Photos were taken at 7 DAI. (D) Disease index curve over the course of time after inoculation. The disease index score (±SE) was determined from 24 inoculated plants per genotype. Asterisks indicate that mean values are significantly different between wild-type and transgenic plants (**$P < 0.01$, SNK test). (E) Trypan blue and DAB staining of cell death and H_2O_2 generation. Leaves were harvested from 4-week-old wild-type and transgenic 35*S*::*BrERF11* plants after 48 h inoculation with *R. solanacearum* by leaf infiltration.

sequence homology with *At*ERF11, *Gm*ERF4, *Sl*ERF3, *At*ERF4 and other VIIIa group ERFs at the amino acid level. One conserved CMVIII-1 (EAR) motif and a conserved CMVIII-2 motif were also found in its C-terminal region (Fig. 1a). Furthermore, *Br*ERF11-GFP transient expressed in onion epidermis cells localized to the nucleus (Fig. 3).

Frequently the production of endogenous phytohormones including SA, JA and ET is coupled with plant response to biotic or abiotic stress. These endogenous hormones modulate the downstream signaling pathway and the expression of defense associated genes [4]. Our study showed that the transcript levels of *BrERF11* were inducible by exogenous applied ETH. This result further confirms that *Br*ERF11 is a member of ERF proteins in Chinese cabbage. In addition, we also found that the transcript levels of *BrERF11* were induced by exogenous applied SA and MeJA, which had been commonly found in the defense signaling against pathogens in plants. These results suggest that *Br*ERF11 may involve in crosstalk between SA-, JA- and ET-mediated signaling pathways against pathogen infection. To further confirm this speculation, we performed gain-of-function analysis of *BrERF11* and found that ectopic expression of *BrERF11* in T_2 tobacco plants conferred significantly enhanced resistance to *R. solanacearum* inoculation, with much lower wilting symptoms than that of wild types plants (Fig. 4). Significant increased HR and burst of H_2O_2 phenotype were further observed in 35*S*::*BrERF11* tobacco plants compared to wild-type plants with trypan blue and DAB staining detection, respectively. Since it is well established that accumulation of H_2O_2 to high concentration lead to the HR [21,32], we envisage the transcript changes of *BrERF11* target genes may trigger the burst of H_2O_2, and ultimately confers hypersensitive disease resistance. Taken together, it seems most likely that *BrERF11* functions as a transcriptional activator. This conclusion is also supported by the in vitro transient assay using the particle gun bombardment method in onion epidermal cells (Fig. A.1). The *Br*ERF11 effector is

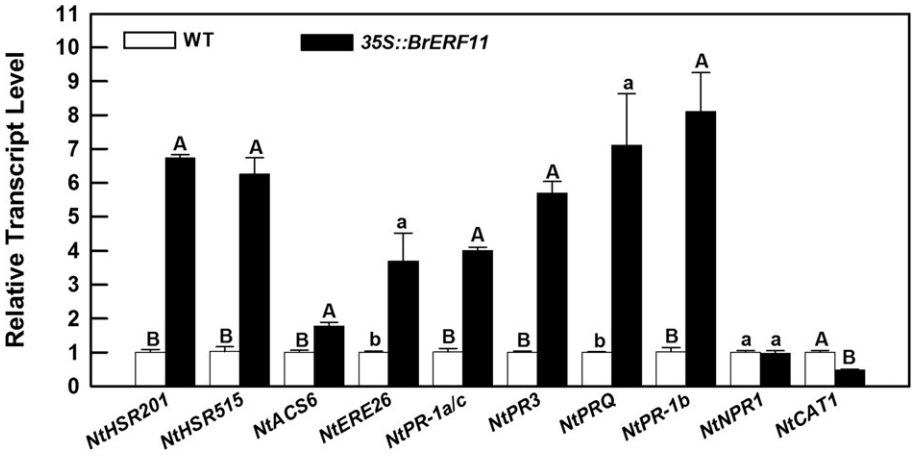

Fig. 5. Analysis of tobacco defense-related marker genes transcripts in wild-type CB1 and *35S::BrERF11* transgenic tobacco plants. The transcript levels of *NtHSR201*, *NtHSR515*, *NtACS6*, *NtEFE26*, *NtPR-1a*/*c*, *NtPR3*, *NtPRQ*, *NtPR-1b*, *NtNPR1* and *NtCAT1* were determined by quantitative real-time PCR. Relative transcript levels were normalized using the transcripts of *NtEF1α*. The transcript levels of each gene in wild-type plants were used as the control and assigned value of 1. Data represents the average of three independent replicates ± SE. Statistically significant differences between wild-type and transgenic plants were determined by SNK test (lower case: $P < 0.05$, upper case: $P < 0.01$) using different letters.

able to activate the reporter plasmid carrying 2×GCC box and exhibited GUS activity.

To elucidate the molecular mechanism underlying the enhanced HR and disease resistance, we performed quantitative real-time PCR analysis to monitor the changes of defense associated genes transcriptional responses in *35S*::*BrERF11* transgenic plants. The expression of *CAT1* was specifically inhibited by SA and 2, 6-Dichloroisonicotinic acid (INA) but induced by the increased concentrations of H_2O_2 in vivo [33]. In our study, less accumulation of *NtCAT1* transcripts were observed in *35S*::*BrERF11* transgenic tobacco plants than in wild-type plants after infection with *R. solanacearum*, which was speculated leading to the H_2O_2 level enhancement in *35S*::*BrERF11* transgenic pants (Fig. 4E).

In addition, we found enhanced transcript levels of ET-responsive *ACC* oxidase gene *NtEFE26* and *NtACS6*, SA-responsive *NtPR-1a*/*c*, *NtPR3* and *NtPRQ*, JA-responsive *NtPR-1b* and HR marker genes such as *NtHSR201* and *NtHSR515* in *35S*::*BrERF11* transgenic pants. The transcript levels of these genes were also significantly increased at least at one of the two tested time points after *R. solanacearum* inoculation. These results were consistent with that of upregulation of *BrERF11* transcript levels by exogenous applied ETH, SA and MeJA (Fig. 2), suggesting that overexpression of *BrERF11* conferred enhanced disease resistance by regulating biosynthesis of endogenous ethylene as well as by acting as an important node in the crosstalk of SA-, JA- and ET-mediated defense signaling pathways. However, we didn't find transcript

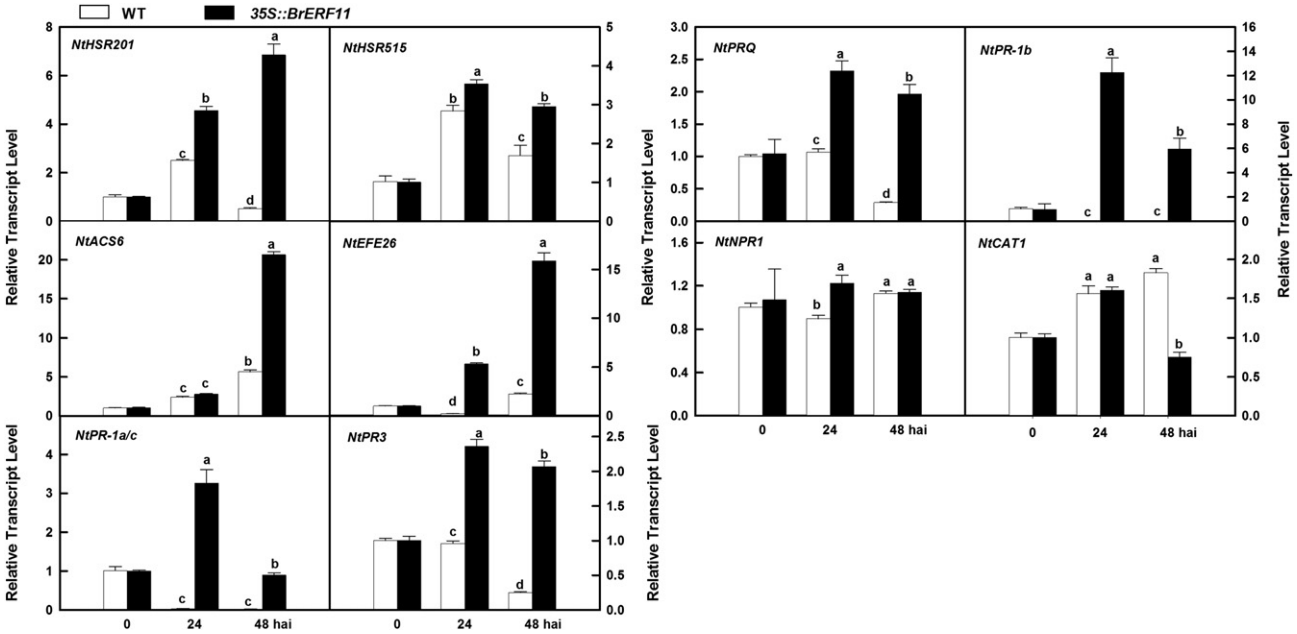

Fig. 6. Analysis of transcript levels of tobacco defense-related marker genes in wild-type CB1 and *35S::BrERF11* transgenic tobacco plants 24 and 48 h after inoculation with *R. solanacearum*. The transcript levels of *NtHSR201*, *NtHSR515*, *NtACS6*, *NtEFE26*, *NtPR-1a*/*c*, *NtPR3*, *NtPRQ*, *NtPR-1b*, *NtNPR1* and *NtCAT1* were determined by quantitative real-time PCR. Relative transcript levels were normalized using the transcripts of *NtEF1α*. The transcript levels of non-treatment wild-type or *35S*::*BrERF11* plants were used as the control and assigned value of 1. Data represents the average of three independent replicates ± SE. Statistically significant differences were determined by SNK test ($P < 0.05$) using different letters.

accumulation of *NPR1* gene in transgenic tobacco constitutive expression of *BrERF11* (Fig. 5). Previous studies show that JA and ET can act synergistically in defense reaction [34]. JA and ET signaling pathways can also act antagonistically [35] or synergistically [36] with SA signaling pathway in PTI or ETI [37]. Since PTI and ETI use these conserved signaling machinery differently, synergistic relationships among the signaling sectors are evident in PTI, compensatory relationships among the sectors dominate in ETI [37]. Previous studies have found that tomato ERF protein Pti4 can be activated by the gene-for-gene avrPto-Pto interaction and involved in *R* gene mediated ETI [15,16]. *At*ERF5 was found involved in chitin-induced innate immunity response [14]. In conclusion, we suggest that *Br*ERF11 may act as important node in the crosstalk between SA and JA/ET dependent signaling pathways in ETI and PTI. However, the mechanism of the activation by *Br*ERF11 is still unknown. We don't find any known activation motif including the EDLL motif in *Br*ERF11 or any unconserved amino acid residue in the EAR motif which is responsible for abolishing the repression capacity described by Trujillo et al. [18] previously. It is likely that *Br*ERF11 functions as a transcriptional activator by interacting with other defense-related proteins or there is unknown activation motif in *Br*ERF11 we don't find yet. Our future work will elaborate on this mechanism.

4. Materials and methods

4.1. Plant materials and growth condition

Wild-type tobacco (*Nicotiana tabacum* .L cv. CB1, a cultivar obtained from Tobacco breeding group in Fujian Agriculture and Forestry University), *BrERF11*-overexpressing tobacco and Chinese cabbage (*B. rapa* L. *ssp. pekinensis*, a local inbred line obtained from Fujian Agriculture and Forestry University) were grown in a growth chamber at 25 ± 2 °C with 70% relative humidity and a 16 h day/8 h night photocycle after germination. Seeds of tobacco were treated with 75% ethanol for 30 s and then surface-sterilized with 10% hydrogen peroxide for 10 min. After washing for five times with sterile water, the seeds were placed on Murashige and Skoog medium (0.6% agar) with 50 mg L^{-1} hygromycin for transgenic lines, or without hygromycin for control plants, under a 16 h day/8 h night photocycle at 25 ± 2 °C. At the two-leaf stage, the survival transgenic and control seedlings were transplanted to plastic trays containing mixed soil (peat moss/perlite, 2/1,v/v) and grown for 2–3 weeks. At four-leaf stage, seedlings were transplanted to plastic pots containing peat moss and general soil (1/1, v/v) for another 3–4 weeks.

4.2. Isolation and sequence analysis of BrERF11

The putative full-length *BrERF11* cDNA clone (GenBank Accession No: DQ887755) was isolated from a SMART™ cDNA library (Clontech Inc., Palo Alto, CA, USA) established from Chinese cabbage seedlings. A contig was assembled from 20 Chinese cabbage EST sequences from GenBank (http://www.ncbi.nlm.gov/) using *At*ERF11 (AB055882) amino acid sequence as a probe. The *Br*ERF11 cDNAs was isolated by a PCR based 96-well screening method [38] with the primer pairs (forward, 5′-CGTTACGCCGCCGAGAT-3′; reverse, 5′-TCAGGCTTGGGAGG-GAG-3′) designed on the basis of the contig sequence. Positive clones were converted from λTriplEx2 phagemid to pTriplEx2 plasmid following the manufacturer's protocol. The full-length cDNA amplification was using specific primers (forward, 5′-CTCGGGAAGCGCGCCATT-GTG-3′; reverse, 5′-ATACGACTCACTATAGGGCGAATTGG-CC-3′) of pTriplEx2. The deduced protein sequence was aligned with its homologs using DNAMAN software (version 6.0) and the BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

4.3. Pathogen and inoculation

R. solanacearum was originally isolated and purified from stem exudates of diseased pepper. Well-separated fluidal colonies were selected from tetrazolium chloride medium and grown at 28 °C in PSA medium for 36 h. For disease resistance studies, intact roots of 6-week-old wild type and transgenic tobacco plants were wounded in the soil by cutting and dipped in 10^8 cfu/mL bacterial suspensions in 10 mM $MgCl_2$. Disease symptoms were evaluated daily according to severity of wilting and using a 0–4 disease scale: 0 = no wilting, 1 = 1–25%, 2 = 26–50%, and 3 = 51–75% of leaves wilted, and 4 = 76–100% wilted or dead. Disease testing using the detached upper third leaves of 6-week-old tobacco plants was performed as previously described [39]. For quantitative real-time PCR analysis, 10 μL bacterial suspensions with 10^8 colony-forming units (cfu) per milliliter (OD600 nm = 0.8) in 10 mM $MgCl_2$ was infiltrated into the lateral nerve of expanded upper third leaves of 6-week-old wild type and transgenic tobacco plants using a syringe. The forth leaves were harvested at the indicated times and immediately frozen in liquid nitrogen.

4.4. Chemical treatments

Two-week-old Chinese cabbage seedlings were sprayed with 5 mM salicylic acid (SA) or 100 μM methyl jasmonate (MeJA) dissolved in 10% ethanol. Control seedlings were sprayed with 10% ethanol. For other chemical treatments, 10 mM ethephon and 10 mM hydrogen peroxide (H_2O_2) were dissolved in distilled water (H_2O). Control seedlings were sprayed with distilled water (H_2O). At various time points, the leaves of treated seedlings were harvested, frozen in liquid nitrogen and stored at −80 °C until used.

4.5. Subcellular localization of BrERF11

The coding region of *Br*ERF11 protein without a stop codon was amplified from the cDNA clone with the primers containing two attB recombination sites (forward, 5′- AAAAAGCAGGCTTCA-TGGCGCCGACAGCTAAAACGAC-3′; reverse, 5′-AGAAAGCTGGGT-CATTCTCAGGCTT-GGGAGGGAG-3′, attB recombination sites are underlined). The cDNA fragment was first introduced into Gateway pDONR™207 donor vector (Invitrogen) by an entry cloning (BP) reaction, and then cloned into the pMDC83 by an LR recombination reaction according to the manufacturer's instructions (Invitrogen). The *Br*ERF11-GFP fuse protein driven by the *2×35S* promoter was used for transformation of onion epidermal cells. The plasmid containing GFP alone was used as control. For transient expression analysis, the inner epidermal peels of onion were placed inside-up on modified MS medium (20 g L^{-1} sucrose). Transient transformation was performed with the plasmid-coated tungsten particles using PDS-1000/He system (Bio-Rad) at 1100 psi. All other parameters through particle bombardment were performed as described [40]. Bombarded onion peels were incubated in the dark at 25 °C for 18 h and analyzed by Olympus fluorescence light microscope at the wavelength of 488 nm.

4.6. Generation of the 35S::BrERF11 plasmid and tobacco transformation

To generate the *35S::BrERF11* plasmid, the full-length *BrERF11* cDNA was first introduced into Gateway pDONR™207 donor vector and then inserted into the destination vector pMDC32 driven by 2×*CaMV 35S* promoter. The *35S::BrERF11* plasmid was transferred

into *Agrobacterium tumefaciens* strain EHA105 and then transformed into tobacco by leaf-disc method as described previously [41]. To confirm transgene integration, the initial transgenic T_0 lines were selected by hygromycin and further confirmed by RT-PCR. The T_2 generation were obtained and used in this study.

4.7. Trypan blue and DAB staining

For detection of cell death, trypan blue staining was performed as described previously [42]. Forty eight hours after inoculated with pathogen, the inoculated tobacco leaves were stained and mounted in 70% glycerol for microscopic observation. To measure the levels of H_2O_2, tobacco leaves were detached at 48 h after inoculated and placed in 1 mg mL^{-1} 3,3′-diaminobenzidine (DAB, Sigma) solution for 15 h at room temperature. The leaves were then boiled for 5 min in a solution of 3:1:1 ethanol/lactic acid/glycerol and placed in absolute ethanol before observation.

4.8. Quantitative real-time PCR

For quantitative real-time PCR analysis, total RNA from the leaves of Chinese cabbage and tobacco plants after each treatment were extracted with TRIzol reagent (Invitrogen™, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was generated by converting 500 ng total RNA using Primescript RT reagent (perfect real time, TaKaRa), and then cDNA was diluted 1:10 with TE prior to use. Real-time PCR using Mastercycler ep *realplex* (Eppendorf, Hamburg, Germany) was performed with SYBR® Premix Ex Taq™ II (perfect real time, TaKaRa).

Each reaction mix (10 μL) contained 5 μL SYBR Premix Ex Taq (2×), 0.2 μL PCR forward/reverse gene specific primers (10 μM) and diluted cDNA (1 μL). For each gene, three experimental replicates were obtained using different cDNAs synthesized from three biological replicates. Amplification conditions were as follow: one cycle of 30 s at 95 °C; 40 cycles of 5 s at 95 °C, 34 s at 60 °C; one cycle of 15 s at 95 °C, 1min at 60 °C, 15 s at 95 °C, 15 s at 60 °C. The specificity of amplification was confirmed by melting curve analysis after 40 cycles. The relative expression level of target gene was calculated using the comparative CT method ($2^{-\Delta\Delta CT}$ method) [43] by normalizing the PCR threshold cycle number (Ct value) of the target gene with that of reference gene. For detection the relative transcript levels of *BrERF11* under different treatments, the Chinese cabbage *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, AB333800) was used as an internal reference. Amplification was performed using following gene-specific primer pairs: for *BrERF11* (forward, 5′-TCAGAGCAGCACCGTGGAGT-3′; reverse, 5′-TCAGGCTTGGGAGGGAGATTA-3′); for *GAPDH* (forward, 5′-ACTGTCTCGCTCCATTCG-3′; reverse, 5′-AGTTTCCCTTTGAGGTTAG -3′). The relative transcript level of *BrERF11* was calculated as follows: $2^{-(\Delta CT\ (sample)-\Delta CT\ (calibration))}$, for each time point ΔCT (sample) = CT (target, sample) − average CT (ref, sample), ΔCT (calibration) = average CT (target, calibration) − average CT (ref, calibration). For detection the relative transcript levels of defense-related marker genes in wild type and *35S*::*BrERF11* transgenic tobacco plants after inoculation with *R. solanacearum*, the tobacco *NtEF1α* (GenBank accession number: D63396) was used as an internal reference. The following gene-specific primer pairs were used: *NtEF1α* (forward, 5′-TGCTGCTGTAACAAGATGGATGC-3′; reverse, 5′-GAGATGGGGACAAAGGGGATT-3′); *NtHSR201* (X95343) (forward, 5′-CAGCAGTCCTTTGGCGTTGTC-3′; reverse, 5′-GCTCAGTTTAGCCGCAGTTGTG-3′); *NtHSR515* (X95342) (forward, 5′-TTGGGCAGAATAGATGGGTA-3′; reverse, 5′-TTTGGTGAAAGTCTTGGCTC-3′); *NtACS6* (AF392978) (forward, 5′-GCATTGTTATGAGTGGAGGGG-3′; reverse, 5′-CAGATTCTAAGGCTTCTTTTGTGAC-3′); *NtEFE26* (Z29529) (forward, 5′-CGGACGCTGGTGGCATAAT-3′; reverse, 5′-CAACAAGAGCTGGTGCTGGATA-3′); *NtPR-1a*/c (X05959) (forward, 5′-AACCTTTGACCTGGGACGAC-3′; reverse, 5′-GCACATCCAACACGAACCGA-3′); *NtPR3* (X51425) (forward, 5′-CAGGAGGGTATTGCTTTGTTAGG-3′; reverse, 5′-CGTGGGAAGATGGCTTGTTGTC-3′); *NtPRQ* (M29868) (forward, 5′-ACCACAGGACAACAAGCCATCT-3′; reverse, 5′-ATCTTCCACTGCGTCATTCCGT-3′); *NtNPR1* (U76707) (forward, 5′-GGCGAGGAGTCCGTTCTTTAA-3′; reverse, 5′-TCAACCAGGAATGCCACAGC-3′); *NtPR-1b* (X66942) (forward, 5′-AACCCATCCATACTATTCCTTG-3′; reverse, 5′-GCCGCTAACCTATTGTCCC-3′); *NtCAT1* (AY128694) (forward, 5′-CAACTTCCTGCTAATGCTCCAA-3′; reverse, 5′-TGCCTGTCTGGTGTGAATGA-3′); The relative transcript levels of defense-related marker genes in wild type or *35S*::*BrERF11* transgenic tobacco plants were calculated as $2^{-\Delta\Delta CT}$. The non-treatment wild-type or *35S*::*BrERF11* plants were used as calibration. ΔΔCT = ΔCT (wild type or *35S*::*BrERF11* sample collected after inoculation with *R. solanacearum*)-ΔCT (calibration), for each time point ΔCT (wild type or *35S*::*BrERF11* plants samples collected after inoculation with *R. solanacearum*) = CT (target, sample) − average CT (ref, sample), ΔCT (calibration) = average CT (target, calibration) − average CT (ref, calibration).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2012.10.010.

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