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Article *in* Physiological and Molecular Plant Pathology · February 2004

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A hypersensitive response was induced by virulent bacteria in transgenic tobacco plants overexpressing a plant ferredoxin-like protein (PFLP)

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Accepted 28 May 2004

Abstract

The hypersensitive response (HR) displayed by resistance plants against invading pathogens is a prominent feature of an incompatible plant pathogen interaction. It has been shown that tobacco cell cultures transgenic for a plant ferredoxin-like protein (PFLP) that functions as an electron acceptor of Photosystem I increased harpin-mediate HR. In this work we report increased bacterial disease resistance of *pflp* transgenic tobacco. Compared to the controls, four distinctive characteristics were found in the *pflp*-transgenics after inoculation with virulent bacterial cells *Erwinia carotovora* subsp. *carotovora* and *Pseudomonas syringae* pv. *tabaci*: (i) instead of typical disease symptoms, an HR-like necrosis was observed; (ii) the proliferation of the virulent pathogen was highly retarded; (iii) the expression of *hsr203j,* an HR marker gene, was apparently induced; (iv)H_2O_2 accumulation was induced immediately. Together, those results demonstrate that enhanced production of PFLP in the transgenic plant conditions the induction of a hypersensitive response during compatible pathogen attack.

Keywords: Ferredoxin-like protein; *Erwinia carotovora* subsp. *carotovora*; *Pseudomonas syringae* pv. *tabaci*; *hsr203j*

1. Introduction

Avirulent pathogens elicit a rapid collapse of the challenged host cells in the so-called hypersensitive response (HR) that results in a restricted necrotic lesion from surrounding healthy tissue. Although some host tissue is damaged during the HR process, the localized host cell death contributes to the limitation of pathogen spread [22,24,26]. A battery of inducible defense-related responses often accompanies an HR, including the generation of antibiotics [5], an oxidative burst [37] and enzymes involved in the general phenylpropanoid pathway [9]. Many defense-related signal molecules, such as salicylic acid, ethylene and jasmonic acid was also induced under HR condition [27]. These molecules have emerged as a key signal in the establishment of *disease resistance* and are able to protect the plant against further pathogen infection [8,11].

Harpin is one group of glycine-rich, cysteine-lacking, heat-stable proteins that can elicit HR in the absence of bacteria [41]. Three genera of plant bacterial pathogens, *Erwinia*, *Pseudomonas*, and *Ralstonia* spp. export harpins via the type III protein secretion system [3,12]. Genetic evidence indicates that harpins may play a minor role in bacterial elicitation of the HR, but it may assist the delivery of other pathogenesis proteins across the plant cell wall [12]. HR induced by harpin from *Erwinia amylovora* ($HrpN_{Ea}$) or *Pseudomonas syringae* pv. *syringae* ($HrpZ_{Pss}$) was prevented by inhibition of calcium influx and ATPase activity in tobacco cell suspensions [16]. Harpin has a pronounced effect on the plasmalemma, affecting H^+-ATPase, ion channels or membrane carriers [30,33]. It also causes

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doi:10.1016/j.pmpp.2004.05.005

K^+ efflux and extracellular alkalization in cell suspension cultures but not in protoplasts [19]. A secret able form of harpin from *P. syringae* pv. *phaseolicola* can elicit HR when expressed endogenously in plants [39]. These results indicate that the site of harpin action is the plant cell membrane/cell wall.

Previously, we reported that PFLP (plant ferredoxin like protein) was able to increase the generation of $harpin_{pss}$-mediated AOS and HR in tobacco suspension cells [7]. However, it is not know whether higher AOS generation in transgenic plant would assist in bacterial pathogen defense. In this paper we generated *pflp* transgenic tobacco to study the effect on defense against plant pathogenic bacteria. The *pflp* transgenic tobacco showed higher sensitivity to harpin than the wild type. When inoculated with the virulent pathogens *Erwinia carotovora* subsp. *carotovora* and *Pseudomonas syringae* pv. *tabaci*, the interaction was incompatible and the *pflp* transgenic tobacco showed disease resistance. Moreover, the accumulation of H_2O_2 and the expression level of HR marker gene *hsr203j* were highly induced in *pflp*-transgenic lines. These results imply that an enhanced amount of PFLP in the transgenic plants condition the induction of a hypersensitive response during virulent pathogen attack.

2. Materials and methods

2.1. Construction of the transformation vector

The coding sequence of *pflp* gene was amplified from the sweet pepper clone [7] by PCR with the following primers: B5-SPF: 5′-CGG GAT CCC GAT GGC TAG TGT CTC AGC TAC CA-3′, and S3-PF: CGA GCT CGT TAG CCC XCG AGT TCT GCT TCT-′3. The PCR product was digested with *Bam*H I and *Sac*I. The full-length *pflp* fragment replaced the GUS protein coding sequence from pBI121 vector with CaMV 35S promoter (Clontech, Palo Alto, CA, USA.), and the insert was verified by DNA sequencing. The resultant plasmid, pBISPFLP, was then transformed into *Escherichia coli* DH5α.

2.2. Generation of transgenic tobacco lines

Agrobacterium tumefaciens C58C1 was transformed with pBISPFLP vectors as described by Holsters et al. [17]. Transformation of tobacco (*Nicotiana tabacum* cv. *Xanthi*) was performed by the standard leaf disc transformation method using kanamycin selection (100 μg/ml) [18]. PCR analysis and DNA gel blotting confirmed six independent transformant lines. All transgenic plants were grown in a growth chamber (16 h light/8 h dark at 30 °C). The irradiances of growth chamber are 48 μmol m^{-2} s^{-1}. Two transgenic lines were self-fertilized and the seeds were collected for seeding and PCR analysis.

2.3. Extraction and gel blot analysis of DNA, RNA, and protein

Genomic DNA was extracted from young leaf tissue by the Qiagen genomic kit protocol (Qiagen). Digestion with restriction enzymes, electrophoretic separation on agarose gels, and transfer to nylon membranes (Boehringer Mannheim) were performed by using standard procedures [35]. Membranes were hybridized at 65 °C with the full length NPT II marker probe PCR-label with digoxigenin-11-dUTP (Boehringer Mannheim) according to the manufacturer's protocols. After hybridization, membranes were washed under high stringency conditions (2×SSC, 0.1% SDS) and detected using the DIG luminescent detection kit (Boehringer Mannheim).

Total RNA was isolated from tobacco leaves using the Qiagen Plant RNA Kit (Qiagen) and quantified by spectrophotometry, assuming $A_{260}=40$ μg/ml [35]. Total RNA (15 μg) was electrophoresed through 1% agarose/formaldehyde gels and then transferred onto nylon membranes. Membranes were hybridized at 55 °C overnight with *hsr203j* probes PCR-label with digoxigenin-11-dUTP (Boehringer Mannheim) according to the manufacturer's protocols. After hybridization, membranes were washed under high stringency conditions and detected using the DIG Luminescent detection kit (Boehringer Mannheim).

Proteins were extracted by homogenizing 0.2 g of fresh leaf tissue in 0.5 ml Tris buffer (150 mM NaCl, 50 mM Tris pH 7.5) using a plastic pestle fitted to a 1.5 ml centrifuge tube. The protein concentrations of the samples were determined with Coomassie brilliant blue dye using a microassay method as recommended by the manufacturer (BioRad). Two micrograms of each protein sample were electrophoresed through gels containing 12.5% polyacrylamide plus SDS (SDS-PAGE). These gels were then either stained with Coomassie blue or electro-transferred onto nitrocellulose membranes using the BioRad blue tank method. PFLP proteins were detected on Western blots using anti-PFLP antibodies followed with mouse anti-rabbit IgG-peroxidase conjugate.

2.4. $Harpin_{Pss}$ preparation and plant hypersensitive response assay

The $harpin_{Pss}$ clone was provided by Dr H.-C. Huang at the Agricultural Biotechnology Laboratories, National Chung-Hsien University, Taiwan. $Harpin_{Pss}$ protein was extracted according to He et al. [16]. *Escherichia coli* DH5α (pSY10) which harbors the $harpin_{Pss}$ gene (hrpZ) was grown in Luria Broth containing ampicillin (50 μg/ml) at 37 °C in the dark with shaking overnight in the presence of isopropylthio-β-D-galactoside. To obtain $harpin_{Pss}$, the bacteria were first washed and sonicated for 30 s in 10 mM phosphate buffer pH 6.5 and boiled for 10 min. After boiling, the extracts were centrifuged at 10,000×*g* for 10 min. Supernatants were desalted by a Microconcentrator (Amicon) and stored at 4 °C.

The HR assay was performed according to Huang et al. [20]. Fully expanded tobacco leaves were wounded to form tiny pricks on the lower surface of the leaves by a 25-gauge needle. $Harpin_{Pss}$ was prepared in 50 mM Tris buffer (pH 7.5) and infiltrated by pressing a 1 ml syringe without a needle to the prick. The infiltrated plant was incubated in a growth chamber (16 h light/8 h dark at 25 °C).

2.5. Transgenic tobacco resistance assays

All transgenic tobacco resistance assays were carried out with T2 progenies of the transgenic plants in independent experiments. Fully expanded upper leaves of plants, approximately 80-days old, were used for infection with pathogen. Inoculations with *Pseudomonas syringae* pv. *tabaci* and *Erwinia carotovora* subsp. *carotovora*were performed. Each inoculation was repeated four times in individual plants of each transgenic line. Bacteria inside the leaf discs were released by grinding the infiltration area in sterile water in a microfuge tube and then plated on Nutrient Broth agar plates. The plates were cultured at 30 °C overnight, and the colonies were counted the following day.

2.6. Determination of H_2O_2

H_2O_2 was determind according to method of Jana and Choudhuri [21]. Tobacco leaves (100 mg) were homogenized with 0.6 ml of 50 mM phosphate buffer (pH 6.5) with 10 mM 3-amino-1, 2, 4-triazole. The homogenates were centrifuged at 6000*g* for 25 min. Titanium sulphate (0.2 ml of 0.1%) in 20% (v/v) H_2SO_4 was added to the supernatant and centrifuged at 6000*g* for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm to determine the level of H_2O_2.

3. Results

3.1. Screening and expression analysis of pflp transgenic tobacco plants

Tobacco plants expressing heterologous *pflp* gene isolated from sweet pepper were generated by *Agrobacterium*-mediated transformation. pBI based plasmid construct containing sweet pepper *pflp* cDNA was prepared for tobacco transformation. (Fig. 1). Viable transformation

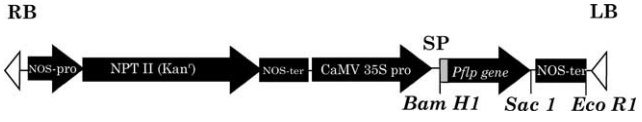

Fig. 1. Map of the relevant portions of the transformation plasmids. *pBR-spflp*, *NOS*-pro = the promoter region of *A. tumefaciens* nopaline synthase, *NOS*-ter = the terminator region of the same gene, *NPT II* = the coding sequence of neomycin phosphotransferase II gene, CaMV 35S-pro = the CaMV 35S promoter sequence, pflp = the coding sequence for the sweet pepper PFLP and SP = signal peptide of sweet pepper PFLP.

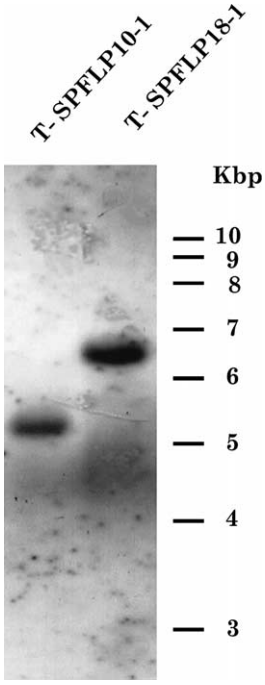

Fig. 2. Gel blots analysis of genomic DNA, DNA gel blot analysis of T-SPFLP transformed tobacco using *NPT II* cDNA as probe. Genomic DNA (15 μg) was digested with *Eco*R I for T-SPFLP10-1 and T-SPFLP18-1 as indicated and subjected to Southern blot analysis.

lines were selected and designated as T-SPFLP. In order to identify the distribution of *pflp* transgene in transgenic tobacco genomes, genomic DNA was extracted from the transgenic lines and subjected to Southern analysis. Given that wild type tobacco contains the ferredoxin gene [7], neomycin phosphotransgeraseII (NPT II) cDNA was used as a probe to identify the transgenic loci. Genomic DNA from T-SPFLP10-1 and T-SPFLP18-1 were digested with *Eco*RI restriction enzyme. Fig. 2 showed that, after probing with the NPTII cDNA probe, DNA of the transgenic tobacco lines T-SPFLP10-1 and T-SPFLP18-1 exhibit one band, respectively. The patterns of Southern blotting suggest that T-SPFLP10-1 and T-SPFLP18-1 result from independent transformation events and incorporate the *pflp* transgene at different chromosomal locations. Western blot analysis showed that PFLP protein levels in T-SPFLP transformants was two to three fold increased compared to the controls (Table 1).

Table 1
Quantitative PFLP expression levels in transgenic tobacco

Transgenic lines	Protein (fold)[a]
T-SPFLP10-1	3.13 ± 0.21
T-SPFLP18-1	2.80 ± 0.58
Wild type	1.00 ± 0.21

[a] Protein levels were determined with western blot. The PFLP protein level of wild type tobacco was defined as 1.

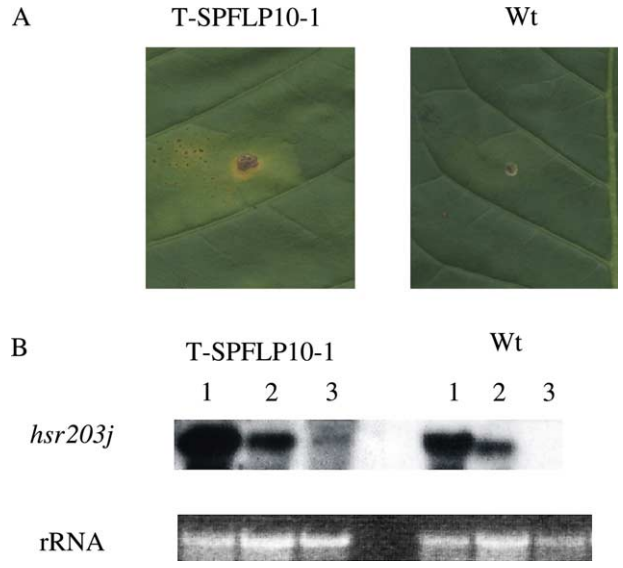

Fig. 3. The harpin$_{Pss}$-mediated hypersensitive response (HR) in transgenic and wild type tobacco. (A) Low concentration of harpin$_{Pss}$ induced slight HR in transgenic tobacco. Harpin$_{Pss}$ (0.1 μg) was infiltrated into transgenic (T-SPFLP10-1) and wild type (Wt) tobacco leaves. Photograph was taken 2 days post infiltration. (B) Induction of *hsr203j* gene expression by harpin$_{Pss}$. The transgenic (T-SPFLP10-1) and wild type (Wt) tobacco were infiltrated with 10 μg (lane 1), 1 μg (lane 2) and 0.1 μg (lane 3) of harpin$_{Pss}$. Total RNA in the infiltration areas of tobacco leaves were extracted after 12 h. RNA blots (15 μg per lane) were probed with the *hsr203j* cDNA probe. Ethidium bromide staining of rRNA was used to verify the loaded amount of total RNA.

3.2. pflp transgenic plants showed a high sensitivity to harpin$_{Pss}$

In order to investigate the relationship between the PFLP and harpin$_{Pss}$-mediated HR in vivo, different dosages of harpin$_{Pss}$ were infiltrated into the intercellular spaces of transgenic and wild type tobacco leaves and HR necroses were subsequently examined. After infiltration with 10 and 1 μg harpin$_{Pss}$ over 24 h, in the transgenic line leaves showed HR necroses almost to the full extent of the infiltration area, but wild type tobacco leaves exhibited much less HR necrosis. Even infiltration using lower concentrations of harpin$_{Pss}$ (0.1 μg) over 24 h in the transgenic line leaves showed a slight HR necrosis but not in wild type leaves (Fig. 3A).

The HR molecular marker gene *hsr203j* [31,32] that accumulates specifically in tissues undergoing HR was examined. After infiltration with 10 and 1 μg of harpin$_{Pss}$ over 12 h, the *hsr203j* messenger accumulation was three fold increase in transgenic lines when compared to the wild type. When infiltrated with 0.1 μg harpin$_{Pss}$, the activation of *hsr203j* could be detected in transgenic tobacco but not in wild type (Fig. 3B). These results implied that the *pflp* transgenic tobacco have higher sensitivity to the HR elicitor.

3.3. pflp transgenic tobacco exhibited resistance to virulent bacterial pathogens

To determine the effects of the *pflp* gene on plant disease resistance, two individual transgenic lines were challenged

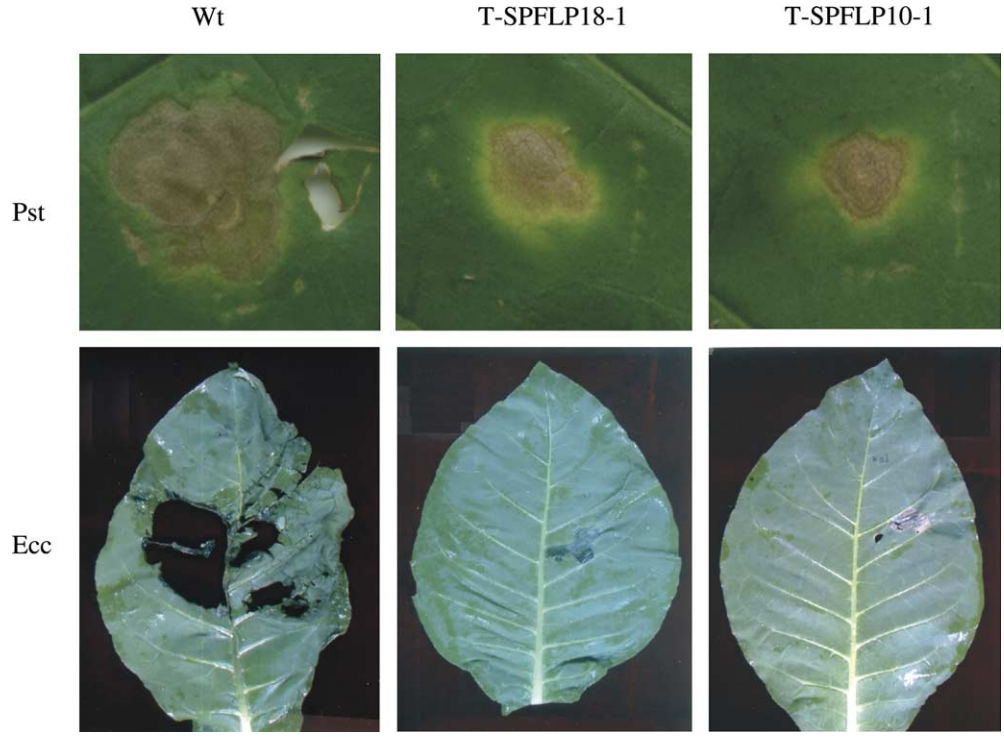

Fig. 4. Resistance of *pflp* transgenic tobacco to virulent pathogens. Fully expanded upper leaves of transgenic (T-SPFLP18-1, TSPFLP10-1) and control tobacco (Wt) were infiltrated with 100 μl of bacterial suspension *P. syringae* pv. *tabaci* (1.0×10^8 cfu/ml)(upper panel) or *E. carotovora* subsp. *carotovora* (1.0×10^8 cfu/ml) (lower panel). Photographs of upper panel were taken 5 days post inoculation and lower panel were taken 2 days post inoculation.

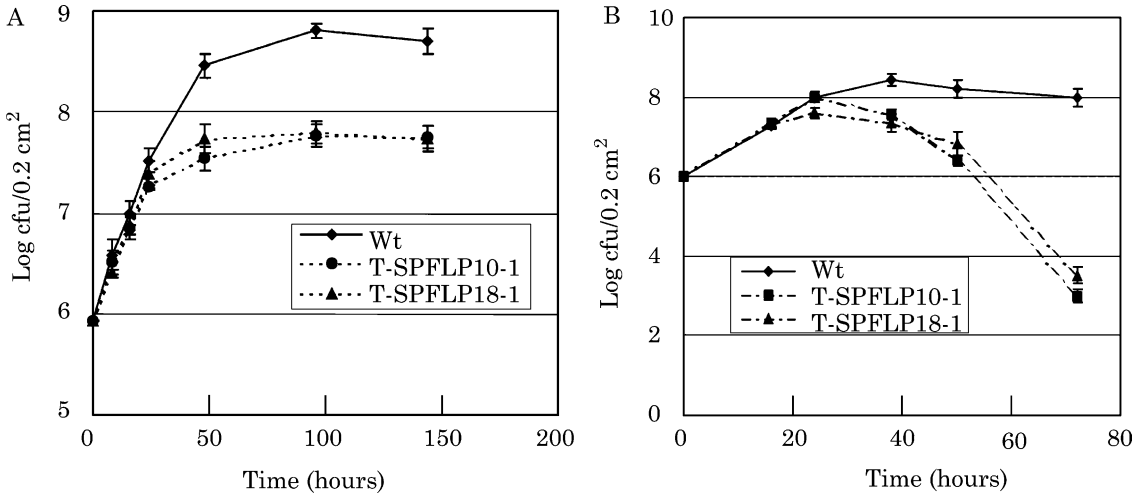

Fig. 5. The multiplication of bacterial pathogens were inhibited in *pflp* transgenic tobacco. Fully expanded upper leaves of two transgenic lines T-SPFLP10-1, T-SPFLP18-1 and control tobacco (wt) were infiltrated with 100 µl of bacterial suspension of *P. syringae* pv. *tabaci* (1.0×10^8 cfu/ml) (A)or *E. carotovora*subsp. *carotovora* (1.0×10^8 cfu/ml). (B) Bacterial populations were detected on successive times post inoculation. Data presented are mean and standard deviation of four individual plants.

with the virulent bacterial plant pathogens *P. syringae* pv. *tabaci* or *E. carotovora*subsp. *carotovora*. The wild fire symptom occurred in wild type 3-days post *P. syringae* pv. *tabaci*(1×10^8 cfu/ml) inoculation and the symptoms expanded continuously. However, the leaves of transgenic lines exhibited HR-like symptoms after 1-day post inoculation and this necrosis was dehydrated and limited in the infection site, even on 7-days post inoculation (Fig. 4A–C). Transgenic plants were also evaluated for resistance to bacterial soft rot disease caused by *E. carotovora* subsp. *carotovora*. *E. carotovora* subsp. *carotovora* (1×10^8cfu/ml) cause tissue maceration in wild type tobacco leaves (Fig. 4D). As shown in Fig. 4E and F, *pflp* transgenic tobacco leaves exhibited HR-like necrosis and the necrosis was limited even after 2-days post inoculation. Bacterial populations in the infiltrated areas were calculated post inoculation at different times. A *t*test demonstrated that differences in pathogen number between transgenic and wild type tobacco 2-days post infections were significant ($P<0.05$). Postinoculation (2-days), the population of *P. syringae* pv. *tabaci* and *E. carotovora* subsp. *carotovora* in the wild-type tobacco was approximately 10 times higher than that found in the transgenic lines (Fig. 5). These results indicated that overexpression of *pflp* in transgenic tobacco could enhances resistance against both kinds of virulent pathogens, *E. carotovora* subsp. *carotovora* and *P. syringae* pv. *tabaci*.

3.4. hsr203j gene expression was induced in transgenic tobacco by virulent pathogen infections

pflp transgenic tobacco plants showed HR-like necrosis when inoculated with compatible pathogens. To make sure those necroses was due to HR, an HR molecular marker gene *hsrs203j* was monitored after virulent pathogen inoculated in transgenic tobacco. The leaves of *pflp* transgenic (SPFLP18-1) and wild type tobacco were infiltrated with 100 µl of *E. carotovora*subsp. *carotovora* and *P. syringae* pv. *tabaci*, respectively (1.0×10^7 cfu/ml). Total RNA in the infiltration areas of tobacco leaves were extracted and probed with the *hsr203j*. The HR marker gene *hsr203j* was highly induced at 6 h post *E. carotovora* subsp. *carotovora* inoculation and this induction was continued over 24 h in the *pflp* transgenic tobacco (Fig. 6A). Northern blot analysis was also performed when tobacco leaves were challenged with compatible

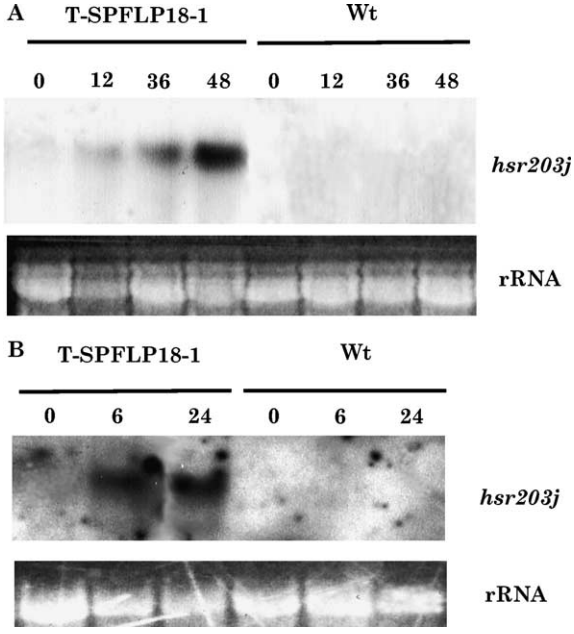

Fig. 6. Induction of *hsr203j* gene expression in transgenic tobacco by virulent pathogen infected. The transgenic (SPFLP18-1) and wild type (wt) tobacco were infiltrated with 100 µl of *E. carotovora* subsp. *carotovora*(A) and *P. syringae* pv. t*abaci* (B) individual (1.0×10^7 cfu/ml). Total RNA in the infiltration areas of tobacco leaves was extracted at the time points indicated. RNA blots (15 µg per lane) were probed with the *hsr203j* cDNA probe. Ethidium bromide staining of rRNA was use to verify the loaded amount of total RNA.

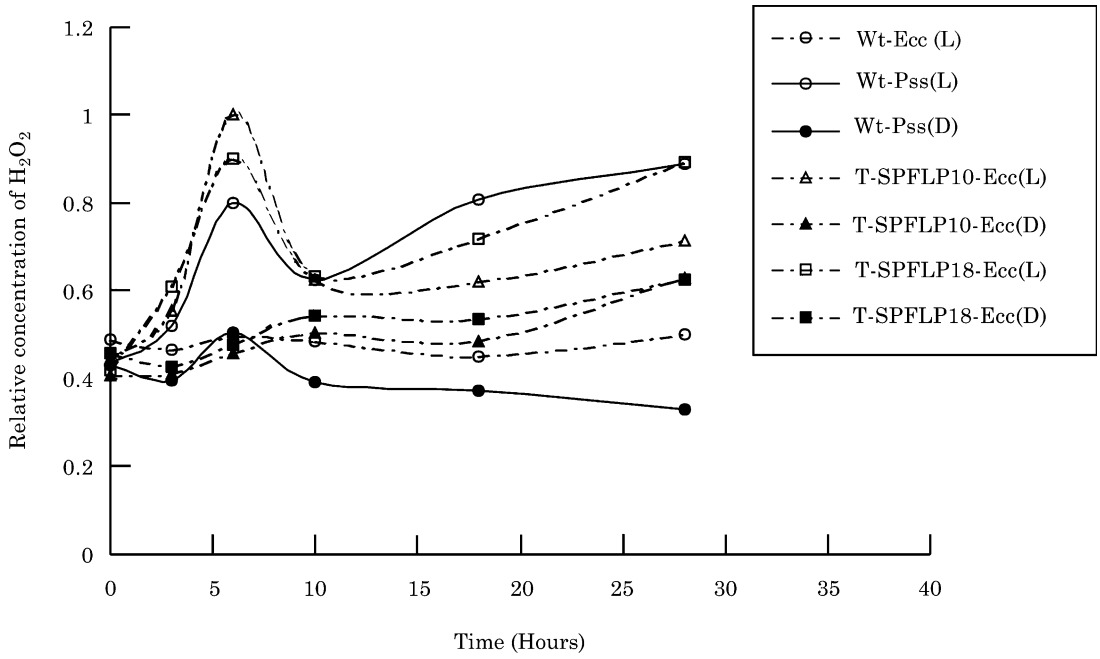

Fig. 7. H_2O_2 was induced in transgenic tobacco by virulent pathogen infections. The wild type (Wt) and transgenic tobacco plants (T-SPFLP10, T-SPFLP18) were infiltrated with 100 μl of *E. carotovora* subsp. *carotovora* (Ecc) and *Pseudomonas syringae* pv. *syringae* (Pss) (1.0×10^6 cfu/ml) under light(L) and dark(D) conditions individual. H_2O_2 in the infiltration areas of tobacco leaves was extracted at the time points indicated. Data were expressed as mean of relative values from four individual plants.

pathogen *P. syringae* pv. *tabaci* (Fig. 6B). The *hsr203j* was induced at 12 h post inoculation and this induction was continued over 48 h. It indicates that a virulent bacterial pathogen indeed induce HR maker gene in the *pflp* transgenic plants.

3.5. H_2O_2 was induced in transgenic tobacco by virulent pathogen infections

The rapid production of peroxide (H_2O_2) by plant is one of the most striking events during the early phase of the HR. To evaluate the production of H_2O_2 in *pflp* transgic tobacco, two different *pflp* transgenic tobacco lines (T-SPFLP18, T-SPFLP10) were infiltrated with 100 μl of *E. carotovora* subsp. *carotovora* (1.0×10^6 cfu/ml) and the yield of H_2O_2 was measured as describe by Jana and Choudhuri [21]. The concentration of H_2O_2 was highly increased at 6 h post *E. carotovora* subsp. *carotovora* inoculation and this induction was continued over 10 h in the *pflp* transgenic under light condition. This accumulation of H_2O_2 was similar to avirulent pathogen *Pseudomonas syringae* pv. *syringae* inoculated in the wild type tobacco (Fig. 7). The concentration of H_2O_2 was estimated to be 80–120 mM at the peak. However, no H_2O_2 accumulation was observed when inoculation with the avirulenct pathogen was followed by incubation in the dark.

4. Discussion

AOS accumulation has been shown to be required for plant pathogen defense [40]. Nevertheless, not all cases of AOS accumulation increase in transgenic plant improve plant disease resistance. For example, AS1 transgenic tobacco that increases AOS accumulation was more sensitive to HR elicitor. However, the lesion cause by the virulent pathogen *P. syringae* pv. *tabaci* was more serious in AS1 transgenic tobacco than in the wild type [28]. Previously, we reported that PFLP was able to increase the generation of $harpin_{pss}$-mediated AOS and HR in tobacco suspension cells [7]. To understand the effect of overexpression *pflp* in transgenic tobacco on disease resistance, *pflp* transgenic tobacco was generated and challenged with virulent bacterial pathogens.

The growth of *P. syringae* pv. *tabaci* in the *pflp* transgenic tobacco was significantly inhibited when compared with wild type tobacco (Fig. 5). The visible necrosis induced by *P. syringae* pv. *tabaci* was restrained significantly in the *pflp* transgenic tobacco (Fig. 4). To further confirm that this necrosis was due to HR, the HR marker gene *hsr203j* expression pattern was monitored. When inoculated with *P. syringae* pv. *tabaci* the *hsr203j* was activated in *pflp* transgenic tobacco but not in wild type (Fig. 6B). Additionally, Southern analysis showed that the *pflp* transgene incorporated at different loci in two independent transgenic lines (Fig. 2). Thus, the disease resistance to the virulent pathogens was due to *pflp* transgene and not as a result from rearrangement of the transgene at the specific locus of integration. These results indicated that *pflp* transgenic tobacco has acquired disease resistance and this disease resistance was obtained by the induction of HR.

Earlier we reported that rice and the orchid *Oncidium* which overexpressed an PFLP transgene from sweet pepper

showed increased disease resistance. [25,38]. In this study, to confirm that this disease resistance was generally specific, another bacterial pathogen, *E. carotovora* subsp. *carotovora* was challenged in *pflp* transgenic tobacco. *E. carotovora* subsp. *carotovora* is a wide host range bacterial plant pathogen that rapidly rots affected tissue. Although it has an inner typeIII secretion system region coding for the elicitor harpin, it never induces HR because the amount of secreted harpin is too low [4,23,29,34]. When *E. carotovora* subsp. *carotovora* inoculated in the *pflp* transgenic tobacco, the HR marker gene *hsr203j* were also activated (Fig. 6A). This result implies that not only *P. syringae* pv. *tabaci* but also *E. carotovora* subsp. *carotovora* could induce HR resistance in *pflp* transgenic tobacco. We also demonstrated that *pflp* transgenic tobacco was more sensitive to low amount of harpin (Fig. 3). We surmised that *pflp* transgenic tobacco could be induced HR more easily by low amounts of harpin and thus show an HR against virulent bacterial pathogens.

Ferredoxin-I plays as a role of photosynthetic electron transport by supplying electrons for the reduction of $NADP^+$ to NADPH and under ertain circumstances can be involved in the production of AOS [1]. PFLP is able to increase the generation of AOS in tobacco suspension cells [7]. In this paper we measure the H_2O_2 generated in *pflp* transgenic tobacco after virulent pathogen infection. When inoculated with the virulent pathogens *Erwinia carotovora* subsp. *carotovora*, the accumulation of H_2O_2 was highly induced in *pflp*-transgenic lines. This result was similar to an avirulent pathogen *Pseudomonas syringae* pv. *syringae* inoculated in the wild type tobacco (Fig. 7). H_2O_2 generation in the HR is usually considered to be highly associated with the light condition [42], In this study, the H_2O_2 accumulation of *pflp* transgenic tobacco plants induced by the virulent pathogen never occurred under dark conditions. These results imply that the HR induced by the virulent pathogen in *pflp* transgenic tobacco is light dependent.

Exploitation of plant endogenous defense mechanism is a useful strategy to create disease resistance traits [6,9]. A number of reports have indicated that expression of elicitors or *avr* gene products in transgenic plants can trigger an HR that generates broad-spectrum disease resistance to virulent pathogens [2,10,13,15,36]. The potential application of this strategy is limited, because HR is a programmed cell death process in which the plant depletes many energy resources to synthesize defense-related compounds. Our strategy has the advantage that overexpression of *pflp* in transgenic plants does not trigger any macroscopic or microscopic spontaneous HR directly before pathogen infection. However, overexpression of *pflp* in transgenic plant to protect from bacterial infection was limited by the internal regulatory elements of PFLP in the young seedling [14]. In young seedlings, ferredoxin could not accumulate very well in the transgenic tobacco even under control of 35S promoter (data not shown).

In summary, we have demonstrated the utility of *pflp* in transgenic tobacco to increase disease resistance. HR was induced in *pflp* transgenic tobacco against two different genera of virulent pathogens. Using this approach, transgenic plants of more broad disease resistance could be generated for the future.

Acknowledgements

We would like to thank Dr Pontier from Laboratoire de Biologie Moleculaire des Relations Plantes/Microorganismes, France for providing the *hsr203j*clone. This work was supported by grants to T.-Y. Feng from Academia Sinica, Taiwan, Republic of China.

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