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ORIGINAL PAPER

Introduction of the harpin$_{Xooc}$-encoding gene *hrf2* in soybean enhances resistance against the oomycete pathogen *Phytophthora sojae*

Lu Niu · Jing Yang · Jinhua Zhang · Hongli He · Guojie Xing · Qianqian Zhao · Dongquan Guo · Li Sui · Xiaofang Zhong · Xiangdong Yang

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Abstract *Phytophthora* root and stem rot (PRR) caused by an oomycete pathogen *Phytophthora sojae* is one of the most devastating and widespread diseases throughout soybean-producing regions worldwide. The diversity and variability of *P. sojae* races make effective control of the pathogen challenging. Here, we introduced an elicitor of plant defense response, the harpin$_{Xooc}$-encoding *hrf2* gene from the rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzicola* into soybean and evaluated resistance to *P. sojae* infection. Molecular analysis confirmed the integration and expression of *hrf2* in the transgenic soybean. After inoculation with *P. sojae*, non-transformed control (NC) plants exhibited typical PRR symptoms, including necrotic and wilting leaves, and plant death, whereas most of the transgenic plants showed slightly chlorotic leaves and developed normally. Through T_3 to T_5 generations, the transgenic events displayed milder disease symptoms and had higher survival rates compared to NC plants, indicating enhanced and stable resistance to *P. sojae* infection, whereas without *P. sojae* inoculation, no significant differences in agronomic traits were observed between the transgenic and non-transformed plants. Moreover, after inoculation with *P. sojae*, significant upregulation of a set of plant defense-related genes, including salicylic acid- and jasmonic acid-dependent and hypersensitive response-related genes was observed in the transgenic plants. Our results indicate that *hrf2* expression in transgenic soybean significantly enhanced resistance to *P. sojae* by eliciting multiple defense responses mediated by different signaling pathways. The potential functional role of the *hrf2* gene in plant defense against *P. sojae* and other pathogens makes it a promising tool for broadening disease resistance in soybean.

Keywords Soybean · *Phytophthora* stem and root rot · *Phytophthora sojae* · *hrf2* · Harpin · Defense response

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L. Niu · J. Yang · J. Zhang · H. He · G. Xing ·
Q. Zhao · D. Guo · L. Sui · X. Zhong · X. Yang (✉)
Jilin Provincial Key Laboratory of Agricultural Biotechnology, Jilin Academy of Agricultural Sciences, Changchun 130033, China
e-mail: xdyang020918@126.com

Introduction

The genus *Phytophthora* comprises over 100 species of parasitic oomycetes, the vast majority of which can cause severe diseases of various agriculturally important plants such as potato and soybean. Among them, *Phytophthora sojae* has a narrow host range and is primarily restricted to soybean, although some plant

species such as lupin are also reported to be susceptible to the pathogen (Tyler 2007; Xiong et al. 2014). *Phytophthora* root and stem rot (PRR) caused by *P. sojae* is one of the most devastating and widespread diseases throughout many soybean-growing regions and is responsible for annual economic loss of 1–2 billion dollars (Sheng et al. 2015; Tyler 2007; Xiong et al. 2014). In recent years, PRR incidence has been increasing in several soybean-producing areas around the world, when soil conditions are suitable for pathogen development (Xiong et al. 2014). *P. sojae* can infect seeds, seedlings, and plants in all growth stages, causing seed decay, collapse of emerging seedlings, and root and stem rot in established plants. Presently, PRR is primarily managed based on host-mediated resistance provided by resistance (*R*) genes. In the *P. sojae*-soybean pathosystem, several types of resistance have been described, including race-specific resistance conferred by single dominant *Rps* (resistance to *P. soja*) genes and partial resistance provided by multiple genes (Qutob et al. 2000; Sugimoto et al. 2012). The resistance mediated by *Rps* genes encoding nucleotide-binding leucine-rich repeat (NB-LRR)-type proteins is race-specific and follows the gene-for-gene model (Bhattacharyya et al. 2005; Lin et al. 2014; Yin et al. 2013). To date, 18 *Rps* genes/alleles from soybean genetic sources have been identified and most of them have been mapped on the soybean genome (Lin et al. 2014; Sugimoto et al. 2012; Tyler 2007). Among them, seven *Rps* genes including *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1 k, *Rps*3a, *Rps*6, and *Rps*8 are employed in commercial soybean production. However, PRR virulence pathotypes are very complex, with at least 55 *P. sojae* physiological races identified, and many virulence combinations occur because of mutations and sexual outcrossing (Abney et al. 2007; Burnham et al. 2003; Ryley et al. 1998; Tyler 2007). The diversity and variability of *P. sojae* races both within and between fields make it challenging to effectively control the pathogen based only on the *Rsp* gene-mediated resistance. Moreover, the use of resistant soybean cultivars with single *Rsp* genes can also promote the accumulation of new virulent pathogen strains, which is particularly evident in fields under continuous cultivation (Wang et al. 2006). Furthermore, although partial resistance which is inherited as quantitative trait loci (Wang et al. 2012), can provide more durable protection against *P. sojae*, it does not always prevent significant crop loss caused by the pathogen (Burnham et al. 2003). Therefore, new strategies for enhancing PRR resistance in soybean are still greatly needed.

To date, several attempts have been made to increase resistance of soybean to *P. sojae* infection by expressing foreign genes which confer resistance to fungal pathogens (Dong et al. 2015; Du et al. 2018; Fan et al. 2015). Overexpression of Gly m 4 l, a pathogenesis-related class 10 protein which plays an important role in the soybean defense system against *P. sojae*, was shown to enhance resistance to PRR (Fan et al. 2015). Expression of a novel ethylene response factor, GmERF5, in soybean significantly enhanced resistance to *P. sojae* and upregulated the expression of pathogenesis-related *PR10*, *PR1-1*, and *PR10-1* genes involved in systemic acquired resistance (SAR) in plants (Dong et al. 2015). Recently, Du et al. (2018) showed that expression of the harpin-encoding gene *hrpZm* from *Pseudomonas syringae* enhanced resistance to *P. sojae* infections in transgenic soybean.

Harpin proteins encoded by the *hrp* (hypersensitive response and pathogenicity) genes are produced by several gram-negative plant pathogenic bacteria (Tampakaki et al. 2010). Harpins are secreted through type III secretion system and are mostly localized to the extracellular space in plant tissues, unlike bacterial effector proteins that act inside plant cells (Choi et al. 2013). Some harpins function as a part of translocator complexes involved in the translocation of effectors into plant cells (Choi et al. 2013). When harpins are directly applied to plants or expressed intracellularly, they trigger diverse beneficial processes such as induction of defense responses and enhancement of plant growth (Choi et al. 2013; Pavli et al. 2011; Peng et al. 2003; Shao et al. 2008; Sohn et al. 2007). Overexpression of harpin-encoding genes can enhance resistance to diseases and insects in plants such as tobacco, rice, rape, and cotton (Fu et al. 2014; Li et al. 2004; Miao et al. 2010; Rong et al. 2010; Wei et al. 1992). Recently, it has been reported that harpin overexpression in rice can even improve plant tolerance to abiotic stresses such as drought (Zhang et al. 2011). Multiple functions of harpin-encoding genes in plant development and defense against biotic and abiotic stresses make them a promising tool for enhancing plant disease resistance and improve crop yield through genetic engineering.

The harpin$_{Xooc}$-encoding *hrf2* gene first isolated from the rice pathogenic bacterium *Xanthomonas*

oryzae pv. *oryzicola* (Rong et al. 2010) has been shown to elicit hypersensitive response (HR)-related programmed cell death (PCD) in non-host plants and significantly enhance resistance to the fungal pathogen *Sclerotinia sclerotiorum* (Rong et al. 2010). In the present study, we generated transgenic soybean plants expressing *hrf2* and assessed their resistance to the oomycete pathogen *P. sojae*.

Materials and methods

Agrobacterium tumefaciens-mediated transformation and generation of transgenic plants

A 414-bp fragment of the coding *hrf2* region (as shown in Fig. S1) from *X. oryzae* pv. *oryzicola* strain RS105 (kindly provided by Prof. Xuewen Gao, Nanjing Agricultural University, China) was cloned into the *Sac*I/*Xba*I sites of the compatible pCAMBIA3300 plasmid to generate the recombinant binary vector pCAMBIA3300-hrf2 (Fig. 1a). In the resulting construct, the total length of the T-DNA is 3.7 kb which covers the *hrf2* coding region between the cauliflower mosaic virus (CaMV) 35S promoter and a *polyA* site, and the *bar* gene expression cassette which confers resistance to the herbicide glufosinate. The construct was then mobilized into *A. tumefaciens* strain EHA105. Soybean cultivar Shennong 9 which is susceptible to *P. sojae*, was used to generate transgenic soybean plants using the *Agrobacterium*-mediated transformation as described previously (Yang et al. 2017). The generated transgenic plants tolerant to glyfosinate (5 mg L^{-1}) were transplanted into a greenhouse and analyzed by molecular screening.

Molecular analysis of transgenic soybean plants

Transgenic plants were first screened using LibertyLink® strips (EnviroLogix Inc., USA) and polymerase chain reaction (PCR) as described previously (Yang et al. 2018). Primers HRF2-F1 (5′-GTGGATTGATGTGATATCTCCACTG-3′) and HRF2-R1 (5′-CAAAGTCGCCGCCGCTGCTG-3′) specific for the CaMV 35S promoter and *hrf2*, respectively, were used to amplify a 492-bp fragment to confirm transgene presence. Glufosinate spray (1500 mg L^{-1}) and PCR analysis were used to select positive progenies (generations T_1 to T_5). For Southern blotting analysis, total DNA was extracted from T_2 transgenic and non-transformed control (NC) plants using a modified cetyltrimethylammonium bromide method (Telzur et al. 1999). Thirty micrograms of genomic DNA were digested with *Sac*I or *Hind*III, subjected to electrophoresis in 0.8% agarose gels and subsequently transferred to positively charged nylon membranes (GE Amersham, USA). The transformation vector and non-transformed plants were used as positive and negative controls, respectively. Hybridization probes were prepared by amplifying the 414-bp *hrf2* and 441-bp *bar* coding regions using primers HRF2-F2/HRF2-R2 (5′-ATGAACTCTTTGAACACAC. AATTC-3′/5′-TTACTGCATTGATGCGCTGTCGTTC-3′) and BAR-F1/BAR-R1 (5′-GCACCATCGTCAACCACTACATCGAG-3′/5′-TGAAGTCCAGCTGCCAGAAACCCAC-3′), respectively, and labeling them with digoxigenin (DIG)-High Prime (Roche, Germany). Hybridization was carried out at 42 °C for 12–16 h and staining was performed at room temperature with BCIP/NBT as substrate.

For western blotting analysis, leaf samples (0.1 g) were collected from T_3 transgenic and NC plants and total proteins were extracted with buffer containing 100 mM NaCl, 10 mM EDTA, 200 mM Tris–HCl, 0.05% Tween-20, 0.1% SDS, 14 mM β-mercaptoethanol, 400 mM sucrose, and 2 mM phenylmethanesulfonyl fluoride. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels, proteins were transferred onto PVDF membranes (GE Healthcare, USA) and probed with a rabbit polyclonal antibody against the recombinant HRF2 protein (with molecular weight of 13.63 kDa) expressed in *E. coli* (1:500 dilution, GenScript Co., Ltd., Nanjing, China), and then with horseradish peroxidase (HRP)-labeled goat-anti-rabbit IgG (1:5000 dilution; Abcam, UK) at room temperature for 4 h. After extensive washing, protein bands were visualized using BiodlightTM Western Chemiluminescent HRP substrate (Bioworld Technology, Inc. USA).

Evaluation of transgenic soybean plant resistance to *P. sojae*

P. sojae race 1, a prevalent strain in China, was cultured in 10% V8 media at 25 °C in the dark as described previously (Erwin and Ribeiro 1996), and used to infect unifoliate soybean leaves fully expanded

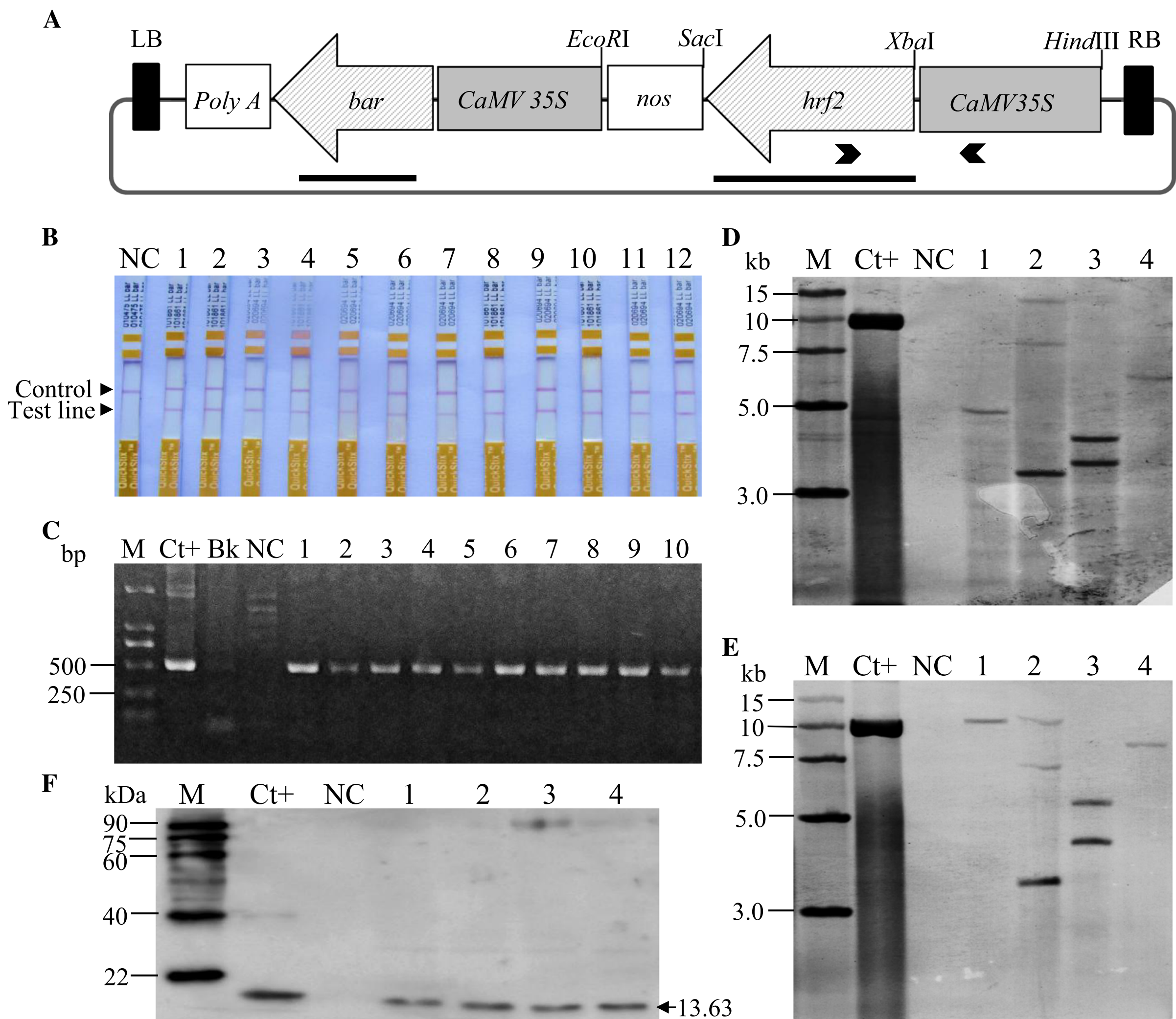

Fig. 1 Generation and molecular analysis of transgenic soybean plants. **a** Schematic diagram of the pCAMBIA3300-hrf2 vector for *Agrobacterium*- mediated genetic transformation. The coding region of *hrf2* was inserted between the constitutive promoter CaMV 35S and a *polyA* site. The construct also contained a selectable marker *bar* for selection of glufosinate-resistant transgenic plants. Solid bars indicate probes (*bar* and *hrf2*) used for Southern blotting. Small arrows indicate primers used for PCR screening. **b, c** Screening of transgenic plants using LibertyLink strip (**b**) and PCR detection (**c**). M, DNA marker (2 K); Ct +, positive control; NC, non-transformed plants; Bk, ddH_2O; 1–12, T_0 transgenic plants. **d**, **e** Southern blotting analysis of transgenic plants. Genomic DNA was digested with *Sac*I or *Hind*III, and hybridized with the corresponding DIG-labeled *hrf2* (**d**) and *bar* (**e**) probes, respectively. M, DNA marker (15 K); Ct +, positive control; NC, non-transformed plants; 1–4, T_2 transgenic events L13, L44, L16, and L32. **f** Western blotting analysis of transgenic plants using a polyclonal antibody raised against the recombinant HRF2 protein; M, protein molecular weight markers; Ct +, recombinant HRF2 protein expressed in *E. coli*. NC, non-transformed plants; 1–4, transgenic events L13, L44, L16 and L32

in the greenhouse. Twenty hypocotyls of 15-day-old soybean seedlings of each transgenic and NC plants were inoculated with macerated mycelia (inoculum) of *P. sojae* using a standard hypocotyl inoculation method (Abney et al. 2007). The inoculated plants were maintained for 18–24 h in the dark, and then switched to the 18-h light/6-h dark regime at 25 °C for symptom development. PRR symptoms were evaluated approximately 7–10 days after inoculation and the percentage of surviving seedlings was calculated. The same number of non-transformed plants were mock-inoculated (wounded) without the pathogen and

used as controls. All experiments were performed independently three times with 20 plants per replicate.

Expression analysis of defense-related genes

Quantitative real time (qRT) PCR was performed to assess transcription levels of defense-related SA-dependent genes such as *GmPR1a* (AF136636), *GmPR2* (β-1, 3-glucanase, M37753), *GmPR3* (chitinase class I, AF202731), *GmPR5* (pathogenesis-related group 5 protein, BU765509), *GmPR12* (defensin, M37753), and *GmPAL* (phenylalanine ammonia lyase, X52953), JA-dependent genes such as *GmAOS* (allene oxide synthase, DQ288260) and *GmPPO* (polyphenol oxidase, EF158428), and HR-related genes such as *GmNPR1-1* (FJ418594), *GmNPR1-2* (FJ418596), *GmSGT1* (NM_001249656), and *GmRAR1* (FJ222386). Three fresh leaves from randomly selected T_3 transgenic and NC plants were harvested and frozen in liquid nitrogen at 0, 4, 8, and 12 h after inoculation with *P. sojae*. Total RNA was isolated using the EasyPure PlantRNA Kit (TransGen Biotech, China) and treated with DNase I to remove genomic DNA according to the manufacturer's protocol. RNA integrity was confirmed by agarose gel electrophoresis and its concentration quantified by spectrophotometry (Nanodrop ND-1000, USA). First-strand cDNA was synthesized with the ThermoScript RT-PCR system (Invitrogen, USA) and used as a template for qRT-PCR performed at the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 2 min, 62 °C for 30 s, and 72 °C for 30 s. Primers used in this study are listed in Supplementary Table S1. Relative transcription levels of the target genes were calculated relative to that of the constitutively expressed native soybean gene *GmACT11* (GenBank No. BW652479) using the $2^{\Delta\Delta}$Ct method as previously described (Yang et al. 2018). The results were statistically analyzed based on at least three independent biological replications.

Agronomic traits of transgenic plants in field conditions

For evaluation of the agronomic performance, the transgenic plants were planted in the field station in Jilin province, China. Three replicates of triple-row plots per transgenic event were grown using a randomized block design, with each row consisting of twenty-thirty plants. At maturity, ten plants from each transgenic event and NC control (without *P. sojae* inoculation) were randomly sampled and evaluated for agronomic traits such as plant height, branch, node, pod, and seed numbers, and 100-seed weights.

Statistical analysis

Least significant differences (LSD) between mean values were analyzed by the *t* test at $P = 0.05$ or 0.01 using SPSS software (v. 17.0) and each transgenic event was compared with the corresponding NC plants.

Results

Generation of transgenic soybean plants and molecular screening

Sixty-four plants of soybean genotype Shennong 9 were produced from glufosinate-tolerant green shoots following the *Agrobacterium*-mediated transformation. Among these, thirty-seven plants were confirmed positive by LibertyLink® strip detection and PCR analysis. LibertyLink strip detection showed expression of the *bar* gene with two red lines appearing simultaneously for transgenic plants (Fig. 1b). PCR analysis of LibertyLink-positive plants showed amplification of the expected 492-bp fragment corresponding to the CaMV 35S-*hrf2* coding sequence (Fig. 1c). After preliminary PRR resistance screening, four independent transgenic events L13, L16, L32 and L44 which exhibited higher resistance, were selected for further molecular analysis and resistance evaluation. Integration of the foreign gene was confirmed by Southern blotting with both DIG-labeled *hrf2* and *bar* probes. The results showed that hybridization signals appeared in the selected transgenic plants and all the bands were greater than the expected 1.56-kb (*Sac*I-*hrf2*-RB, Fig. 1d) and 3.44-kb (*LB-bar-hrf2-Hind*III, Fig. 1e) fragments, respectively. In contrast, no hybridization signals were observed in NC plants. Moreover, low copy numbers of T-DNA insertions (single copies for L13 and L32, two copies for L16, three copies for L44) were detected in the four independent transformation events. Western blotting analysis revealed the presence of a 13.63-kDa polypeptide corresponding to the HRF2 monomer in

transgenic plants, whereas no signals were observed in NC plants (Fig. 1f), confirming expression of the HRF2 protein in transgenic events.

Transgenic plants exhibited stably enhanced resistance to PRR under greenhouse conditions

Phenotypic reactions of the transgenic events and their corresponding NC plants to *P. sojae* were evaluated in the greenhouse. At 7 days after inoculation with *P. sojae*, typical PRR symptoms, including necrotic and wilting leaves and plant death, were observed in most NC plants of each experimental replicate. In contrast, most of transgenic plants showed no visible symptoms or only slightly chlorotic leaves and developed normally, and mock-inoculated transgenic seedlings were asymptomatic (Fig. 2a). The percentage of surviving seedlings calculated to assess disease severity caused by *P. sojae* inoculation was higher in transgenic events (73.35–92.86%) compared to NC plants (38.50–40.37%) (Fig. 2b), although slight variations in the proportion of surviving seedlings for each transgenic event across the three replicates were observed, which might be attributed to minor differences in environmental conditions. Furthermore, transgenic events consistently exhibited enhanced resistance to *P. sojae* compared to NC over three generations (from T_3 to T_5) (Fig. 2b). To analyze the influence of *hrf2* expression on agronomic characteristics of transgenic plants, we evaluated several traits such as leaf morphology, flower and hilum color, plant and podding height, node numbers, weight of 100 seeds, and maturity period under field conditions without *P. sojae* inoculation. The data showed that there were no significant differences between transgenic and NC plants, indicating no visible influence on agronomic performance caused by *hrf2* expression (Table 1).

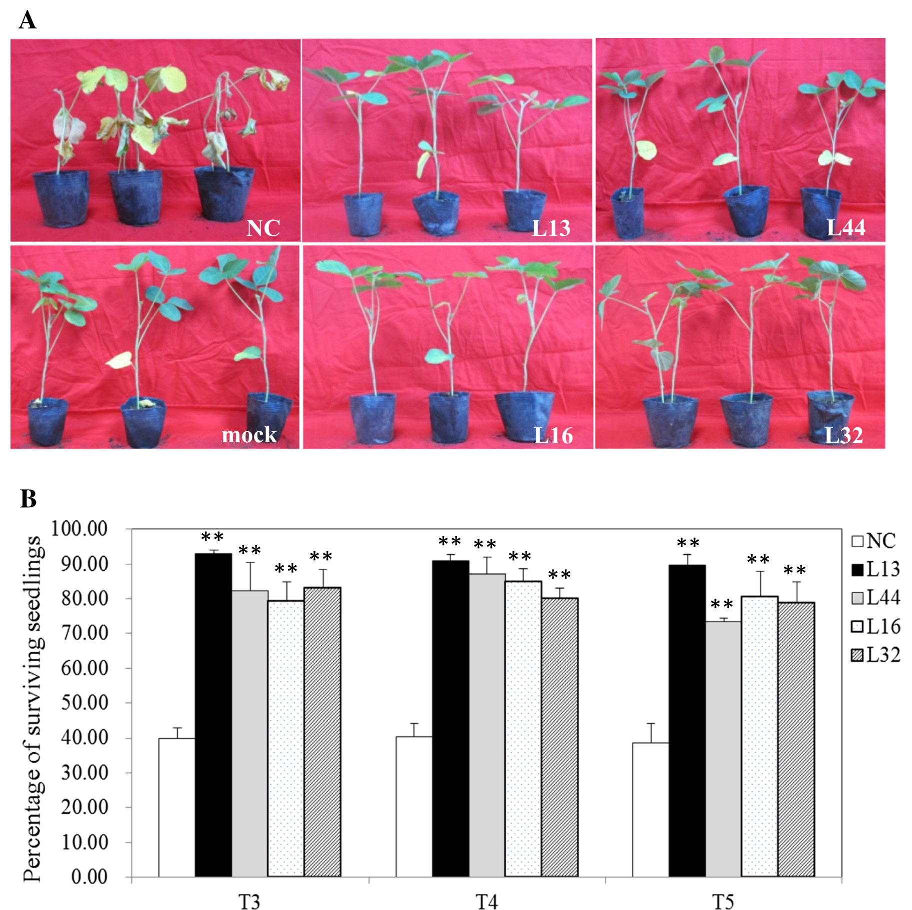

Fig. 2 PRR resistance evaluation of transgenic soybean plants expressing *hrf2*. **a** Resistance response of T_3 transgenic plants to *P. sojae* infection. **b** Increased survival rate of three consecutive generations (T_3–T_5) of transgenic plants inoculated with *P. sojae*. NC, non-transformed plants; mock, non-transformed plant wounded without *P. sojae* inoculation. L13, L44, L16, and L32, transgenic events. The data are presented as the mean ± SE of three independent experiments (20 plants per each replicate); $^{**}P < 0.01$ compared to corresponding NC plants

Table 1 Agronomic performance of transgenic soybean events in the field

Agronomic traits	Transgenic events				
	NC	L13	L44	L16	L32
Leaf shape	Round	Round	Round	Round	Round
Flower color	White	White	White	White	White
Seed coat color	Yellow	Yellow	Yellow	Yellow	Yellow
Hilum color	Black	Black	Black	Black	Black
Maturity period (d)	128	128	128	128	128
Plant height (cm)	90.33 ± 5.24a	95.32 ± 9.9 a	93.54 ± 11.3a	91.29 ± 5.31a	95 ± 6.65a
Branching number (/plant)	4.06 ± 1.06a	4.08 ± 2.04a	4.54 ± 1.71a	4.71 ± 0.49a	4.3 ± 1.95a
Node number (/plant)	18.67 ± 1.88a	17.92 ± 3.25a	20.08 ± 2.75a	18.43 ± 2.07a	20.5 ± 3.06a
Podding height (cm)	6.11 ± 2.3a	6.68 ± 2.63a	6.69 ± 2.21a	5.29 ± 1.5a	7.6 ± 2.63a
100-seed weight (g)	16.79 ± 0.95a	17.14 ± 1.37a	16.65 ± 1.21a	16.49 ± 0.87a	16.81 ± 1.99a

The data were collected from the field station in Jilin province, China. Ten plants from each transgenic event and 10 corresponding NC plants were randomly sampled and measured at maturity

Letters in each row indicate significant differences (LSD, $P > 0.05$)

hrf2-expression upregulated multiple defense-related genes in transgenic plants

To investigate the molecular mechanisms underlying enhanced resistance to *P. sojae* in *hrf2*-expressing plants, we analyzed the transcription of 12 defense-related genes after *P. sojae* infection by qRT-PCR. First, we evaluated the time course of *hrf2* expression in transgenic plants after inoculation with *P. sojae*. The results showed that *hrf2* mRNA levels peaked at 4 h and remained high at 8 and 12 h post inoculation in all three examined transgenic soybean plants (Fig. 3a), although the abundance of the *hrf2* transcript was slightly different among different transgenic events. Then, we compared mRNA levels of six SA-dependent genes (*GmPR1*, *GmPR2*, *GmPR3*, *GmPR5*, *GmPR12* and *GmPAL*), two JA-dependent genes (*GmAOS* and *GmPPO*), and four HR-related genes (*GmNPR1-1*, *GmNPR1-2*, *GmSGT1*, and *GmRAR*) in transgenic and NC plants 4 h after *P. sojae* challenge. The results showed that the expression of all the 12 defense genes was significantly upregulated in response to *P. sojae* infection in transgenic plants but remained unchanged or increased to a lesser extent in NC plants (Fig. 3b–d). Especially high expression levels of the 12 defense-related genes were observed in transgenic event L13, which was consistent with the results of PRR resistance evaluation. Our data suggest that the expression of the *hrf2* gene in soybean could elicit multiple resistance responses mediated by different signaling pathways, thus enhancing plant resistance to *P. sojae* infection and protecting soybean against PRR.

Discussion

Harpin proteins are known to increase resistance of plants to fungi and insects, improve their tolerance to abiotic stresses, and promote growth (Fu et al. 2014; Li et al. 2004; Miao et al. 2010; Rong et al. 2010; Wei et al. 1992; Zhang et al. 2011). It has been reported that the introduction of the harpin$_{Xooc}$-encoding *hrf2* gene in rapeseed could effectively enhance its resistance to *S. sclerotiorum*, an oxalate-secreting necrotrophic fungal pathogen (Rong et al. 2010). In the present study, we showed that the transgenic plants constitutively expressing the *hrf2* gene also exhibited significantly enhanced resistance to the oomycete pathogen *P. sojae*, as demonstrated by the hypocotyl inoculation assay. Compared with non-transformed plants, the transgenic events showed milder PRR symptoms and had higher survival rates. These results are consistent with the multiple functional activity of harpin proteins which were reported to induce plant resistance against

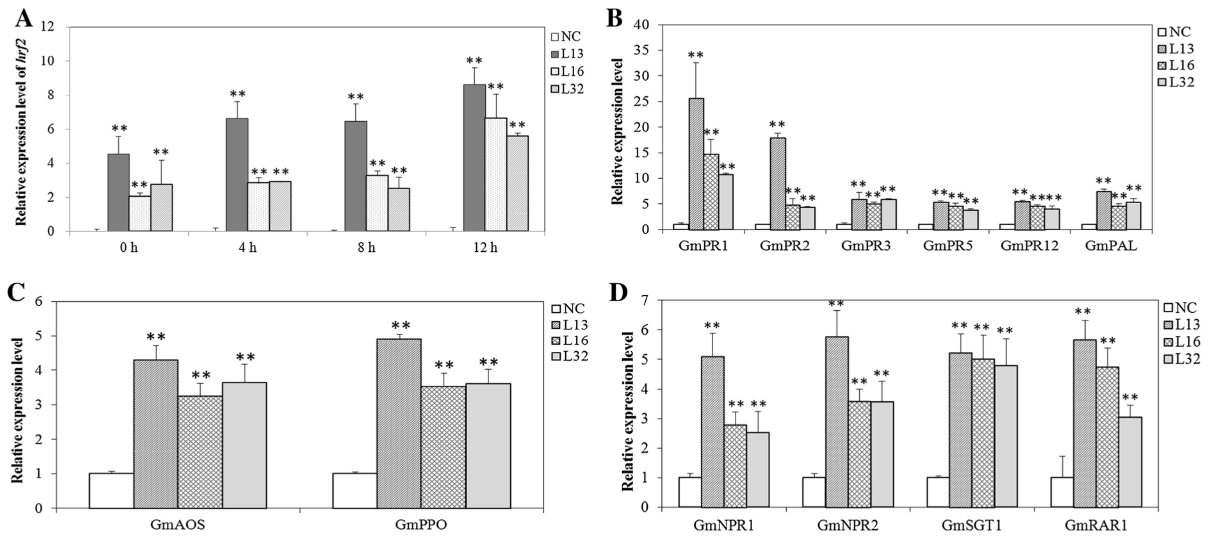

Fig. 3 Relative expression of defense-related genes in transgenic soybean plants after *P. sojae* inoculation. **a** Expression changes of *hrf2* mRNA in transgenic plants. Total RNA was extracted from fully grown leaves of T_3 transgenic plants at 0, 4, 8, and 12 h after inoculation with *P. sojae* and mRNA levels of *hrf2* were calculated relative to that of the *GmACT11* gene. **b**–**d** Relative expression of six salicylic acid-dependent genes (**b**), two jasmonic acid-dependent genes (**c**), and four hypersensitive response-related genes (**d**) at 4 h after *P. sojae* inoculation. NC, non-transformed plants; L13, L16, and L32, transgenic events. The data are presented as the mean ± SE of three biological replicates; $^{**}P < 0.01$ compared to corresponding NC plants

diverse plant pathogens including fungi, bacteria, and viruses either after external application or stable expression (Choi et al. 2013; Pavli et al. 2011; Peng et al. 2003; Shao et al. 2008; Sohn et al. 2007). The potential function of *hrf2* in plant defense against *P. soja* and other pathogens such as *S. sclerotiorum* makes this gene a promising candidate for engineering disease resistance in soybean breeding.

Mechanisms underlying increased resistance of harpin-expressing plants to various infections have to be characterized for future combination with other defense and resistance components. Phytohormones SA and JA are known to play crucial roles in regulating defense mechanisms in plants (Pieterse et al. 2009; 2012; Robert-Seilaniantz et al. 2011; Sugano et al. 2014). It was reported that an extensive crosstalk existing between phytohormone-controlled signaling pathways contributes to the induction of strong defense responses in plants (Koornneef and Pieterse 2008). SA plays a crucial role in the activation of defense mechanisms in response to biotrophic and hemibiotrophic pathogens, whereas JA is typically associated with defense against necrotrophic pathogens, and the respective pathways are often mutually antagonistic. However, synergistic interactions have also been reported in some pathosystems. In this study, we observed that six SA-dependent genes (*GmPR1*, *GmPR2*, *GmPR3*, *GmPR*, *GmPR12*, and *GmPAL*) and two JA-dependent genes (*GmAOS* and *GmPPO*) were simultaneously upregulated in *hrf2*-expressing transgenic plants compared with NC plants which showed no or little transcriptional changes in response to *P. sojae* infection. Moreover, transgenic plants also exhibited significantly increased expression of genes involved in HR signaling. These findings suggest that the enhanced resistance to *P. sojae* infection in transgenic soybean constitutively expressing *hrf2* may be attributed to significant transcriptional upregulation of a panel of genes involved in different plant defense-related signaling pathways. In addition, it was also reported that external application of harpins such as $HrpN_{Ea}$ to plants or constitutive expression of harpin-encoding genes promote plant growth through the ethylene-mediated signaling pathway (Chuang et al. 2010; Dong et al. 2004; Oh and Beer 2007). However, in this study, we did not observe significant differences in agronomic traits such as plant height in transgenic soybean events compared to NC plants in our preliminary field experiment. This discrepancy may be attributed to distinct signaling pathways mediated by HRF2 and other harpin proteins. The influence of expression of *hrf2* on the agronomic

performance of transgenic soybean needs to be further conducted larger field experiments.

Compared to exogenous application of harpins which can independently activate HR-related PCD and the SA-mediated SAR pathway in plants (Choi et al. 2013; Peng et al. 2003), endogenous expression of harpins may elicit more complicated responses to pathogens. Thus, transgenic plants expressing *hrp* genes show stronger reactivity to pathogens manifested by substantial increase in the expression of defense-related genes mediated by multiple signaling pathways (Pavli et al. 2011; Peng et al. 2004; Shao et al. 2008; Sohn et al. 2007), however, it should be noted that gene expression patterns might be distinct for different harpin proteins (Du et al. 2018). For example, although NPR1 is thought to be a key transcriptional regulator of multiple signaling pathways involved in plant defense (Peng et al. 2004), no changes in *npr1* gene expression were detected in *hrpN*-transgenic plants inoculated with *B. cinerea* (Sohn et al. 2007). In contrast, in the present study, we observed a significant increase of *npr1* expression in transgenic soybean after *P. sojae* infection. Similarly, transcriptional upregulation of the *npr1* gene in response to pathogen infection was reported in transgenic tobacco plants expressing $hpa1_{Xoo}$ but not in those expressing hpa_{GEP} (Peng et al. 2004; Sohn et al. 2007). This difference in *npr1* regulation among harpin-expressing plants may be due to distinct receptors targeted by harpins of various origins (Miao et al. 2010).

In conclusion, our study suggests that the expression of the *hrf2* gene encoding $\text{harpin}_{\text{Xooc}}$ in soybean enhances plant resistance to *P. sojae* infection and protects against PRR through transcriptional upregulation of multiple defense-related genes. However, it should also be noted that transgenic soybean event expressing *hrf2* are not entirely immune to *P. sojae* under our experimental conditions. For sustainability of PRR resistance, it is important to rely on multiple components of defense by pyramiding complementary resistance genes thorough breeding or genetic transformation.

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Author's Contributions XY designed the experiments. LN conducted the experiments and wrote the manuscript. GX, DG, LS, and QZ performed *Agrobacterium*-mediated transformation experiments. JZ conducted the hypocotyl inoculation assay. HH, JY, and XZ participated in molecular screening and qRT-PCR analyses. All authors have read the manuscript and contributed to its revision.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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