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Ferredoxin from sweet pepper (*Capsicum annuum* L.) intensifying harpin$_{pss}$-mediated hypersensitive response shows an enhanced production of active oxygen species (AOS)

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Abstract

The hypersensitive response (HR) is a form of cell death associated with plant resistance to pathogen infection. Harpin$_{pss}$, an elicitor from the bacterium *Pseudomonas syringae* pv. *syringae*, induces a HR in non-host plants. Previously, we reported an amphipathic protein from sweet pepper interfering with harpin$_{pss}$-mediated HR. In this report, we isolated and characterized a cDNA clone encoded that amphipathic protein from sweet pepper. This protein is designated as PFLP (plant ferredoxin-like protein) by virtue of its high homology with plant ferredoxin protein containing an N-terminal signal peptide responsible for chloroplast targeting and a putative 2Fe-2S domain responsible for redox activity. Recombinant PFLP obtained from *Escherichia coli* was able to significantly increase active oxygen species (AOS) generation when mixed with harpin$_{pss}$ in tobacco suspension cells. It also showed enhanced HR when co-infiltrated with harpin$_{pss}$ in tobacco leaves. We used a transgenic tobacco suspension cells system that constitutively expresses the *Pflp* gene driven by the CaMV 35S promoter to study the function of PFLP in enhancing harpin$_{pss}$-mediated hypersensitive cell death *in vivo*. In response to harpin$_{pss}$, suspension cells derived from *Pflp* transgenic tobacco showed a significant increase both in the generation of AOS and in cell death as compared to the wild type. AOS inhibitors diphenylene iodonium chloride (DPI) and lanthanum chlorate ($LaCl_3$) were used to study the involvement of AOS in harpin$_{pss}$-induced cell death. Our results demonstrate enhanced generation of AOS is necessary to cause enhanced hypersensitive cell death in *Pflp* transgenic tobacco cells and it is plasma membrane-bound NADPH-oxidase-dependent. Sub-cellular localization studies showed that PFLP is present in the cytoplasm and chloroplast of *Pflp* transgenic tobacco cells, but only in the chloroplast, not in the cytoplasm, of wild-type tobacco cells. It is possible that PFLP can change the redox state of the cell upon harpin$_{pss}$ inoculation to increase AOS generation and hypersensitive cell death. Overall, this study will provide a new insight in the functional properties of ferredoxin in hypersensitive cell death.

Abbreviations: AOS, active oxygen species; DPI, diphenylene iodonium chloride; FPLC, fast-performance liquid chromatography; HR, hypersensitive response; $LaCl_3$, lanthanum chlorate; PCR, polymerase chain reaction; PK, polynucleotide kinase reaction; PFLP, plant ferredoxin-like protein; RT-PCR, reverse transcriptase polymerase chain reaction.

Introduction

Plants employ a wide array of defense mechanisms against pathogen attack. Among those, hypersensitive response (HR) is an induced resistance mechanism, characterized by rapid, localized cell death upon their encounter with a microbial pathogen (Dangl *et al.*, 1996; Alvarez *et al.*, 1998). The HR cell death forms a physical barrier to prevent further pathogen infection (Mehdy, 1994). In addition, a local HR is often associated with activation of plant defense responses in the surrounding and even distal uninfected parts of the plants leading to the development of systemic acquired resistance (SAR) (Xie and Chen, 2000).

The cDNA nucleotide sequence data reported will appear in the EMBL and GenBank Nucleotide Sequence Databases under the accession number AF039662.

Various kinds of defense-related responses are often accompanied by HR, including induction of MAP kinase activity (Desikan *et al.*, 1999), systemic acquired resistance-mediated increase of salicylic acid and *NIM1* gene activity (Dong *et al.*, 1999), activation of phenylalanine ammonia-lyase and glutathione *S*-transferase (Desikan *et al.*, 1998) and ion influxes across the membranes, alkalinization of the growth medium, cell membrane depolarization and production of active oxygen species (AOS) (Wei *et al.*, 1992; Baker *et al.*, 1993; He *et al.*, 1994; Popham *et al.*, 1995). Several lines of evidence suggest that the plant cell death during HR results from the activation of a programmed cell death (PCD) pathway that shares a number of mechanistic features with animal apoptosis (Xie and Chen, 2000). Signaling events such as protein phosphorylation and generation of AOS involved in animal apoptosis have also been found in plant associated HR (Levine *et al.*, 1994; Jabs *et al.*, 1996; Alvarez *et al.*, 1998).

Oxidative burst, the rapid release of active oxygen species (AOS), is considered one of the earliest events during plant HR (Lamb and Dixon, 1997). AOS have direct antimicrobial activities and can therefore reduce pathogen viability. They have also been implicated in the destruction of the challenged plant cells, either through lipid peroxidation or function as a key factor mediating PCD (Greenberg, 1997; Bethke and Jones, 2001). H_2O_2, the most stable AOS, functions as a local trigger for the programmed death of challenged cells and as a diffusible signal to induce protectant genes in surrounding cells (Levine *et al.*, 1994). Furthermore, H_2O_2 plays a role in the phenomenon of cross-tolerance, in which exposure to one stress can induce tolerance to other stresses (Bowler and Fluhr, 2000). Several possible sources of AOS in plants and a number of biotic and abiotic stresses can stimulate oxidative burst. Potential enzymatic sources include plasma membrane-located NADPH oxidase (Desikan *et al.*, 1998) and cell wall-bound peroxidases (Bolwell and Wojtaszek, 1997). Besides these, electron transport processes in chloroplasts, mitochondria and peroxisomes are considered as other sources of AOS (Noctor and Foyer, 1998; Dat *et al.*, 2000). Several reports suggested that AOS could be a common factor regulating various cell signaling pathways (Lamb and Dixon, 1997; Neill *et al.*, 1999; Dat *et al.*, 2000).

Harpins, bacterial proteinaceous elicitors from *Pseudomonas syringae* pv. *syringae* ($harpin_{pss}$) (He *et al.*, 1993) and *Erwinia amylovora* ($harpin_{Ea}$) (Wei *et al.*, 1992), induce HR in non-host plants, which is governed by the *hrp* (HR and pathogenicity) gene cluster (Bonas, 1994). This *hrp* gene-governed pathway is used to deliver the proteins from the bacterial cytoplasm either to the culture medium or directly into the host cell (Galan and Collmer, 1999). Some plant pathogenic bacterial proteins, such as *Avr*-encoded proteins, harpins and PopA1 delivered by the type III secretion system, are able to trigger HR alone. However, harpins and PopA1 elicit HR only when infiltrated at relatively high concentrations into plant intercellular spaces (Arlat *et al.*, 1994; Galan and Collmer, 1999). The role of harpin in the interaction between plant and pathogen remains obscured. Reports suggested that harpin is either necessary or unnecessary for bacterial pathogenicity (Bauer *et al.*, 1995; Charkowski *et al.*, 1998). Reports have shown that $harpin_{pss}$-elicited plant cells induce the rapid generation of AOS (Desikan *et al.*, 1996) and mitogen-activated protein kinase activity during defense responses (Desikan *et al.*, 1999). Recent evidence suggests that harpins may interact with membranes directly and form an ionophore *in vitro* which may facilitate the delivery of virulent factors into host plant cells in the type III secretion system (Lee *et al.*, 2001). Harpin expressed endogenously in tobacco causes HR only when it is produced in a secretable form (Tampakaki and Panopoulos, 2001). These reports suggested that the site of action of harpins may be the cell wall, but no plant cell surface proteins have been reported which react with harpin and transmit signals to plant cells.

Previously, we had reported a plant amphipathic protein (formerly reported as AP1) from sweet pepper which influences the HR mediated by *Pseudomonas syringae* pv. *syringae* and harpin $harpin_{pss}$ (Lin *et al.*, 1997). Here, we report a cDNA clone of AP1 from sweet pepper. We designate the cDNA clone *Pflp* (plant ferredoxin-like protein) because of its high homology with plant ferredoxins, this cDNA clone encodes a protein identified as ferredoxin with N-terminal signal peptide targeting to chloroplast. An *in vitro* bioassay showed that recombinant PFLP enhances AOS generation in tobacco suspension cells and increases hypersensitive cell death on tobacco leaves when co-infiltrated with $harpin_{pss}$. We used a transgenic tobacco suspension cell system expressing the *Pflp* gene driven by the (CaMV) 35S promoter to study the function of PFLP in enhancing $harpin_{pss}$-mediated hypersensitive cell death *in vivo*. Our results indicated that the enhanced hypersensitive cell death induced by $harpin_{pss}$ in *Pflp* transgenic tobacco is

dependent on AOS and Ca^{+}-mediated signaling pathways. Overall, these results suggest that PFLP can increase $harpin_{pss}$-induced hypersensitive cell death through increasing AOS production.

Materials and methods

Plant materials

Sweet pepper (*Capsicum annuum* L. cv. ECW) was obtained from Dr C-F. Wang, Asian Vegetable Research Development Center, Tainan, Taiwan. Tobacco (*Nicotiana tabacum* cv. WC38) was obtained from laboratory collection. All plants were used in this study grown in a greenhouse with a photoperiod of 16 h at 23–25 °C.

Reverse transcriptase PCR and cDNA cloning

Total RNA was isolated from sweet pepper leaves according to Nelson (1994). First-strand cDNA synthesis was carried out with an oligo(dT) primer and Superscript Reverse Transcriptase (Life Technologies) performed according to the manufacturer's instructions. First-strand cDNA was subjected to PCR by using a degenerate primer (5′-GCIACITAC/TAAA/GGTIAAA/G-3′) and an anchor primer of oligo 5′ (dT) with 1 mM dNTP and 2.5 units of *Taq* polymerase (Life Technologies). The second nested PCR was performed by adding 1 μl of purified PCR product (with the Qiagen PCR purification Kit), 0.4 μm of a nested degenerate primer (5′-ACICCIGAC/TGGICC-3′), 0.4 μm of 3′ oligo $(dT)_{18}N$ anchor primer and other PCR components. RT-PCR products were eluted from agarose gel by the Qiaquick Gel Extraction Kit (Qiagen). The purified cDNA fragments were treated by polynucleotide kinase (PK) reaction and then cloned into pT7Blue Blunt-end Vector (Novagen) and followed by DNA sequence analysis.

PCR amplification of the 5′ region of the cDNA clone

mRNA was isolated from total RNA by oligo(dT) affinity magnetic particles with the Straight A mRNA isolation System (Novagen). PCR amplification of the 5′ cap region was performed in a 30 μl reaction mixture containing 2 μl single-stranded cDNA, the 5′ PCR universal caps primer (Clontech) and a 3′-specific primer (5′-CATCTTGGTCAAAGTTTGAATC-3′) corresponding to the 3′ non-coding region of the *pflp* cDNA clone. Other components were added according to the manufacturer's protocol. Amplification was carried out with 35 cycles: 45 s denaturation at 94 °C; 1 min annealing at 53 °C; 1 min extension at 72 °C; and 5 min final extension at 72 °C. After PCR amplification the purified Klenow cDNA fragments were treated with PK reaction and ligated into pT7 Blue Blunt-end Vector (Novagen).

DNA sequencing and analysis

Sequences of cDNA fragments were determined by the dideoxynucleotide chain termination method by using a Sequenase Kit (PE ABI) and performed in an ABI Prism 377 DNA Sequence Analyzer (ABI Applied Bio systems, Foster city, CA). Database searches and sequence comparisons were done with the BLAST search program (Altschul *et al.*, 1997).

Expression of recombinant Pflp protein

A mature peptide-coding region of *Pflp* cDNA clone was subcloned into *Bam*HI and *Hin*dIII sites of the pQE-30 vector system (Qiagen) and transformed to the host strain *Escherichia coli* M15. *E. coli* transformants harboring the plasmid were grown in LB broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. When the culture reached an absorbance of 0.6 at A_{600}, isopropylthio-β-D-galactoside (IPTG) induction was realized to a final concentration of 1 mM, followed by 4 h incubation at 37 °C. Cells were harvested and N-terminal 6× His-tagged proteins were purified by the Ni-NTA resin spin kit (Qiagen) according to the manufacturer's instructions. Finally, PFLP was eluted from the column with elution buffer containing 250 mM imidazole. A P6 gel spin column (BioRad) was used to remove the salt from the eluate.

Western blot analysis

Proteins were separated on 12% SDS-PAGE and electrophoretically transferred onto PVDF-plus membrane (Micron Separations). Blots were probed with anti-PFLP polyclonal antibody and treated with the western blot AP system (Boehringer Mannheim) to visualize the signal. All antibodies were used at dilution 1:1000. Antibody was developed with the *E. coli* overexpressed PFLP recombinant protein by injecting in rabbits with immunization adjuvant (TiterMax gold) provided by Cytrx. Antiserum was collected 28 days

after the injection without any booster dose. Anti-$(His)_6$ antibodies were obtained commercially from Qiagen.

Southern blot analysis

Genomic DNA was isolated from tobacco suspension cells with the Qiagen Genomic tip protocol. Freeze-dried suspension cells (2 g) were ground in liquid nitrogen, suspended in lysis buffer (20 mM EDTA, 0.5 mg/ml cellulase, 1% TritonX-100, 500 mM guanidine-HCl, 200 mM NaCl, 10 mM Tris-HCl, pH 7.9) and centrifuged at 12 000 rpm for 20 min. Supernatants of lysate were transferred onto an ion-exchange column (Qiagen Genomic tip) to isolate DNA. The eluted DNA was precipitated with isopropanol, washed with 70% ethanol and air-dried. The pellet was re-suspended in TE buffer. About 20 μg DNA was digested with *Hin*dIII, *Bam*HI and *Eco*RI, fractionated in 0.7% agarose gel and Southern-transferred onto Nylon membrane (Boehringer Mannheim) by standard procedures (Sambrook *et al.*, 1989). Membranes were hybridized at 65 ˚C with the full-length *Pflp* cDNA probe and labeled with digoxigenin-11-dUTP (Boehringer Manneheim) by PCR according to the manufacturer's protocol. After hybridization, membranes were washed in high-stringency conditions and detected with the Dig luminiscent detection kit (Boehringer Mannheim).

Preparation of elicitor harpin$_{pss}$

Harpin$_{pss}$ isolation was carried out by the method described by He *et al.* (1993). *E. coli* DH5α (pSYH10) harboring the harpin$_{pss}$ gene was grown in Luria-Bertani broth amended with ampicillin (50 μg/ml) at 37 °C in dark conditions at 225 rpm in the presence of IPTG. To obtain harpin$_{pss}$ the bacterial cells were washed, sonicated for 30 s in 0.01 M phosphate buffer pH 7.5 and boiled for 10 min. After boiling the extracts were centrifuged at 10 000 rpm for 10 min. Supernatants were desalted by using Microconcentrators (Amicon) and stored at −20 °C.

Plant hypersensitive response assay

The HR assay was performed according to Huang *et al.* (1995). Fully expanded tobacco leaves were wounded to form tiny holes on the lower surface of the leaves with a 25-gauge needle. Harpin$_{pss}$ was infiltrated with a 1 ml blunt-end syringe through the hole along with recombinant PFLP protein. The infiltrated plant was incubated at 28 °C, 12 h light/12 h dark conditions. The HR after 24 h was recorded by digital photography.

Generation of Pflp *transgenic tobacco lines*

The coding sequence of *Pflp* gene (includes N-terminal signal peptide) was amplified from *Pflp* cDNA clone by polymerase chain reaction with the following primers: AgSP5, 5′-CGGGATCCCGATGG CTAGTGTCTCAGCTACCA-3′; Ag3, 5′-CGAGCTC GTTAGCCCACGAGTTCTGCTTCT-3′. The PCR product was digested with *Bam*HI and *Sac*I and was purified. The full-length *pflp* fragment replaced the GUS protein coding sequence from pBI121 vector (Clontech, Palo Alto, CA) and the insert was verified by DNA sequencing. *Agrobacterium tumefaciens* C58 was transformed with pBI121 vector containing *Pflp* gene as described by Holsters *et al.* (1978). Transformants of tobacco (*Nicotiana tabacum* cv. Xanthi) were obtained by the standard leaf disk transformation method with kanamycin selection (100 mg/l) (Horsch *et al.*, 1985).

Plant cell culture

Wild-type and *Pflp* transgenic tobacco suspension cells were maintained in Murashige and Skoog (MS) medium supplemented with sucrose (30 g/l), thiamine (1 mg/l), myo-inositol (100 mg/l), KH_2PO_4 (180 mg/l) and 2,4- dichlorophenoxyacetic acid (0.2 mg/l). Cells (2 ml) were transferred to 25 ml of fresh MS medium every 7 days. The cells were grown in an incubator at 26 °C at 130 rpm. For AOS and cell death assays, suspension cells showing more than 80% viable cells were used. Cell concentrations were determined spectrophotometrically as described by James and Lee (2001). Equal cell concentration was taken in both wild-type and *Pflp* transgenic tobacco suspension cells in all experiments.

Measurement of AOS and cell death

Cellular AOS production was measured by a method dependent on intracellular deacylation and oxidation of 2′-7′-dichlorofluorescein diacetate (DCFH-DA) to the fluorescent compound 2′-7′-dichlorofluorescein (DCF) (Buxser *et al.*, 1999). AOS production was measured at intervals after addition of harpin$_{pss}$ and other chemicals to the medium of tobacco suspension cells. DCFH-DA (Sigma Aldrich, USA) was used at

a concentration of 10 mM (dissolved in dimethylformamide). The production of AOS was determined by fluorescence activity using DCFH-DA as probe with a Fluoroimager (wavelength 485/535 nm). Cell death was measured by using tryptan blue staining. The cell death rate was measured with the following formulae: % cell death = (number of stained cells)/total number of cells) × 100.

Immunolocalization

Tobacco leaf tissues were used for localization of PFLP protein. Leaf tissues were fixed with 0.1% gluteraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.0, dehydrated in a grade alcohol series and embedded in Lowicryl HM20. Ultrathin sections were taken with a diamond knife by the Ultracut-E ultramicrotome (Reichert Jung, Vienna, Austria) and collected on nickel grids coated with Formvar and stained as described by Lend *et al.* (1988). Tissues were incubated with primary antiserum against the PFLP protein (1:500 dilutions) at 37 °C for 1 h. After washing the sections were treated with 1:20 diluted good anti-rabbit colloid gold conjugates (12 nm colloidal gold-Affinipure Goat Anti-Rabbit IgG, Jackson Immuno Research Lab.) for 30 min at room temperature. Sections were counterstained with uranyl acetate and lead citrate before examining under a microscope (Philips CM 100 electron microscope) at 80 kV.

Results

Isolation and characterization of the pflp *cDNA clone*

The cDNA clone encoding native PFLP protein was isolated from sweet pepper. Two nested degenerate and inosine containing oligonucleotide primers were designed based on the N-terminal sequence of native PFLP protein and an anchor primer of oligo 5′ (dT_{18})N were used for PCR amplification. The cDNA fragments were cloned, sequenced and used to screen for candidate *Pflp* cDNA clones, containing N-terminal putative amino acid sequences identical to the native PFLP protein. A 5′-RACE RT-PCR was performed with a global 5′ cap primer and 3′ gene-specific primer derived from the 3′ non-coding region of the *Pflp* cDNA clone to amplify the 5′-end region of the candidate clones. Finally, we isolated a 662 bp full-length *Pflp* cDNA clone containing identical N-terminal amino acid sequences to the native

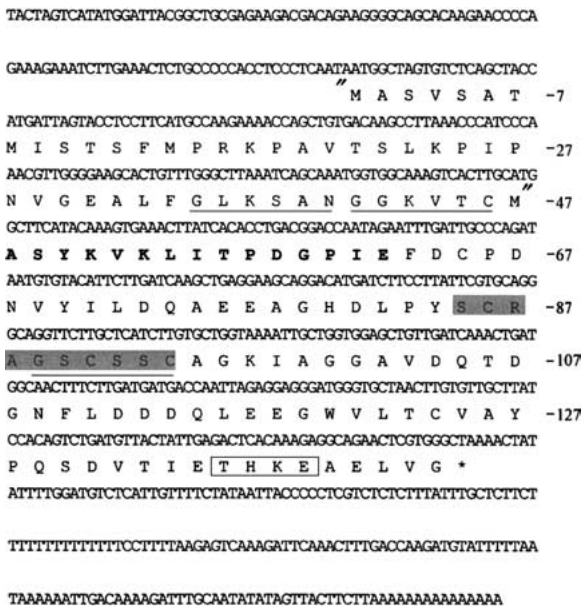

Figure 1. Nucleotide and deduced amino acid sequence of *Pflp* cDNA from sweet pepper. The 144 deduced amino acid sequence indicates the 432 bp open reading frame. The shaded region shows the putative 2Fe-2S domain. The box area indicates the casein kinase II phosphorylation site. Underlined area indicates the N-myristoylation site. The stop codon is indicated by *. " " indicates a putative signal peptide region.

PFLP protein (Figure 1). The *Pflp* gene was also sub-cloned in an *E. coli* expression vector tagged with $(His)_6$ and the expressed recombinant protein was purified by affinity spin column chromatography. Purified recombinant PFLP protein was used to develop the anti-PFLP polyclonal antibody. Anti-PFLP polyclonal antibody recognized the recombinant PFLP protein of 22 kDa in SDS-PAGE analysis. Similarly, anti-PFLP antibody recognized the native PFLP protein and shows the same molecular mass of 22 kDa in SDS-PAGE as previously observed by Lin *et al.* (1997) (Figure 2). This result indicates that the recombinant PFLP is the same as the native PFLP. The actual molecular weight of the native PFLP protein and recombinant PFLP protein in mass spectrophotometric analysis and based on amino acid sequence analysis was evaluated as 12.58 kDa (data not shown).

The PFLP protein sequence showed identity to *Lycopersicon esculentum* ferredoxin-I (72%), pea ferredeoxin-I (52%), *Arabidopsis thaliana* ferredoxin A (54%), *Spinacia oleracea* ferredoxin-I (52%), *Oryza sativa* ferredoxin (56%) and maize ferredoxin (48%). These ferredoxins are thought to be electron carriers in photosynthetic tissues. Among these se-

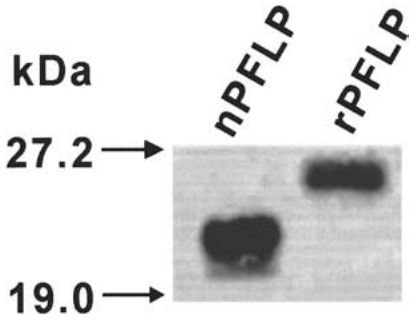

Figure 2. Western blot showing native PFLP protein (nPFLP) from sweet pepper and $(His)_6$-tagged PFLP recombinant protein (rPFLP). Blots were probed with anti-PFLP antibody and signal detected by using the Boehringer Mannheim AP system.

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AAD02175    "-MASVSATMISTSFMPRKPAVTSLKPIP-NVGEALFGLKSANGGK--VTCMASYKVKLI
CAA99756    --MASISGTMISTSFLPRKPAVTSLKAIS-NVGEALFGLKSGRNGR--ITCMASYKVKLI
CAA35754    MASTALSSAIVGTSFIRRSPAPISLRSLPSANTQSLFGLKSCTARCGRVTAMATYKVKFI
FESP1       MAATTTTMNGMATTFVPKPQAPMMAALPSNTGRSLFGLKTGSRGG--RMTMAAYKVTLV
                 :: :   :.*:*: :  *   : .:.    .:*****:.          **:***.::

AAD02175    TPDGPIEFDCPDNVYILDQAEEAGHDLPYSCRAGSCSSCAGKIAGGAVDQTDGNFLDDDQ
CAA99756    TPEGPIEFECPDDVYILDQAEEEGHDLPYSCRAG-SSSCAGKVTAGSVDQSDGNFLDEDQ
CAA35754    TPEGELEVECDDDVYVLDAAEEAGIDLPYS-RAGSCSSCAGKVVSGSVDQSDQSFLDDEQ
FESP1       TPTGNVEFQCPDDVYILDAAEEEGIDLPYSCRAGSCSSCAGKLKTGSLNQDDQSFLDDDQ
            ** * .:*.:* *.**:** *** * ***** *** .******:  *:::* * .***::*

AAD02175    LEEGWVLTCVAYPQSDVTIETHKEAELVG
CAA99756    EAAGFVLTCVAYPKGDVTIETHKEEELTA
CAA35754    IGEGFVLTCAAYPTSDVTIETHKEEDIV-
FESP1       IDEGWVLTCAAYPVSDVTIETHKEEELTA
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Figure 3. Comparison of the deduced amino acid sequence of sweet pepper ferredoxin (AAD02175) with *Lycopersicon esculentum* ferredoxin I (CAA99756), *Arabidopsis thaliana* ferredoxin I (CAA35754) and spinach ferredoxin I (FESP1). The identical amino acids are shown by * and conserved substitutions are shown by : . " " indicates the putative N-terminal signal peptide targeting to chloroplast.

quence comparisons, it shows maximum positional identity with *L. esculentum* ferredoxin-I. Sequence comparison showed the highest amino acid homology with ferredoxin-I of *Lycopersicon esculentum*, sharing an identity of 72% and a similarity of 81%. Identity values for other plant ferredoxins fell below 59%. Sequence comparison in database searches suggests that the PFLP protein is a ferredoxin-I containing a Ser- and Thr-rich N-terminal signal peptide of 47 amino acids targeting to chloroplast and putative 2Fe-2S domain (Figure 3).

Recombinant PFLP enhances harpin$_{pss}$-mediated HR

Lin *et al.* (1997) have reported that native PFLP protein interfered with harpin$_{pss}$-mediated HR. To confirm the HR-interfering activity of recombinant PFLP, a mixture of harpin$_{pss}$ and PFLP was infiltrated into tobacco leaves. All treatments were infiltrated in the same leaf panel and three leaves from three different

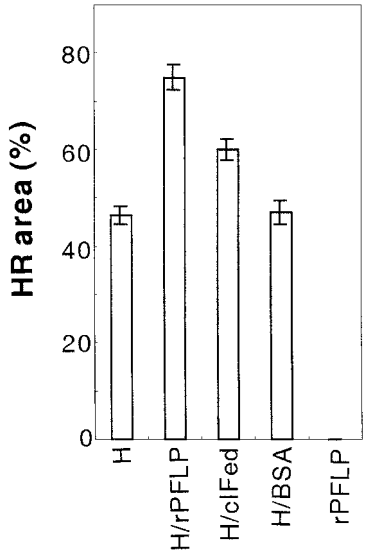

Figure 4. Hypersensitive response bioassay. Expression of the HR like necrosis in tobacco leaves was photographed 24 h after the infiltration of 50 μl containing 10 μg harpin$_{pss}$ (H), 10 μg recombinant PFLP protein (rPFLP), a mixture of 10 μg harpin$_{pss}$ and 10 μg recombinant PFLP protein (H/rPFLP), a mixture of 10 μg harpin$_{pss}$ and 10 μg *Clostridium pasteurianum* ferredoxin protein (H/clFed) and a mixture of 10 μg harpin$_{pss}$ and 10 μg bovine serum albumin (H/BSA) served as control. Percentage of HR was measured against the total infiltration area at 24 h after infiltration. Values are means of $\pm$ SE of three replicates. Values for the treatments 'H/PFLP' and 'H/clFed' are significant when compared to 'H' alone ($P < 0.05$).

plants at the same age were used. The HR area was measured against the total infiltration area after 24 h. The HR area was significantly increased (75%) in the presence of recombinant PFLP protein infiltrated with harpin$_{pss}$ when compared to harpin$_{pss}$ (47%) alone (Figure 4). We tested the same phenomenon with another ferredoxin from *Clostridium pasteurianum* (purchased from Sigma Aldrich, USA); this also showed a significant increase (60%) in the HR area when co-infiltrated with harpin$_{pss}$. This result suggests that PFLP protein intensifies the harpin$_{pss}$-mediated HR *in vitro*.

PFLP enhances AOS generation in vitro

The production of AOS is one of the earliest events during HR and considered as a hallmark of plant defense responses (Levine *et al.*, 1994). Moreover, PFLP protein is a ferredoxin that is involved in many redox reactions leading to the production of AOS. It is possible that PFLP will enhance AOS production and intensify the harpin$_{pss}$-mediated HR. To measure the AOS *in vitro* a fluorescence assay was car-

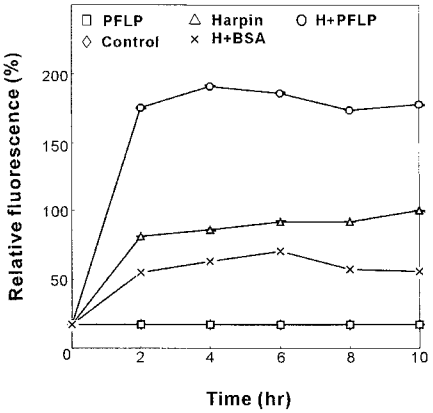

Figure 5. Harpin-induced oxidative burst. Wild-type tobacco cells were treated with buffer as control, 25 μM recombinant PFLP protein (PFLP), 100 nM $harpin_{pss}$ (Harpin), 100 nM $harpin_{pss}$ and 25 μM recombinant PFLP protein (H+PFLP) and 100 nM $harpin_{pss}$ and 25 μM bovine serum albumin (H+BSA) as negative control. At indicated time points of incubation with $harpin_{pss}$, the AOS level was detected with a fluorimager as mentioned in Materials and methods. Data are means of ± SE of 9 replicates from three independent experiments at each time point. The treatment value of 'H/PFLP' at each time point is significant as compared to treatment 'H' alone ($P < 0.05$).

ried out. Inoculation of wild-type tobacco suspension cells with $harpin_{pss}$ and recombinant PFLP protein showed significant increase in AOS generation when compared to $harpin_{pss}$ and $harpin_{pss}$/BSA treatments (Figure 5), and this increase was sustained for some hours. This result shows that PFLP cannot only intensify $harpin_{pss}$-mediated HR but also enhance the production of AOS *in vitro*.

Transgenic tobacco suspension cells show an enhanced production of AOS and cell death

A transgenic system was developed to evaluate, if over-expressed PFLP can also enhance $harpin_{pss}$-mediated HR *in vivo*. Transgenic tobacco expressing the *Pflp* gene was obtained through *Agrobacterium*-mediated plant transformation. The gene constructed in the pBI121vector for transformation is presented in Figure 6A. Viable transformants were selected by kanamycin at 100 μg/ml. Transformation with the *Pflp* gene into kanamycin-resistant lines was confirmed by polymerase chain reaction. Finally, we got seven viable transgenic lines. Among those, transgenic line 16-8 showed stronger PFLP protein expression. We used this line for further experimental analyses. We could develop the suspension cells system from this transgenic line by virtue of the ease of AOS and cell death measurements induced by $harpin_{pss}$. Genomic DNA and protein blot assays suggested that the *Pflp* transgene is present as a single copy (Figure 6B) and protein expression is stronger (1.5-fold) than wild-type tobacco suspension cells (Figure 6C). These results confirmed the presence of the *Pflp* gene and its expression in transgenic tobacco suspension cells.

The level of AOS generation and the degree of hypersensitive cell death of the *Pflp* transgenic tobacco suspension cells were found to be significantly higher than in the wild type under elicitor $harpin_{pss}$ treatment (Figure 7). To reveal the AOS signaling pathway in more detail, we added NADPH oxidase inhibitor like DPI or Ca^{2+} channel blocker ($LaCl_3$) into tobacco cell culture during $harpin_{pss}$-induced hypersensitive cell death. In the presence of DPI or $LaCl_3$ the generation of AOS was highly inhibited in both wild-type and *Pflp* transgenic cells (Figure 7A). The cell death rate is not different between $harpin_{pss}$, $harpin_{pss}$ plus DPI, and $harpin_{pss}$ plus $LaCl_3$ treatments in wild-type cells. In contrast, in *Pflp* transgenic tobacco suspension cells, $harpin_{pss}$-induced cell death was significantly inhibited in the presence of DPI and $LaCl_3$ (Figure 7B). These results suggest that the enhanced $harpin_{pss}$-induced cell death was dependent on AOS and Ca^{2+} in *Pflp* transgenic tobacco suspension cells. However, $harpin_{pss}$-induced cell death was independent on AOS and the Ca^{2+} signaling pathway in wild-type tobacco suspension cells.

Subcellular localization of PFLP protein

Localization studies were performed to investigate the subcellular distribution of PFLP in tobacco mesophyll cells. Immunogold labeling of PFLP protein was detected in the chloroplast and cytoplasm of *Pflp* transgenic tobacco cells but only in the chloroplast, not in the cytoplasm, of wild-type tobacco cells (Figure 8). The presence of gold particles is higher in transgenic cells than in wild-type tobacco cells.

Discussion

In this study, we have demonstrated the function of PFLP in $harpin_{pss}$-induced hypersensitive cell death in tobacco suspension cells. Results of protein sequence comparisons suggest that PFLP is a ferredoxin with characteristic Ser- and Thr-rich N-terminal signal peptide targeting to chloroplast and the putative 2Fe-2S domain. Upon $harpin_{pss}$ infection, *Pflp* transgenic tobacco suspension cells showed increases in

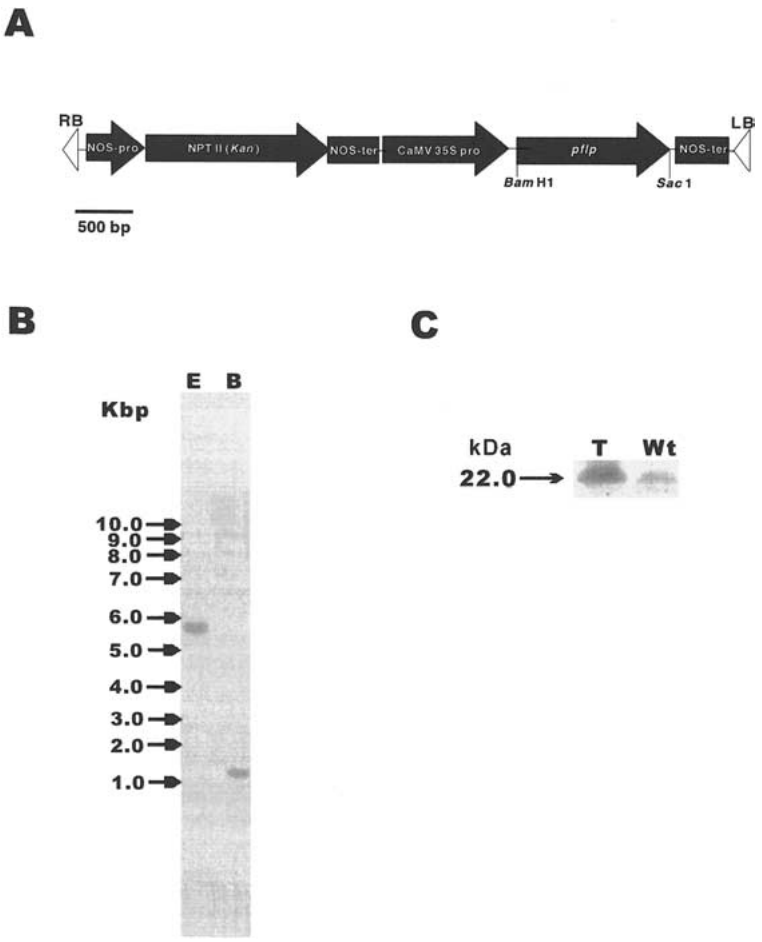

Figure 6. *Pflp* transgenic tobacco suspension culture cells analyses. A. Map of the relevant part of the transformed plasmid containing the sweet pepper *Pflp* gene. NOS-pro, the promoter region of the *Agrobacterium tumefaciens* nopalin synthase gene; NOS-ter, the terminator region of the *A. tumefaciens* nopalin synthase gene; NPTII, coding sequence of the neomycin phosphotransferase II gene; CaMV 35S-pro, CaMV 35S promoter sequence; *Pflp*, the coding sequence for the full-length PFLP protein including the N-terminal signal peptide. B. Southern blot analysis of *Pflp* transgenic tobacco suspension culture cells showing the incorporation of *Pflp* gene by using Dig-labeled NPTII cDNA as a probe. Each lane containing 15 μg genomic DNA was digested with *Eco*RI (E) and *Bam*HI (B) before being subjected to southern blot analysis. C. Western blot analysis showing the expression of PFLP protein in *Pflp* transgenic tobacco suspension culture cells. Total protein (20 μg) from wild-type tobacco suspension cells (Wt) and *Pflp* transgenic tobacco suspension cells (T) was purified and subjected to gel blot analysis. The blot was probed with anti-PFLP antibody and signal detected by using Boehringer Manneheim AP system.

AOS generation and hypersensitive cell death. The use of AOS inhibitors like DPI and $LaCl_3$ suggested that enhanced generation of AOS is the possible cause of increasing $harpin_{pss}$-induced hypersensitive cell death. Overall, these studies suggest that cellular AOS caused by over-expressed ferredoxin influences $harpin_{pss}$-induced hypersensitive cell death.

A previous report described that native PFLP (AP1) could delay the $harpin_{pss}$-mediated HR. In this study, we show that recombinant PFLP enhances $harpin_{pss}$-mediated HR. Generally, ferredoxin can act as reductants or oxidants depending on the redox environment in which their function is studied. This may be one of the possible reasons to cause the difference between the functions of native PFLP and recombinant PFLP. However, our data showed the possible explanation for these contradictory results was *n*-octanol, which was used as organic phase in the amphipathic extraction method of native PFLP protein but not in the recombinant PFLP protein purification. Wimley *et al.*, (1998) reported that *n*-octanol could modify the secondary structure of membrane bound hydrophobic proteins, especially in β-sheet formation. Moreover, recombinant PFLP purified under denaturing condi-

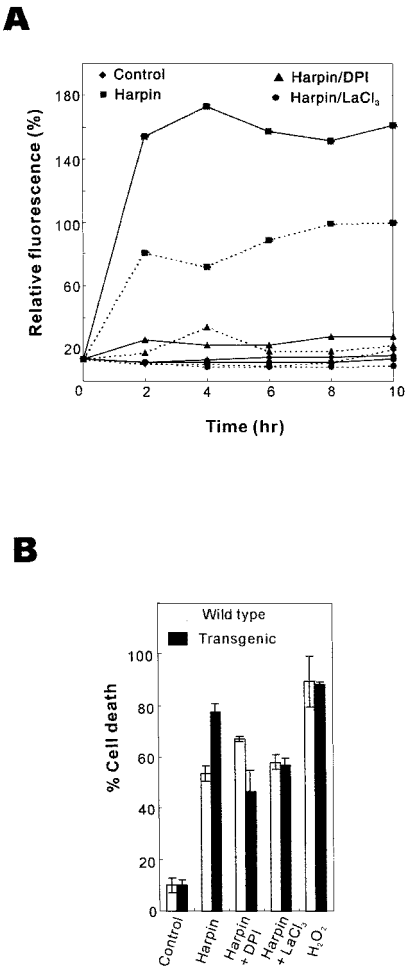

Figure 7. A. Harpin-induced oxidative burst and its inhibition by DPI and $LaCl_3$. Both *Pflp* transgenic and wild-type tobacco cells were treated with $harpin_{pss}$ (100 nM final concentration). Inhibitors DPI (20 μM/final concentration) and $LaCl_3$ (0.5 mM final concentration) were added 20 min prior to $harpin_{pss}$ treatment. At indicated time points of incubation with $harpin_{pss}$, the AOS level was detected with a fluoroimager as mentioned in Materials and methods. Solid lines indicate *Pflp* transgenic tobacco suspension cells and dotted line indicate wild-type tobacco suspension cells. Data are means of $\pm$ SE of 9 replicates from three independent experiments. Values are significant at each time point when comparing *Pflp* transgenic tobacco suspension cells with wild-type tobacco suspension cells in response to $harpin_{pss}$. B. Hypersensitive cell death induced by $harpin_{pss}$. Both *Pflp* transgenic and wild-type tobacco cells were incubated with $harpin_{pss}$ (100 nM final concentration) and cell death was monitored after 24 h by the trypan blue staining method. DPI (20 μM final concentration) and $LaCl_3$ (0.5 mM/ final concentration) were added 20 min prior to $harpin_{pss}$ treatment. H_2O_2 (100 nM final concentration) was used as a positive control. Data are means $\pm$ SE calculated from three independent assays. Values are significant when comparing wild-type and *Pflp* transgenic suspension culture cells in response to $harpin_{pss}$ treatment. In *Pflp* transgenic suspension culture cells significant reduction was observed in harpin + DPI and harpin + $LaCl_3$treatments as compared to $harpin_{pss}$ alone ($P < 0.05$).

tions with urea and *n*-octanol also showed a HR delay phenomenon (data not shown) as reported previously by Lin *et al.* (1997). We confirmed this enhancing phenomenon with another ferredoxin type. Ferredoxin from *Clostridium pasteurianum* also showed a significant increase in $harpin_{pss}$-mediated HR (Figure 4). These results suggest that the PFLP has a real enhancing function in $harpin_{pss}$-mediated hypersensitive cell death.

Generally, ferredoxin gene expression is driven in an organ-specific and development-dependent fashion by the native promoter (Vorst *et al.*, 1990; Gallo-Meagher *et al.*, 1992; Bringloe *et al.*, 1995). Native promoter-driven ferredoxin mRNA was able to be expressed in leaves or cotyledons but never in roots (Vorst *et al.*, 1990; Gallo-Meagher *et al.*, 1992). We had used the CaMV 35S promoter to express the *Pflp* gene constitutively in a tobacco cell system. The level of PFLP in transgenic tobacco suspension cells is higher (1.5-fold) than in the wild type (Figure 6C). An earlier report has shown that parsley *4CL-1* gene expression (regulated by light in native condition) driven by the CaMV 35S promoter was neither sensitive to light induction nor to elicitor treatment (Douglas *et al.*, 1991). Another report has shown that CaMV 35S promoter-driven ferredoxin mRNA accumulated even in roots (Gallo-Meagher *et al.*, 1992). This implies that CaMV 35S promoter-driven PFLP is neither tissue-specific nor light-sensitive. Due to the presence of N-terminal signal peptide the mature PFLP will be directed into the chloroplast. Sub-cellular localization studies have confirmed that PFLP is present in the chloroplast region but due to over-expression, PFLP is also present in the cytoplasm of *Pflp* transgenic tobacco cells (Figure 8).

In this study, we have developed a system containing wild-type and transgenic tobacco suspension cells differed only in the expression level of ferredoxin. The transgenic tobacco suspension cells containing elevated level of PFLP could significantly enhance the $harpin_{pss}$-induced generation of AOS and hypersensitive cell death. The involvement of AOS in enhancing $harpin_{pss}$-induced cell death was investigated by means of specific inhibitors. AOS generation was inhibited in the presence of DPI (NADPH oxidase inhibitor) and $LaCl_3$ (a Ca^{2+} channel blocker) in both *Pflp* transgenic and wild-type tobacco suspension cells. In the presence of DPI and $LaCl_3$, $harpin_{pss}$-induced hypersensitive cell death is not inhibited in wild-type cells (Figure 7B). This result is consistent with a recent report in which DPI did not block harpin-

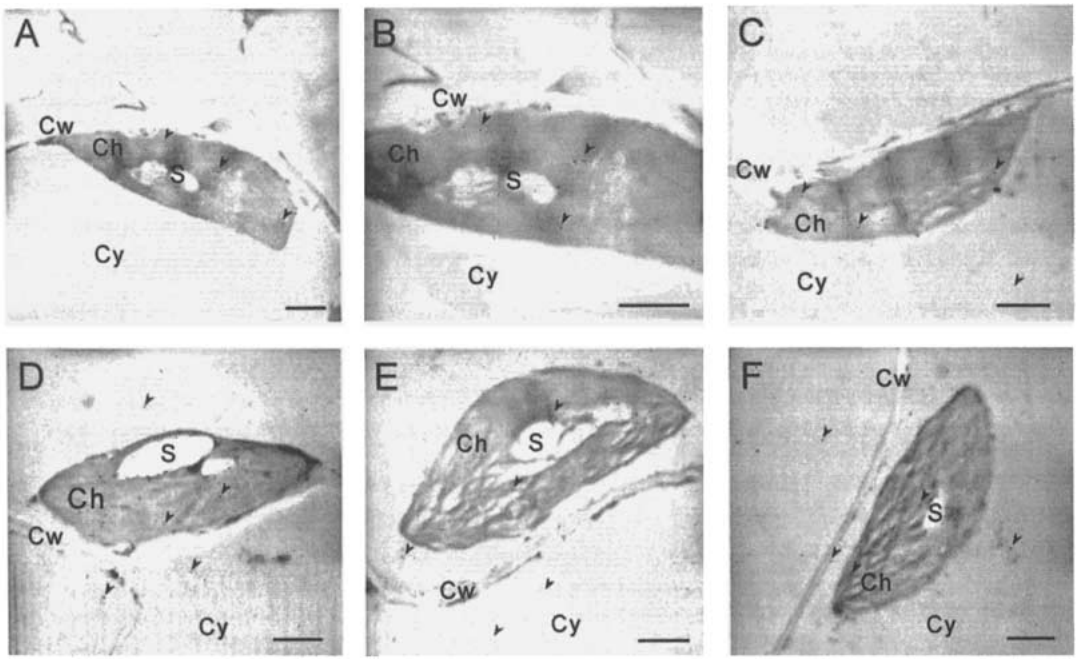

Figure 8. Localization of PFLP protein in tobacco cells by the immunogold labeling method. Leaf sections were labeled with anti-PFLP polyclonal antibodies. A–C. Wild-type tobacco cells. D–F. *Pflp* transgenic tobacco cells. Cell wall (Cw), chloroplast (Ch), starch granule (S) and cytosol (Cy) of tobacco cells are labeled in the photograph. The position of the 12 nm gold particles is indicated by arrows. Scale: the bar represents 1 μM.

induced cell death (Xie and Chen, 2000). In contrast, in *Pflp* transgenic cells, the cell death was significantly inhibited in the presence of DPI and $LaCl_3$. This result suggests that the significant increase of $harpin_{pss}$-induced hypersensitive cell death was dependent on AOS production and Ca^{2+}-mediated signaling pathways. Reports are available that elicitor induced cell death is independent on AOS and Ca^{2+} signaling pathways (Able *et al.*, 2000; Sasabe *et al.*, 2000; Xie and Chen, 2000). Here, our data showed that enhanced $harpin_{pss}$-induced hypersensitive cell death caused by the elevated level of ferredoxin (PFLP) in transgenic tobacco cells is AOS-dependent.

Chloroplasts are the most powerful source of AOS in plants (Foyer *et al.*, 1994). Photosynthesis is a combination of successive redox reactions occurred in the chloroplast. Photosynthetic electron transport system includes Fe-S centers, ferredoxin and thioredoxin on the reducing side of photosystem I. These electron transport components are auto-oxidisable and produce superoxide radicals and H_2O_2 (Dat *et al.*, 2000). Ferredoxin plays a role in the electron transport chain by reducing $NADP^+$ to NADPH leading to the production of AOS (Biehler, 1996). The reduction of ferredoxin in the chloroplast is tightly regulated via the electron transport chain. Moreover, chloroplasts have a robust network of constitutive antioxidants, both enzymatic and non-enzymatic and wide range of inducible defenses to enable efficient photosynthesis to be performed over a wide range of environmental conditions (Foyer and Halliwell, 1976; Salin, 1987; Asada, 1994; Kozaki and Takeba, 1996). It seems that ferredoxin localized in the chloroplast cannot influence the enhancing AOS generation upon $harpin_{pss}$ inoculation. Our present data clearly indicate that the enhanced AOS generation in *Pflp* transgenic tobacco cells is plasma membrane-bound NADPH-oxidase-dependent (Figure 7A). Plasma membrane-bound NADPH-oxidase has recently been considered as a source of AOS for the oxidative burst, which is typical of plant-pathogen incompatible interactions (Desikan *et al.*, 1996; Lamb and Dixon, 1997). Subcellular localization data also support the notion that heterologous PFLP is present in the cytoplasm of *Pflp* transgenic tobacco cells (Figure 8). Based on these results, it is possible that PFLP action lacks some endogenous controls in cytosol and some molecules in cytosol can trigger PFLP to change the redox state of cells. This redox state change will lead to enhanced AOS generation and cell death upon $harpin_{pss}$ inocula-

tion. On the other hand, the molecules triggering PFLP to be auto-oxidative in the cytosol should be expressed in response to $harpin_{pss}$ treatment because the basal AOS level shows no difference in both *Pflp* transgenic and wild-type tobacco cells.

Based on our results the following conclusions can be drawn. First, *Pflp* transgenic tobacco suspension cells show increasing $harpin_{pss}$-induced cell death through enhanced production of AOS. Second, the enhanced $harpin_{pss}$-mediated hypersensitive cell death caused by heterologous PFLP is through an AOS-dependent pathway. Third, it is possible that overexpressed PFLP localized to the cytosol can change the redox state of the cell in response to $harpin_{pss}$ inoculation, leading to enhanced generation of AOS and hypersensitive cell death. To our knowledge, this is the first study showing that ferredoxin can function in enhancing $harpin_{pss}$-mediated HR. It would be of great interest to elucidate further the action of PFLP in the cytosol in enhancing AOS and $harpin_{pss}$-induced hypersensitive cell death.

Acknowledgments

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