PATHOGEN-INDUCED EXPRESSION OF HARPIN$_{PSS}$ INCREASES RESISTANCE IN TOBACCO AGAINST *FUSARIUM OXYSPORUM* f. sp. *NICOTIANAE*

D. Dey, B. Uma, G. Padmaja and A.R. Podile

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, Andhra Pradesh, PIN-500046, India

SUMMARY

Harpin$_{Pss}$ (encoded by the *hrpZ* gene), a proteinaceous elicitor produced by *Pseudomonas syringae* pv. *syringae*, induces cell death in plants through hypersensitive response (HR). With an aim to generate transgenic tobacco resistant to fungal diseases, *hrpZ* was expressed in a secretable form, tagged with the signal peptide (*SP*) of *PR1a*, under the constitutive 35S promoter (*35S*) or pathogen-inducible promoters (PIPs) like phenylalanine ammonia lyase (PAL), osmotin (OSM), and hypersensitive-related (HSR) promoters. The constitutive expression of the secretable form of *hrpZ* did not permit regeneration of transformed cells due to harpin$_{Pss}$-induced cell death. Transformants were recovered at a low frequency (2-6%) from leaf discs infected with *Agrobacterium* harbouring the *SP-hrpZ* driven by PIPs due to wound-induced leaky expression of harpin$_{Pss}$. The transgenic lines were confirmed by PCR using transgene-specific primers for *SP-hrpZ*. The expression of *hrpZ* under PIPs in transgenic lines was confirmed by Western blotting after challenging the leaves with *Fusarium oxysporum* f. sp. *nicotianae*. RT-PCR analysis also confirmed the expression of *SP-hrpZ* driven by PIPs in transgenic tobacco upon infection with *F. oxysporum* f. sp. *nicotianae*. The expression of harpin$_{Pss}$ in these transgenic lines was accompanied by expression of defense-response genes such as *PR1*, *PR2*, *PR3*, *HSR* and *HIN1*. Transgenic tobacco plants showed enhanced resistance to *F. oxysporum* f. sp. *nicotianae*. Our findings suggest the potential use of an elicitor gene (*hrpZ*), driven by PIPs (PAL, OSM, and HSR) for the development of resistant plants.

Key words: disease resistance, hypersensitive response, pathogen-inducible promoter, signal peptide

Corresponding author: A.R. Podile
Fax: +91.4023010120
E-mail: arpsl@uohyd.ernet.in

INTRODUCTION

Harpins are elicitors produced and exported by type III secretion pathway of Gram- negative phytopathogenic bacteria from genera like *Erwinia*, *Pseudomonas*, *Ralstonia* and *Xanthomonas*. Harpins are acidic, hydrophilic in nature, rich in glycine, lack cysteine, and are heat stable with no reported enzymatic activity. Harpins induce plant defense by activating hypersensitive response (HR) (He *et al*., 1993; Wei *et al*., 1992) which is a form of programmed cell death in plants. Infiltration of an aqueous solution of harpin into plants activates plant defense accompanied by expression of pathogenesis-related (PR) proteins and systemic acquired resistance (SAR) (Dong *et al*., 1999; Strobel *et al*., 1996). *P. syringae* pv. *syringae* 61 *hrpZ* gene encodes harpin$_{Pss}$, a 34.7 kDa extracellular protein that rapidly elicits HR upon infiltration in tobacco. Immunolocalization studies revealed a Ca^{2+}-dependent association of harpin$_{Pss}$ with tobacco cell walls (Hoyos *et al*., 1996). Further, harpin treatment leads to plasma membrane depolarization (Pike *et al*., 1998; Hoyos *et al*., 1996) suggesting that the cell wall could be the harpin binding site. However, both the apoplastic and cytoplasmic localization of harpin is able to confer pathogen resistance in plants (Sang *et al*., 2012).

Several strategies were adopted to develop transgenic plants resistant to fungal diseases (Collinge *et al*., 2010) but very limited success has been achieved with fungus-resistant transgenic plants for field application. Initial attempts to engineer fungal disease resistance involved constitutive expression of transgenes under the 35S promoter. Overexpression of lactoferrin conferred enhanced resistance to *Fusarium* head blight in wheat (Han *et al*., 2012). This approach, however, poses a severe burden on the plant, as it leads to reduced growth and to abnormal and lethal phenotypes (Dong *et al*., 1999). Exploitation of HR to induce disease resistance requires specific expression of transgene only during pathogen attack under tightly regulated promoters. Pathogen-inducible promoters (PIPs) restrict the expression of transgenes only at the pathogen's infection sites (Gurr and Rushton, 2005), thereby reducing the burden on the plant, and boosting defense. This is even more advantageous while expressing genes coding for toxic proteins (e.g., barnase, HR elicitors etc.) which reduces the chances of leaky expression that leads to undesired necrosis.

A few PIPs, such as phenylalanine ammonia lyase (PAL), win3.12T, GST1 (glutathione S-transferase), HSR203J (hypersensitive-related), HIN1 (hypersensitive-induced), osmotin (OSM) promoter, etc. have been reported from plants (Yevtushenko *et al*., 2004; Pontier *et al*., 1994; Raghothama *et al*., 1993). However, most of the PIPs drive transgene expression during different developmental stages or in different parts of the plants as well as leaky or wound-inducible expression. The expression of a cecropin A-melittin hybrid peptide under the win3.12T promoter resulted in transgenic tobacco plants resistant to *Fusarium solani* (Yevtushenko *et al*., 2005). Pathogen-inducible expression of cryptogein in transgenic tobacco, under HSR or PAL promoters, conferred broad spectrum resistance to fungal pathogens (Donghua *et al*., 2004; Keller *et al*., 1999).

HR induction and defense gene activation by harpin has been exploited for the development of transgenic plants resistant to fungal diseases. Constitutive expression of harpin in an intracellular form resulted in the development of healthy plants without morphological deformities. The transgenic plants were resistant, showing HR-like local lesions after pathogen attack (Takakura *et al*., 2004; Tampakaki *et al*., 2000). Extracellular accumulation of high level of harpin (through signal peptide) in a constitutive manner would lead to systemic necrosis. Contrary to this, transgenics were developed with constitutive expression of extracellular form (SP-hrpZ) that showed resistance to viral (Pavli *et al*., 2011) and fungal pathogens (Li and Fan, 1999). Harpin accumulation at a low level due to improper processing of SP resulted in recovery of transgenics without necrosis (Pavli *et al*., 2011; Li and Fan, 1999) because, below a critical level, harpin can elicit disease resistance without HR induction (Greenberg, 1997). Similarly, constitutive and stable expression of harpin led to disease resistance without macroscopic damage in tobacco (hrpN), rice (hrf1), *Brassica* (hrf-2), and cotton ($hpa1_{Xoo}$) (Huo *et al*., 2010; Miao *et al*., 2010; Shao *et al*., 2008; Sohn *et al*., 2007; Jang *et al*., 2006). The pathogen-inducible expression of an intracellular form of harpin under PAL1 promoter resulted in normal-looking transgenic plants resistant to bacterial pathogens, which showed HR-like lesions only upon pathogen attack (Takakura *et al*., 2004). Similar results were obtained with transgenic potato expressing the *hrpN* gene under the prp1-1 promoter that conferred resistance to *Phytophthora infestans* (Li and Fan, 1999). Citrus transgenics expressing secretable form of harpin (SP-hrpZ) under the potato Gst1 promoter showed limitations in regeneration of transgenics due to wound-induced expression of harpin (Barbosa-Mendes *et al*., 2009).

In the present study, extracellular form of $harpin_{Pss}$ was expressed under PIPs *viz*., PAL, OSM, and HSR for development of transgenics resistant to *Fusarium oxysporum* f. sp. *nicotianae* (*FON*).

MATERIALS AND METHODS

Bacterial strains. *Agrobacterium tumefaciens* EHA105 harbouring binary constructs (described below) was used for tobacco transformation. Bacterial cultures were grown overnight at 28°C in Luria-Bertani (LB) medium supplemented with kanamycin (50 mg/l) and rifampicin (50 mg/l), sub-cultured, grown to OD_{600} 0.6, centrifuged and suspended in half-strength MS medium for transformation.

Generation of binary constructs. Secretable form of harpin (SP-hrpZ) was generated by in-frame fusion of the coding regions of hrpZ and SP of *PR1a* gene from tobacco (Madhuri *et al*., 2012) and cloned in pGEM-T vector. The SP-hrpZ was cloned downstream of the constitutive 35S promoter available in the pRT100 vector, and PIPs like PAL, OSM and HSR. The tomato PAL (GenBank accession No. M83314, 232 bp), tobacco osmotin (GenBank accession No. S68111, 1 kb) and HSR203J (GenBank accession No. X77136, 700 bp) promoters lacking the silencer elements were used to drive the expression of SP-hrpZ. The tomato PAL promoter was received from Prof. M.V. Rajam, Department of Genetics, University of Delhi-South Campus in the pRT101 vector. The OSM and HSR promoters were PCR-amplified from genomic DNA of tobacco using respective sequence-specific primers (Table 1).

pCAMBIA1300-35S-SP-hrpZ-nos (CSH). The *SP-hrpZ* gene was PCR-amplified from pGEM-T-(*SP-hrpZ*) using gene-specific primers and cloned in the *Nco*I-*Bam*HI sites of pRT100 vector. The entire cassette (*35S-SP-hrpZ-nos*) was cloned into the *Pst*I site of pCAMBIA1300 generating CSH construct (Fig. 1).

pCAMBIA1300-PAL-SP-hrpZ-nos (PSH). The *SP-hrpZ* gene was PCR-amplified (as above) and cloned into *Xho*I-*Bam*HI sites of pRT101 vector harbouring the PAL promoter. The cassette (*PAL-SP-hrpZ-nos*) was cloned into *Pst*I sites of pCAMBIA1300 generating the PSH construct (Fig. 1).

pCAMBIA1300-OSM-SP-hrpZ-nos (OSH). The OSM promoter was PCR-amplified using sequence-specific primers and cloned upstream of *SP-hrpZ* gene in pGEM-T vector. The nos terminator was also PCR-amplified and cloned downstream of *SP-hrpZ* gene generating the cassette (*OSM-SP-hrpZ-nos*) which was cloned in *Pst*I site of pCAMBIA1300 generating the OSH construct (Fig. 1).

pCAMBIA1300-HSR-SP-hrpZ-nos (HSH). Similarly, the HSR promoter was PCR-amplified using sequence-specific primers and cloned upstream of *SP-hrpZ* gene in pGEM-T vector. The nos terminator was also PCR-amplified and cloned downstream of *SP-hrpZ* gene generating the cassette (*HSR-SP-hrpZ-nos*) which was cloned in the *Pst*I site of pCAMBIA1300 generating the HSH construct (Fig. 1).

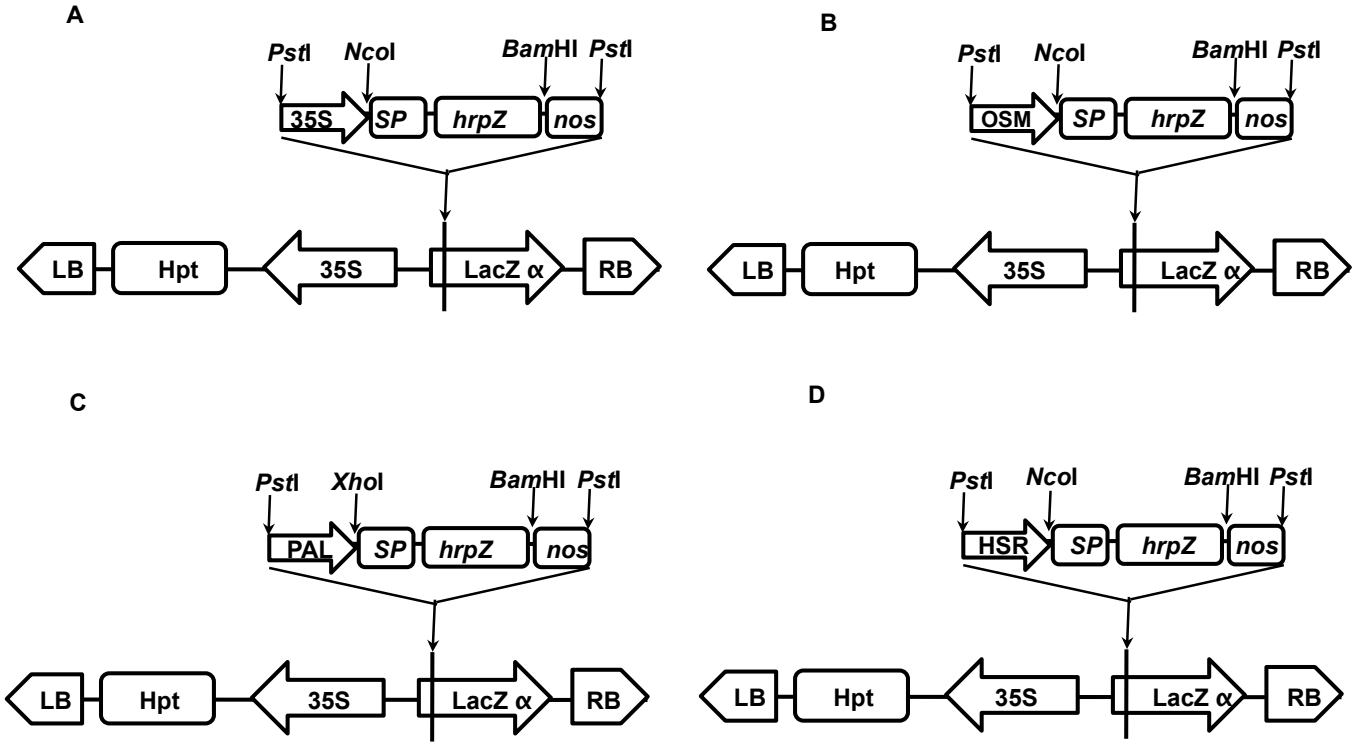

Fig. 1. Constructs used in present study. The *SP-hrpZ* gene was placed downstream of four different promoters. A. 35S CaMV; B. OSM; C, PAL; D, HSR in pCAMBIA1300 with terminator and hygromycin (*hpt*) as selectable marker.

All the four constructs were confirmed by automated DNA sequencing at CDFD (India) or MWG Biotech (India), before mobilizing to *A. tumefaciens* EHA105.

***A. tumefaciens*-mediated transformation of tobacco.** The leaves from 4-week-old, greenhouse-grown plants of *Nicotiana tabacum* cv. Xanthi were surface-sterilized with 70% ethanol for 1 min followed by 0.1% $HgCl_2$ for 5 min and rinsed 4-5 times with sterile double distilled water. The surface-sterilized leaf explants were infected with *Agrobacterium* harbouring the binary constructs using leaf disk transformation. The transformants were selected on MS medium supplemented with hygromycin (25 mg/l), and maintained in a greenhouse at 22±2°C (night) and 30±2°C (day).

Molecular characterization of transgenic plants. Genomic DNA from young leaves of transformed and non-transformed control (NT) plants was isolated using the CTAB (cetyl trimethyl ammonium bromide) method (Rogers and Bendich, 1994). The presence of *SP-hrpZ* in the putative transformants was confirmed by PCR at 94°C for 5 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 90 sec and 72°C for 10 min. Western blot analysis was performed with the protein isolated from the leaves of transgenic lines along with NT plants challenged with *FON* spores (5×10^5 spores/ml water). Total soluble proteins were extracted from the leaves using the TRIzol reagent. Protein samples (70 µg) were separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, USA) and detected as described earlier (Podile *et al.*, 2001). The immunoblot was visualized by the alkaline phosphatase-catalysed color reaction using 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (BCIP-NBT) as substrate (Sripriya *et al.*, 2009).

Bioassay for resistance against *Fusarium oxysporum* f. sp. *nicotianae*. Leaves from 6-week-old transgenic lines (PSH, OSH and HSH) and NT plants were inoculated using a detached leaf bioassay (Bhargava *et al.*, 2007). *FON* was grown on potato dextrose agar for 7 days at 25°C. Agar plugs (5 mm) with fully grown fungal mycelium were placed on adaxial surface of the leaves on both sides of the midrib. After inoculation, the trays were covered with transparent polyethylene sheet to maintain high humidity and incubated at 24°C with 16 h light/8 h dark photoperiod. Symptoms were evaluated and lesion diameter was measured seven days post inoculation (dpi). Three leaves from each transgenic plant, repeated once, were used for the analysis and representative images were acquired.

Semi-quantitative RT-PCR. Leaf samples from NT and transgenic lines (representatives of PSH, OSH and HSH) were drop-inoculated with a *FON* spore suspension (5×10^5 spores/ml water) (Hemelrijck *et al.*, 2006). Leaf samples were collected from treated and untreated plants 12 h post inoculation (hpi) and stored at −80°C. Total RNA was isolated using TriZol (Sigma, USA) and converted into cDNA using BluePrint 1st strand cDNA synthesis kit (TaKaRa, Japan) following the manufacturer's protocol. The cDNA

was used as template in a PCR to amplify *SP-hrpZ* and different defense response genes (*PR1*, *PR2*, *PR3*, *HSR203J* and *HIN1*) using the respective gene-specific primers (Table 1) at 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1-2 min and 72°C for 10 min. EF1α was used as loading control for RNA samples.

RESULTS

Transgenic tobacco plants expressing *SP-hrpZ*. Transgenic tobacco plants expressing *SP-hrpZ* under different PIPs were generated by *Agrobacterium*-mediated leaf-disk transformation. Transgenics with the CSH construct could not be generated due to constitutive expression of *SP-hrpZ* resulting in extracellular accumulation of harpin$_{Pss}$ and killing all explants/shoot buds due to HR. We also faced a major problem in recovering transformed explants to raise transgenics with other constructs. The normal procedure of cutting shoot buds or shoots during subculture could not be adopted as it led to wound-induced expression of harpin from PIPs, and necrosis of the shoots/buds. Thus, the shoots were allowed to grow as a bunch until rooting. This approach helped in recovering transgenics with all constructs, except for CSH. Rooted plants were acclimatized and established in soil in the greenhouse. From a total of *ca.* 750 explants (250 for each construct) used for co-cultivation, 15 different lines for PSH, 5 lines for OSH and 7 lines for HSH were generated (Fig. 2). Transformation efficiency was 6% for PSH, 2% for OSH and 2.8% for HSH. The transgenic lines (PSH, OSH and HSH) were positive for the *hpt* gene (data not shown) and *SP-hrpZ* in a PCR-based screening. The presence of *SP-hrpZ* gene in four transgenic lines for each construct in a multiplex PCR with *virG* is shown in Fig. 3. All transgenic lines were morphologically similar to NT plants and no growth difference was observed except for the case of PSH, where abnormalities (crinkling) in some leaves of a few lines were observed, possibly due to leaky expression of the harpin from the PAL promoter.

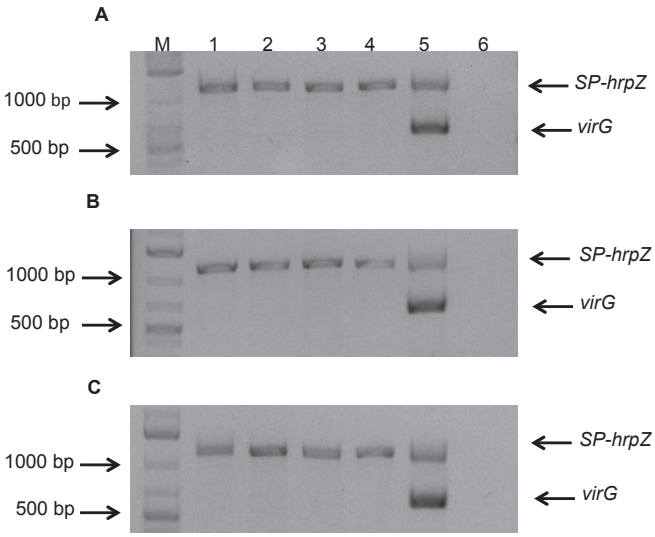

Fig. 2. PCR confirmation of putative tobacco transgenics of PSH, OSH and HSH constructs (A, B, and C, respectively) for the presence of *SP-hrpZ* (1200 bp) and *virG* (590 bp). Lanes 1, 2, 3, and 4: representatives from respective transgenic lines. Lane 5: *A. tumefaciens* EHA105 transformed with one of the binary constructs (PSH/OSH/HSH) used as a positive control for *SP-hrpZ* and *virG*. Lane 6: non-transformed (NT) control tobacco plant used as negative control. Lane M: 1 kb DNA ladder plus (New England Biolabs, USA).

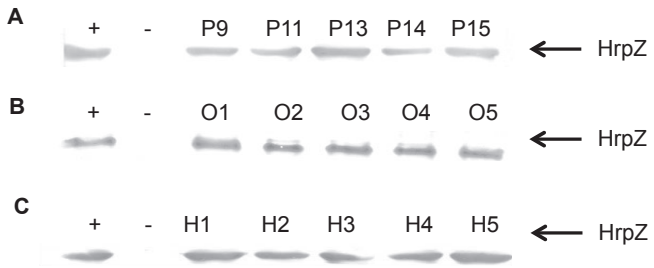

Fig. 3. Western blot analysis of harpin$_{Pss}$ from transgenic lines of PSH (P), OSH (O) and HSH (H) plants (A, B, and C, respectively). Harpin$_{Pss}$ was used as positive control (+) while protein from NT was used as negative control.

Western blotting was performed to confirm the pathogen-inducible expression of harpin$_{Pss}$ in different transgenic lines. *FON*-challenged leaves from PSH, OSH and HSH transgenics were collected after 12 hpi and the protein was isolated. Different transgenic lines of PSH, OSH and HSH constructs showed positive signal for harpin$_{Pss}$ (Fig. 3).

Bioassay of harpin$_{Pss}$ transgenics for resistance to *F. oxysporum* f. sp. *nicotianae*. *FON*-challenged transgenic tobacco lines (PSH, OSH and HSH) showed mild disease symptoms as compared to the NT plants after seven days. The NT plants showed disease symptoms by 2nd dpi. The HSH transgenics showed the least symptoms at the end of seven days, whereas disease symptoms appeared late in OSH and PSH tobacco plants (Fig. 4A). PSH15, OSH3 and HSH1 lines showed maximum resistance to *FON* in their respective group. The resistance of transgenic lines was evaluated by measuring the necrotic lesion diameter around the site of inoculation compared with NT. Although chlorotic halos beyond the necrotic lesions were observed in the leaves of NT plants, only the necrotic regions were taken into consideration. The HSH transgenics showed maximum resistance (*ca.* 50%) among the transgenic lines as evidenced by the milder symptoms shown, compared to symptoms expressed by PSH (*ca.* 25%) and OSH (*ca.* 30%) transgenics (Fig. 4B).

Semi-quantitative RT-PCR analysis. RT-PCR analyses revealed the expression of harpin$_{Pss}$ and defense-response genes (*PR1*, *PR2*, *PR3*, *HSR* and *HIN1*) in putative transgenic plants. The cDNA prepared from the RNA isolated from leaf samples of NT and transgenic tobacco lines challenged with *FON* was used as template to amplify

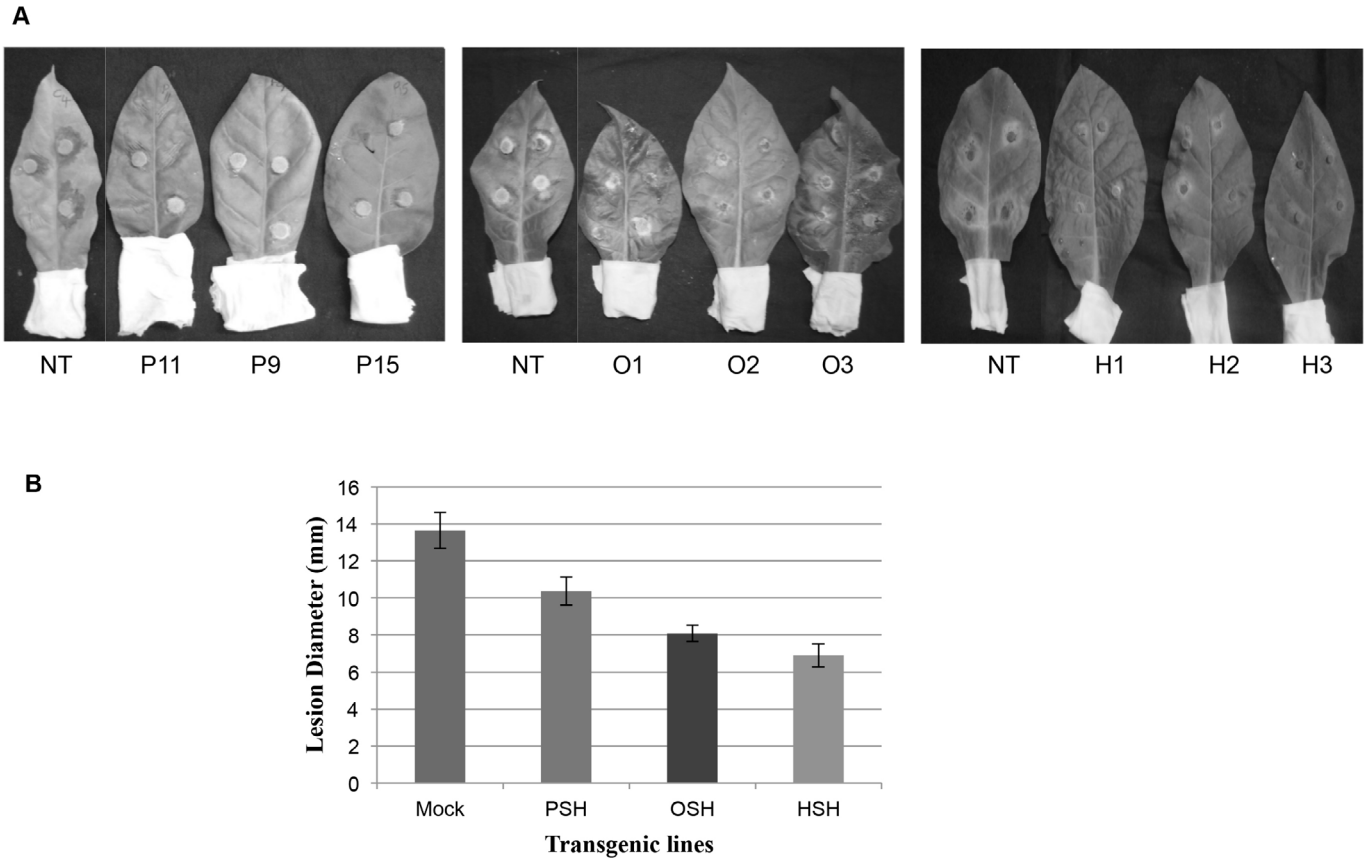

Fig. 4. A. Bioassay of harpin transgenics for resistance against *F. oxysporum* f. sp. *nicotianae*. Three transgenic lines from PSH (P11, P9, and P15); OSH (O1, O2, and O3); HSH (H1, H2, and H3) were used for the bioassay against *F. oxysporum* f. sp. *nicotianae*. Photographs were taken seven days post inoculation. B. Quantitative measurement of disease severity in different transgenic lines against *F. oxysporum* f. sp. *nicotianae*. Detached leaf bioassay was used to assess the resistance observed in different transgenic lines and the lesion diameter was measured 7 days post inoculation. Three leaves from each plant and three plants from each transgenic line were used for the quantitative measurement of lesion diameter. NT plants served as negative control. Lesion diameter is expressed in mm and vertical bars represent the standard deviation of the mean of replicates.

the *SP-hrpZ*, whose expression was detectable in all PSH, OSH and HSH transgenics treated with fungal spores (Fig. 5A). The transcripts of *PR1*, *PR2*, *PR3*, *HSR* and *HIN1* genes were detected in different harpin transgenics upon challenge with *FON* (Fig. 5B). The expression of defense-response genes was relatively high in all transgenics compared to that of NT plants. A low expression of *SP-hrpZ* and defense-response genes was observed in unchallenged leaves of PSH transgenics probably due to leaky expression from PAL promoter.

DISCUSSION

The use of PIPs to drive the expression of HR-inducing genes like *hrpZ* is a promising approach for genetic engineering of plants for disease resistance. Transgene expression only upon pathogen infection avoids undesired accumulation of gene products and abnormal phenotypes (Malnoy *et al.*, 2003, 2006). A pathogen-derived molecule like harpin, used for induction of broad-spectrum disease resistance, has a potential to induce plant disease resistance when expressed in transgenic plants, as it triggers HR in non-host plants. For example, transgenic tobacco plants expressing *hrpZ* from *P. syringae* pv. *syringae* conferred resistance to *Erysiphe cichoracearum* DC (Takakura *et al.*, 2004).

In the present study, we expressed a secretable form of *SP-hrpZ* under 35S promoter and three different PIPs like PAL from tomato, HSR and OSM from tobacco to induce defense responses in transgenic tobacco plants against *FON* (Fig. 1). Tobacco transformants were recovered after *Agrobacterium*-mediated leaf disk transformation. Transformation studies with CSH construct did not allow the selection of transformants possibly due to the constitutive expression and release of harpin$_{Pss}$ into the apoplast causing necrotic cell death of the transformed cells. Constitutive expression of harpin in transgenic plants with or without SP did not result in necrosis of explants or interfere with regeneration process (Pavli *et al.*, 2011; Li and Fan, 1999). This may be due to the low level of harpin accumulation (Li and Fan, 1999) or to the fact that the harpin was not secreted out. It was reported that harpin induces disease resistance but not cell death at a low concentration (Pavli *et al.*, 2011).

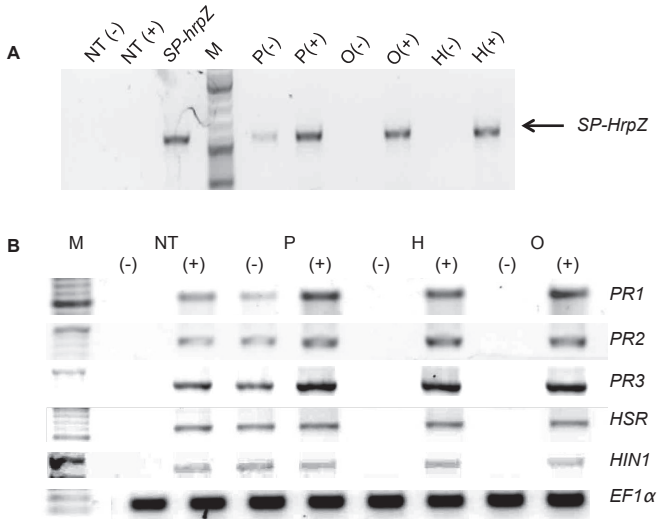

Fig. 5. Pathogen-inducible expression of harpin$_{Pss}$ and defense-response genes in harpin transgenics. A. RT-PCR analysis of expression of pathogen-inducible expression of *SP-hrpZ* in transgenics in the mock-inoculated (–) and *F. oxysporum* f. sp. *nicotianae*-challenged (+) leaves from PSH (P), OSH (O) and HSH (H) transgenics and NT plants. pCAMBIA 1300 having *SP-hrpZ* (lane) was used as positive control. B. RT-PCR analysis of PR genes (*PR1*, *PR2* and *PR3*) and HR-cell death marker genes (*HSR* and *HIN1*) in *F. oxysporum* f. sp. *nicotianae*-challenged (+) and mock-challenged (–) leaves of NT and PSH (P), OSH (O) and HSH (H) transgenics. *EF1α* was used as a loading control.

Inducible promoters have been successfully used in various studies, but drawbacks with respect to inducibility, a relatively high back-ground expression level, or detrimental effects to the host plant are known (Zuo *et al*., 2000). In the present study, the wound-induced expression of harpin$_{Pss}$ resulted in necrosis of the buds during the initial stages of the regeneration process. Though the *hrp*Z gene was placed downstream the PIPs, leaky/wound-induced expression was possible as the pathogen/wound signaling occurs through the same *cis*-acting elements (Rushton *et al*., 2002). To avoid necrosis of the explants due to wounding, the shoots were allowed to grow until rooting without repeated sub-culturing, and the rooted plants were carefully transferred to greenhouse.

We observed that the transformation efficiency of harpin$_{Pss}$ transgenics was lower than the transformation efficiency reported earlier for tobacco (Madhuri *et al*., 2012). A similar low transformation efficiency had been reported with harpin transgenics in citrus (Barbosa-Mendes *et al*., 2009) suggesting the loss of explants due to leaky expression of harpin and harpin-induced necrosis. The harpin$_{Ea}$ (*hrpN*) inhibited shoot regeneration and plantlet development during development of transgenics. A majority of the citrus transgenics showed reduced growth with leaf deformities/curling whereas only a few plants had normal growth (Barbosa-Mendes *et al*., 2009). Similarly, transgenic *Arabidopsis* plants expressing *hrp*N under the Nos promoter exhibited macroscopic damage (Bauer *et al*., 1999) and the damage was greater with *SP-hrpZ* transgenics. Conversely, tobacco plants expressing *hrpG* (Peng *et al*., 2003), pear or tobacco plants expressing *hrpN* (Sohn *et al*., 2007; Malnoy *et al*., 2005) and cotton plants expressing *hpa*$_{Xoo}$ (Miao *et al*., 2010) had a normal appearance and resisted to the diseases. We have also raised male sterile tobacco transgenics using the same *SP-hrpZ* gene under *TA29* promoter without limitations in plant regeneration (Madhuri *et al*., 2012).

Harpin confers broad-spectrum resistance against a wide array of pathogens such as bacterial, fungal (Dong *et al*., 1999) and viral (Strobel *et al*., 1996) pathogens as well as insects. Harpin$_{Ea}$ controlled diseases such as *Fusarium* wilt and *Verticillium* wilt of cotton (Zhang *et al*., 2004) and harpin$_{Xoo}$ conferred resistance to *Ralstonia solanacearum*, *Alternaria alternata* and *Tobacco mosaic virus* (TMV) (Peng *et al*., 2003). The disease resistance response of the harpin$_{Pss}$ transgenics in this study was assessed by detached leaf bioassay against *F. oxysporum* f. sp. *nicotianae*. The PSH, OSH and HSH transgenic lines showed mild disease symptoms compared to NT plants (Fig. 3).

Harpins stimulate the accumulation of SA and ethylene (Zhang *et al*., 2004) and the expression of acidic and basic PR genes in *Arabidopsis* and tobacco (Peng *et al*., 2003; Dong *et al*., 1999). The defense-response genes like *GST1*, *Chia5*, *PR-1a*, *PR-1b etc.* were activated by exogenous application of harpins to plants during the induction of pathogen resistance (Peng *et al*., 2003; Dong *et al*., 1999; Strobel *et al*., 1996). In the present study, *F. oxysporum* f. sp. *nicotianae*-challenged leaves from harpin transgenics showed expression of harpin and defense-response genes like *PR1*, *PR2*, *PR3*, *HSR* and *HIN1* genes that might contribute to the observed resistance (Fig. 5). HSH transgenics displayed superior defense response as compared with OSH and PSH transgenics (Fig. 4). This could be due to the rapid, high level and localized activation of the HSR promoter in response to pathogen infection, limiting the spread of the disease (Pontier *et al*., 1994). Transgenic plants expressing harpin$_{Ea}$ and harpin$_{Xoo}$ either intracellularly or in a secretable form downstream of 35S and other PIPs, conferred resistance to several fungal and bacterial pathogens (Barbosa-Mendes *et al*., 2009; Sohn *et al*., 2007; Malnoy *et al*., 2005; Peng *et al*., 2003; Li and Fan, 1999). Both the constitutive or pathogen-induced expression of harpin reduced the lesions and growth rate of *Phytophthora infestans* in transgenic potato (Li and Fan, 1999).

Here we report that harpin$_{Pss}$ expression under PIPs (PAL, HSR and OSM) resulted in milder symptoms in tobacco challenged with *F. oxysporum* f. sp. *nicotianae*. Constitutive expression of harpin$_{Pss}$ under the 35S promoter resulted in necrosis of the explants whereas the use of PAL promoter caused leaky expression affecting transformation efficiency. Our results support the possible use of PIPs to drive expression of HR-inducing genes like *hrpZ* to raise transgenic plants for fungal disease resistance. Evaluation

of these transgenic lines against other necrotrophs and biotrophs will give more useful information on the use of PIPs to drive expression of HR-inducing genes.

ACKNOWLEDGEMENTS

We thank Prof. P.B. Kirti and Dr. K. Gopinath, University of Hyderabad for providing the binary vector pCAMBIA1300 and *A. tumefaciens* EHA105, respectively. We thank DST-FIST-level II, Department of Plant Sciences, DBT-CREBB and UGC-CAS programmes of School of Life Sciences for the infrastructural facilities. Research fellowships provided by CSIR to DD and DBT-CREBB to BU are gratefully acknowledged.

REFERENCES

Barbosa-Mendes J.M., Mourao F.D.A., Bergamin A., Harakava R., Beer S.V., Mendes B.M.J., 2009. Genetic transformation of *Citrus sinensis* cv. Hamlin with *HrpN* gene from *Erwinia amylovora* and evaluation of the transgenic lines for resistance to citrus canker. *Scientia Horticulturae* **122**: 09-115.

Bauer D.W., Garr E.R., Beer S.V., Norelli J.L., Aldwinckle H.S., 1999. New approaches to the development of transgenic plants resistant to fire blight. *Acta Horticulturae* **489**: 301-306.

Bhargava A., Osusky M., Hancock R.E.W., Forward B.S., Kay W.W., Misra S., 2007. Antiviral indolicidin variant peptides: Evaluation for broad-spectrum disease resistance in transgenic *Nicotiana tabacum*. *Plant Science* **172**: 515-523.

Collinge D.B., Jorgensen H.S.L., Lund S.O., Lyngkjaer M.F., 2010. Engineering pathogen resistance in crop plants: current trends and future prospects. *Annual Review of Phytopathology* **48**: 269-291.

Dong H., Delaney T.P., Bauer D.W., Beer S.V., 1999. Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. *The Plant Journal* **20**: 207-215.

Donghua J., Zejian G., Xujun C., Zhiqiang C., Zhong Z., 2004. Study on tobacco disease resistances mediated by the elicitor gene cryptogein from *Phytophthora cryptogea*. *Chinese Journal of Agricultural Biotechnology* **1**: 125-131.

Greenberg, J.T., 1997. Programmed cell death in plant-pathogen interactions. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 525-545.

Gurr S.J., Rushton P.J., 2005. Engineering plants with increased disease resistance: what are we going to express? *Trends in Biotechnology* **23**: 275-282.

Han J., Lakshman D.K., Galvez L.C., Mitra S., Baenziger P.S., Mitra A., 2012. Transgenic expression of lactoferrin imparts enhanced resistance to head blight of wheat caused by *Fusarium graminearum*. *The Plant Journal* **12**: 33.

He S.Y., Huang H.C., Collmer A., 1993. *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted *via* the Hrp pathway and elicits the hypersensitive response in plants. *Cell* **73**: 1255-1266.

Hemelrijck W.V., Wouters P.F.W., Brouwer M., Windelinckx A., Goderis I.J.W.M., Bolle M.F.C.D., Thomma B.P.H.J., Cammue B.P.A., Delaure S.L., 2006. The *Arabidopsis* defense response mutant esa1 as a model to discover novel resistance traits against *Fusarium* diseases. *Plant Science* **171**: 585–595.

Hoyos M.E., Stanley C.M., He S.Y., Pike S., Pu X-.A., Novacky A., 1996. The interaction of harpin$_{Pss}$ with plant cell walls. *Molecular Plant-Microbe Interactions* **9**: 608-616.

Huo R., Wang Y., Ma L., Qiao J., Shao M., Gao X., 2010. Assessment of inheritance pattern and agronomic performance of transgenic rapeseed having harpin$_{Xoo}$-encoding *hrf2* gene. *Transgenic Research* **19**: 841-847.

Jang Y.S., Sohn S.I., Wang M.H., 2006. The *hrpN* gene of *Erwinia amylovora* stimulates tobacco growth and enhances resistance to *Botrytis cinerea*. *Planta* **223**: 449-456.

Keller H., Pamboukdjian N., Ponchet M., Poupet A., Delon R., Verrier J.L., Roby D., Pierre R., 1999. Pathogen-induced elicitin production in transgenic tobacco generates hypersensitive response and nonspecific disease resistance. *Plant Cell* **11**: 223-235.

Li R., Fan Y., 1999. Reduction of lesion growth rate of late blight plant disease in transgenic potato expressing harpin protein. *Science in China (Series C)* **40**: 96-101.

Madhuri B., Raut S., Dey D., Nazneen A., Uma B., Podile A.R., 2012. Tapetum-specific expression of harpin$_{Pss}$ causes male sterility in transgenic tobacco. *Biologia Plantarum* **56**: 628-634.

Malnoy M., Venisse J.S., Reynoird J.P., Chevreau E., 2003. Activation of three pathogen-inducible promoters of tobacco in transgenic pear (*Pyrus communis* L.) after abiotic and biotic elicitation. *Planta* **216**: 802-814.

Malnoy M., Venisse J.S., Chevreau E., 2005. Expression of a bacterial effector, harpin N, causes increased resistance to fire blight in *Pyrus communis*. *Tree Genetics and Genomics* **1:** 41-49.

Malnoy M., Reynoird J.P., Borejsza-Wysocka E.E., Aldwinckle H.S., 2006. Activation of the pathogen-inducible Gst1 promoter of potato after elicitation by *Venturia inaequalis* and *Erwinia amylovora* in transgenic apple (*Malus domestica*). *Transgenic Research* **15**: 83-93.

Miao W., Wang X., Li M., Song C., Wang Y., Hu D., Wang J., 2010. Genetic transformation of cotton with a harpin-encoding gene *hpaXoo* confers an enhanced defense response against different pathogens through a priming mechanism. *BMC Plant Biology* **10**: 67.

Pavli O.I., Kelaidi G.I., Tampakaki A.P., Skaracis G.N., 2011. The *hrpZ* gene of *Pseudomonas syringae* pv. *phaseolicola* enhances resistance to rhizomania disease in transgenic *Nicotiana benthamiana* and sugar beet. *PLoS ONE* **6**: e17306.

Peng J-.L., Dong H-.S., Dong H-.P., Delaney T.P., Bonasera B.M., Beer S.V., 2003. Harpin-elicited hypersensitive cell death and pathogen resistance requires the *NDR1* and *EDS1* genes. *Physiological and Molecular Plant Pathology* **62**: 317-326.

Pike S.M., Adam A.L., Pu X-.A., Hoyos M.E., Laby R.J., Beer S.V., Novacky A., 1998. Effects of *Erwinia amylovora* harpin on tobacco leaf cell membranes are related to leaf necrosis and electrolyte leakage and distinct from perturbations caused by inoculated *Erwinia amylovora*. *Physiological and Molecular Plant Pathology* **53:** 39-60.

Podile A.R., Lin H-.J., Stainforth V., Sripriya P., Chen L-.F.O., Feng T-.Y., 2001. Conditional expression of harpin$_{Pss}$ causes yeast cell death that shares features of cell death pathway with harpin$_{Pss}$-mediated plant hypersensitive response (HR). *Physiological and Molecular Plant Pathology* **58**: 267-276.

Pontier D., Godiard L., Marco Y., Roby D., 1994. *Hsr*203J, a tobacco gene whose activation is rapid, highly localized and specific for incompatible plant/pathogen interactions. *The Plant Journal* **5**: 507-521.

Raghothama K.G., Liu D., Nelson D.E., Hasegawa P.M., Bressan R.A., 1993. Analysis of an osmotically regulated pathogenesis-related osmotin gene promoter. *Plant Molecular Biology* **23**: 1117-1128.

Rogers S.O., Bendich A.J., 1994. Extraction of total cellular DNA from plants, algae and fungi. In: Gelvin S.B., Schilperoort R.A. (eds). Plant Molecular Biology Manual. pp. 1-8. Kluwer Academic Press, Dordrecht, The Netherlands.

Rushton P.J., Reinstadler A., Lipka V., Lippok B., Somssich I.E., 2002. Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. *Plant Cell* **14**: 749-762.

Sang S., Li X., Gao R., You Z., Lü B., Liu P., Ma Q., Dong H., 2012. Apoplastic and cytoplasmic location of harpin protein Hpa1Xoo plays different roles in H_2O_2 generation and pathogen resistance in Arabidopsis. *Plant Molecular Biology* **79**: 375-391.

Shao M., Wang J.S., Dean R.A., Lin Y.G., Gao X.W., Hu S.J., 2008. Expression of a harpin-encoding gene in rice confers durable nonspecific resistance to *Magnaporthe grisea***.** *Plant Biotechnology Journal* **6:** 73-81.

Sohn S., Kim Y., Kim B., Lee S., Lim C.K., Hur J.H., Lee J., 2007. Transgenic tobacco expressing the *hrpN*$_{EP}$ gene from *Erwinia pyrifoliae* triggers defense responses against *Botrytis cinerea***.** *Molecules and Cells* **24**: 232-239.

Sripriya P., Vedantam L.V., Podile A.R., 2009. Involvement of mitochondria and metacaspase elevation in harpin$_{Pss}$-induced cell death of *Saccharomyces cerevisiae*. *Journal of Cellular Biochemistry* **107**: 1150-1159.

Strobel N.E., Ji C., Gopalan S., Kuc J.A., He S.Y., 1996. Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 HrpZ$_{Pss}$ protein. *The Plant Journal* **9**: 431-439.

Takakura Y., Ishida Y., Inoue Y., Tsutsumi F., Kuwata S., 2004. Induction of a hypersensitive response-like reaction by powdery mildew in transgenic tobacco expressing harpin$_{Pss}$. *Physiological and Molecular Plant Pathology* **64**: 83-89.

Tampakaki A.P., Panopoulos N.J., 2000. Elicitation of hypersensitive cell death by extracellularly targeted *HrpZ*$_{Psph}$ produced in planta. *Molecular Plant-Microbe Interactions* **13**: 1366-1374.

Wei Z-.M., Laby R.J., Zumoff C.H., Bauer D.W., He S.Y., Collmer A., Beer S.V., 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora. Science* **257**: 85-88.

Yevtushenko D.P., Romero R., Forward B.S., Hancock R.E., Kay W.W., Misra S., 2005. Pathogen-induced expression of a cecropin A-melittin antimicrobial peptide gene confers antifungal resistance in transgenic tobacco. *Journal of Experimental Botany* **56**: 1685-1695.

Yevtushenko D.P., Sidorov V.A., Romero R., Kay W.W., Misra S., 2004. Wound-inducible promoter from poplar is responsive to fungal infection in transgenic potato. *Plant Science* **167**: 715-724.

Zhang S., Miao W.G., Nurziya Li. J.H., Jin W., Tan Z.H., 2004. The study of a new biotic product to cotton *Fusarium* wilt and *Verticillium* wilt in field control. *Journal of Shihezi University (Natural Science)* **22**: 71-73.

Zuo J., Niu Q.W., Chua N.H., 2000. An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *The Plant Journal* **24**: 265-273.

Received October 11, 2013
Accepted February 20, 2014