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Ectopically expressed sweet pepper ferredoxin PFLP enhances disease resistance to *Pectobacterium carotovorum* subsp. *carotovorum* affected by harpin and protease-mediated hypersensitive response in Arabidopsis

MANG-JYE GER[1], GUAN-YU LOUH[2], YI-HSIEN LIN[3], TENG-YUNG FENG[4] AND HSIANG-EN HUANG[2,*]

[1]*Department of Life Science, National University of Kaohsiung, Kaohsiung 811, Taiwan*
[2]*Department of Life Science, National Taitung University, 684, Sec. 1, Chunghua Rd., Taitung 950, Taiwan*
[3]*Department of Plant Medicine, National Pingtung University of Science and Technology, Pingtung 912, Taiwan*
[4]*Department of Plant and Microbial Biology, Academia Sinica, Taipei 115, Taiwan*

SUMMARY

Plant ferredoxin-like protein (PFLP) is a photosynthesis-type ferredoxin (Fd) found in sweet pepper. It contains an iron–sulphur cluster that receives and delivers electrons between enzymes involved in many fundamental metabolic processes. It has been demonstrated that transgenic plants overexpressing PFLP show a high resistance to many bacterial pathogens, although the mechanism remains unclear. In this investigation, the *PFLP* gene was transferred into Arabidopsis and its defective derivatives, such as *npr1* (*nonexpresser of pathogenesis-related gene 1*) and *eds1* (*enhanced disease susceptibility 1*) mutants and *NAHG*-transgenic plants. These transgenic plants were then infected with the soft-rot bacterial pathogen *Pectobacterium carotovorum* subsp. *carotovorum* (*Erwinia carotovora* ssp. *carotovora*, ECC) to investigate the mechanism behind PFLP-mediated resistance. The results revealed that, instead of showing soft-rot symptoms, ECC activated hypersensitive response (HR)-associated events, such as the accumulation of hydrogen peroxide (H_2O_2), electrical conductivity leakage and expression of the HR marker genes (*ATHSR2* and *ATHSR3*) in *PFLP*-transgenic Arabidopsis. This PFLP-mediated resistance could be abolished by inhibitors, such as diphenylene iodonium (DPI), 1-L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E64) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), but not by myriocin and fumonisin. The *PFLP*-transgenic plants were resistant to ECC, but not to its harpin mutant strain ECCAC5082. In the *npr1* mutant and *NAHG*-transgenic Arabidopsis, but not in the *eds1* mutant, overexpression of the *PFLP* gene increased resistance to ECC. Based on these results, we suggest that transgenic Arabidopsis contains high levels of ectopic PFLP; this may lead to the recognition of the harpin and to the activation of the HR and other resistance mechanisms, and is dependent on the protease-mediated pathway.

*Correspondence: Email: hhn@nttu.edu.tw

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INTRODUCTION

Ferredoxin (Fd) contains a [2Fe–2S] cluster with a $CX_4CX_2CX_nC$ pattern and regulates the activity of several reductases, such as Fd-NADP reductase (FNR), Fd-thioredoxin reductase (FTR), Fd-dependent glutamate synthase (Fd-GOGAT), Fd-dependent bilin reductases and Fd-dependent sulphite reductase (SiR). These enzymes are involved in various fundamental metabolic activities, such as photosynthesis, photorespiration, phytochrome biosynthesis, reduction of nitrite to ammonium and sulphite to sulphide, the Calvin cycle, starch synthesis, antioxidation, amino acid conversion and lipid saturation via alteration of the activity of the above enzymes. Fd also regulates the enzymes involved in carbon hydride metabolism, such as phosphoribulokinase, fructose-1,6-bisphosphatase, ADP-glucose pyrophosphorylase (AGPase), glucose-6-phosphate dehydrogenase and α-amylase (Arnon, 1989; Balmer *et al*., 2006; Beinert *et al*., 1997; Bertini *et al*., 2002; Hanke and Mulo, 2013; Joliot and Joliot, 2006; Meyer, 2001; Morales *et al*., 2002; Schürmann, 2003; Zurbriggen *et al*., 2008). More than 200 different Fd isoproteins have been identified in plants, such as tomato (Aoki and Wada, 1996; Aoki *et al*., 1998; Green *et al*., 1991), maize (Kimata and Hase, 1989; Matsumura *et al*., 1999; Onda *et al*., 2000), sunflower (Venegas-Calerón *et al*., 2009), sweet pepper (Dayakar *et al*., 2003) and Arabidopsis (Hanke *et al*., 2004). They are classified into photosynthetic-type Fd and non-photosynthetic-type Fd according to the expressing tissue and their potential reductive capacity.

In sweet pepper, one of the photosynthetic Fd types has been identified as plant ferredoxin-like protein (PFLP) because it assists the harpin-mediated hypersensitive response (HR). This PFLP shares 48%–75% identity with photosynthetic-type Fd found in

other plants, such as tomato, pea, spinach, rice, maize and Arabidopsis. PFLP contains two putative functional domains that have been revealed as the 2Fe–2S domain and the casein kinase II phosphorylation (CK2P) site (Dayakar *et al*., 2003). The N-terminal signal peptide has been predicted to carry protein into the chloroplast and the 2Fe–2S domain has been predicted to chelate the iron ion and is required by PFLP to inhibit bacterial growth (Huang *et al*., 2006). Previous studies have also revealed that the mutant PFLP defective in the CK2P domain fails to enhance the harpin-mediated HR in Arabidopsis (Lin *et al*., 2011). The overexpression of PFLP should enhance disease resistance to bacterial pathogens, such as *Pectobacterium carotovorum* ssp. *carotovorum* (*Erwinia carotovora* ssp. *carotovora*, ECC), *Pseudomonas syringae* pv. *tabaci* and *Ralstonia solanacearum*, in different transgenic plants (Huang *et al*., 2004, 2006, 2007b; Liau *et al*., 2003; Lin *et al*., 2010; Namukwaya *et al*., 2012; Tang *et al*., 2001; Yip *et al*., 2007). Infection by the bacterial pathogen activates certain HR events, such as the accumulation of hydrogen peroxide (H_2O_2), and expression of the HR marker gene in *PFLP*-transgenic tobacco (Huang *et al*., 2004).

HR is a plant defence mechanism that accelerates cell death in order to restrict the growth of the infecting pathogen (van Doorn *et al*., 2011; Greenberg and Yao, 2004; Heath, 2000). The morphology of the cells undergoes an HR and shows rapid and transient generation of reactive oxygen species (ROS), including superoxide radicals, H_2O_2 and singlet oxygen (Apel and Hirt, 2004; O'Brien *et al*., 2012). The induced ROS can act as secondary messengers in the activation of signal transduction pathways, reinforcing cell walls or restricting directly pathogen growth (Bolwell *et al*., 2002; Doke *et al*., 1996; Kawano, 2003; Lamb and Dixon, 1997). Previous studies have shown that the plant generation and ROS antioxidant systems are regulated by Fd via photosynthetic electron flow or NADPH generated in the oxidative pentose phosphate pathway (Tognetti *et al*., 2006). Knocking out the major Fd in Arabidopsis led to the up-regulation of the alternative NADPH-requiring redox-regulatory antioxidant system (Voss *et al*., 2008). Consequently, the oxidase/nitric oxide synthase (NOS) inhibitor, diphenylene iodonium (DPI), was able to stop the oxidative burst induced by the avirulent pathogen, *P. syringae*, in Arabidopsis (Alvarez *et al*., 1998; Bolwell *et al*., 1998, 2002), although there were some exceptions when the plant was infected by *Botrytis* spp. (Govrin *et al*., 2006). The HR in plants can be inactivated by thiol protease inhibitors, such as 1-L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E64) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) (Bonneau *et al*., 2008; Elbaz *et al*., 2002; Okita *et al*., 2007; del Pozo and Lam, 1998; Slee *et al*., 1996; Tiwari *et al*., 2002; Yang and Schnellmann, 1996).

Harpin is an HR elicitor that is secreted via the type III secretion system (Galán and Collmer, 1999) and by many different pathogenic bacteria, such as *Erwinia*, *Pseudomonas*, *Xanthomonas* and *Ralstonia*. Pathogen mutations of harpin usually simultaneously reduce pathogenesis and HR (Choi *et al*., 2013; Clarke *et al*., 2005; El-Maarouf *et al*., 2001; He *et al*., 1993; Kariola *et al*., 2003; Kim *et al*., 2004; Li *et al*., 2010; Mukherjee *et al*., 1997; Reboutier *et al*., 2007; Wei *et al*., 1992), and harpin is able to elicit the accumulation of pathogen-related gene transcripts in non-host plants and activates salicylic acid (SA)-responsive mitogen-activated protein kinase (Dong *et al*., 1999; Lee *et al*., 2001). Recent investigations have also revealed that harpin produced by the rice bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae*, Hpa1(Xoo), is able to induce apoplastic H_2O_2 production via NADPH oxidase in the plasma membrane, and cytoplasmic H_2O_2 in a NO-dependent manner in the cytoplasm, and both are needed to induce resistance (Sang *et al*., 2012). However, other studies have revealed that the accumulation of ROS in the mitochondria is necessary for harpin-mediated HR (Garmier *et al*., 2007). The soft-rot pathogen, ECC, also produces harpin, which leads to the induction of HR and systemic resistance at concentrations of about 2–20 μM in both Arabidopsis and tobacco. Both SA- and jasmonate (JA)-regulated defence genes, such as *PR1* (*PATHOGENESIS-RELATED GENE 1*) and *PDF1.2* (*PLANT DEFENSIN 1.2*), are induced by harpin in Arabidopsis. However, harpin fails to induce disease resistance in all *NAHG*-transgenic plants and pathway-specific mutants, such as *ndr1* (*non-race-specific disease resistance 1*), *eds1* (*enhanced disease susceptibility 1*), *coi1* (*coronatine insensitive 1*) and *jar1* (*jasmonate resistant 1*) (Kariola *et al*., 2003; Mukherjee *et al*., 1997; Peng *et al*., 2003).

The SA-dependent signal transduction pathway plays an important role during the plant response to biotic and abiotic stresses, such as drought, chilling, heavy metal toxicity, heat and osmotic stress, and in the regulation of physiological and biochemical processes during plant growth and development (Alvarez, 2000; Delaney *et al*., 1994; Durrant and Dong, 2004; Fragnière *et al*., 2011; Gaffney *et al*., 1993; Koornneef and Pieterse, 2008; Kunkel and Brooks, 2002; Lawton *et al*., 1995; Loake and Grant, 2007; Malamy *et al*., 1990; Mëtraux *et al*., 1990; Rivas-San Vicente and Plasencia, 2011; Uknes *et al*., 1992; Ward *et al*., 1991). The genes involved in the SA-mediated pathway have been investigated, such as *NPR1* (*NONEXPRESSER OF PATHOGENESIS-RELATED GENE 1*) (also known as *NIM1* or *SAI1*), which encodes a protein containing the multiple ankyrin repeat domain and interacts with the TGA transcription factor family. Mutation of *NPR1* reduces the sensitivity to SA and increases the susceptibility to infection by pathogens (Cao *et al*., 1994, 1997; Dong, 2004; Gopalan *et al*., 1996; Pieterse and van Loon, 2004; Pieterse *et al*., 1998; Ton *et al*., 2002). In addition to controlling the expression of PR genes, NPR1 also controls directly the expression of the protein secretory pathway genes that are essential for systemic acquired resistance (SAR) (Wang *et al*., 2005). The *EDS1* gene is another SA-mediated pathway gene which encodes a lipase-like protein that activates disease-resistant TIR-NB-LRR receptors (a Toll-like nucleotide-binding leucine-rich repeat domain). EDS1 is able to combine

many proteins into a complex, such as the TIR-NB-LRR disease resistance proteins RPS4 and RPS6, the negative immune regulator SRFR1, phytoalexin-deficient 4 (PAD4) protein and senescence-associated gene 101 (SAG101). The EDS1 complex is necessary for basal resistance involving the transcriptional up-regulation of PAD4 and the mobilization of SA defences, and is disrupted by the bacterial effectors AvrRps4 and HopA1 (Bhattacharjee *et al*., 2012; Feys *et al*., 2001; Glazebrook *et al*., 1997; Heidrich *et al*., 2011; Rietz *et al*., 2011). The *eds1* mutant is susceptible to infection by *Peronospora parasitica*, which induces defence responses in wild-type Arabidopsis (Aarts *et al*., 1998; Falk *et al*., 1999; Heidrich *et al*., 2011; Jirage *et al*., 1999; Wiermer *et al*., 2005). The *NAHG* gene encodes a salicylate hydroxylase which converts SA to catechol. Transgenic Arabidopsis that overexpresses this *NAHG* gene has no non-host resistance to *P. syringae* pv. *phaseolicola* strain 3121 and *P. syringae* pv. *tomato* (Heck *et al*., 2003; van Wees and Glazebrook, 2003).

Arabidopsis thaliana is susceptible to ECC, even though the defence genes are activated during the infection process (Aguilar *et al*., 2002; Kariola *et al*., 2003; Mysore and Ryu, 2004). In order to investigate the mechanism behind PFLP-mediated resistance against ECC, the *PFLP* gene was overexpressed, together with the *Cauliflower mosaic virus* (CaMV) 35S promoter, in Arabidopsis and its defective derivatives. These transgenic Arabidopsis plants were inoculated with ECC, the ECC harpin mutant strain and HR inhibitors in order to investigate the mechanism behind PFLP-mediated resistance.

RESULTS

Characterization of *PFLP*-transgenic Arabidopsis

In order to investigate PFLP-mediated resistance, *PFLP*-transgenic Arabidopsis plants were generated using *Agrobacterium tumefaciens* C58C1, which contains the pBI121-*PFLP* plasmid. Southern blot analysis, after probing with the *NPTII* (*NEOMYCIN PHOSPHOTRANSFERASE II*) gene, revealed that two individual bands of 8.3 and 5.8 kb were observed in the genomic DNA of the *PFLP-1* transgenic line cut by *Eco*RI, and two bands of 7.9 and 4.4 kb when cut by *Sac*I. Southern blot analysis of the transgenic line, *PFLP-2*, revealed that one 7.8-kb band was cut by *Eco*R1 and a 10-kb band was cut by *Sac*I (Fig. 1A). These results imply that *PFLP-1* and *PFLP-2* are two independent *PFLP*-transgenic lines. In order to estimate the transcription level of the *PFLP* gene in these

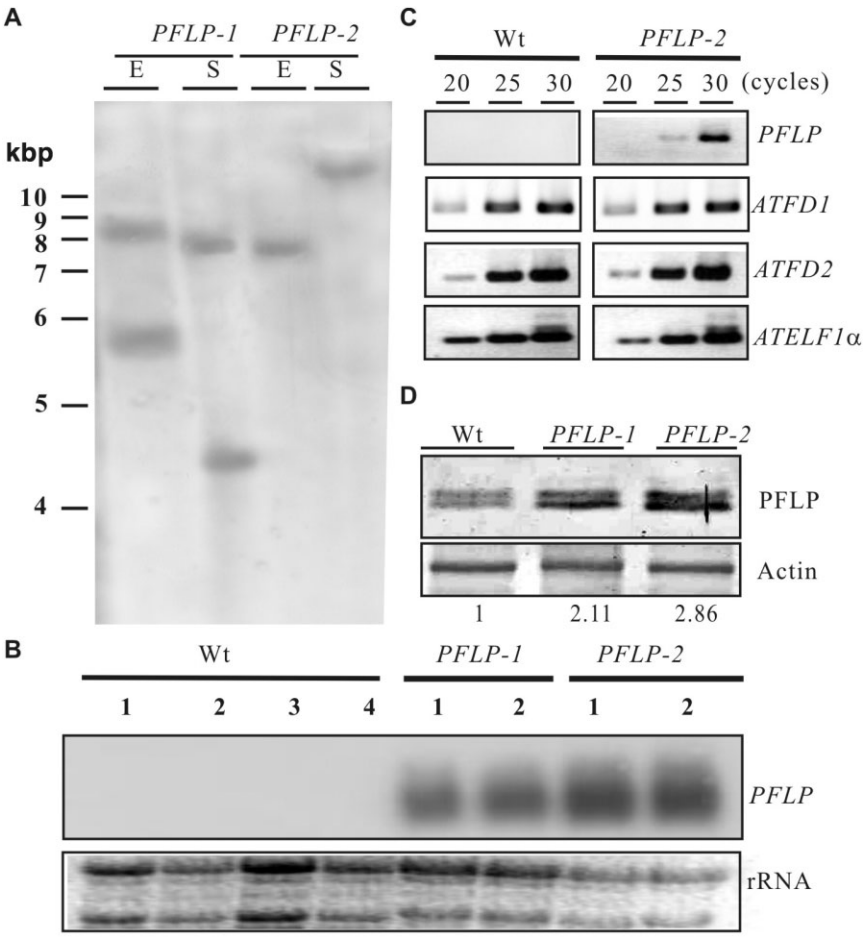

Fig. 1 Characterization of *plant ferredoxin-like protein* (*PFLP*)-transgenic Arabidopsis. The genomic DNA was digested with *Eco*RI (E) or *Sac*I (S) and detected by Southern blot analysis using a *NPTII* (*NEOMYCIN PHOSPHOTRANSFERASE II*) gene probe (A). The total RNAs isolated from non-transgenic Arabidopsis (Wt 1–4) and transgenic lines (*PFLP-1* 1–2 and *PFLP-2* 1–2) were investigated by Northern blot analysis. The ribosomal RNA (rRNA), stained by ethidium bromide (EtBr), was used as the loading control (B). The total RNA (1 μg) was used in the semi-quantifying reverse transcription-polymerase chain reaction RT-PCR) analysis together with specific primers for the *PFLP*, *ATFD1*, *ATFD2* and *ATELF1α* genes. (C). The crude extract proteins (10 μg) of Wt, *PFLP-1* and *PFLP-2* were identified by Western blot analysis with antiserum against PFLP. The monoclonal antiserum against actin was used as a loading control. The number indicated is the relative ratio of the PFLP signal after normalization by actin (D).

transgenic lines, the expression level of the mRNA was estimated by Northern blot analysis with a probe specific for the *PFLP* gene. The results showed that *PFLP* gene transcription was detected in both the *PFLP-1* and *PFLP-2* transgenic lines, but did not occur in the non-transgenic line (Fig. 1B). The expression levels of the *ATFD1* and *ATFD2* genes were compared between transgenic and non-transgenic Arabidopsis by semi-quantifying reverse transcription-polymerase chain reaction (RT-PCR), so that the expression level of endogenous Fd could be ascertained. The results revealed that the amplicon of the *PFLP* gene was only detected in the 25th and 30th cycles in *PFLP*-transgenic Arabidopsis, but was not detected in the non-transgenic line. In contrast, the amplicon intensities of both the *ATFD1* and *ATFD2* genes were the same in transgenic and non-transgenic Arabidopsis (Fig. 1C). This result shows that the expression levels of endogenous Fd genes are not altered by the expression of the *PFLP* gene in the transgenic lines. In order to compare the total protein amounts of photosynthetic-type Fd, the cured extracts of transgenic and non-transgenic Arabidopsis were detected by Western blot analysis using antiserum against PFLP. It was revealed that the *PFLP-1* and *PFLP-2* transgenic lines contained 2.11–2.86 more photosynthetic-type Fd relative to the non-transgenic line (Fig. 1D).

The location of photosynthetic-type Fd inside Arabidopsis was tracked by antiserum against PFLP that had been conjugated with fluorescein isothiocyanate (FITC) and was observed by confocal microscopy. In the confocal microscopy images, the green pseudo-coloured fluorescence corresponding to FITC revealed photosynthetic-type Fd, and the presence of thylakoids was clearly indicated by red pseudo-coloured autofluorescence. Figure 2A,B shows that the green fluorescence, representing Fd, is located in the inner thylakoids of non-transgenic Arabidopsis (Fig. 2C). However, Fd is detected at a higher intensity and with a wider distribution outside the thylakoids in the *PFLP*-transgenic lines (Fig. 2D–F).

Inoculation by ECC of *PFLP*-transgenic Arabidopsis

The transgenic plants were inoculated with ECC by two different methods in order to reveal PFLP-mediated resistance. In the intact plant analysis, ECC caused chlorotic and soft-rot symptoms in non-transgenic Arabidopsis at 72 h post-treatment (hpt), but this did not appear in the *PFLP*-transgenic lines (Fig. 3A,B). In the detached leaf assay, the leaf tissue of the non-transgenic line was macerated by ECC, but was intact in the *PFLP-1* and *PFLP-2* transgenic lines (Fig. 3C). The degree to which the leaves were macerated was estimated via the absorption of a mixture of leaf debris and bacterial suspension using a spectrophotometer at OD_{600} (optical density at 600 nm). The results revealed that absorption by the non-transgenic line reached 0.12 at 60 hpt, but only 0.022 in the two transgenic lines (Fig. 3D).

Activation of HR events in *PFLP*-transgenic Arabidopsis

The activation of HR events, such as electrolyte leakage, expression of HR marker genes and accumulation of H_2O_2, were compared between transgenic and non-transgenic Arabidopsis after ECC inoculation. The results revealed that inoculation with ECC induced the expression of HR marker gene, *ATHSR2*, in *PFLP*-transgenic Arabidopsis at 6–24 hpt, but not in the non-transgenic line. However, expression of the HR marker gene, *ATHSR3*, increased in *PFLP*-transgenic Arabidopsis at 6 hpt (Fig. 4A). Electrolyte leakage was also estimated after ECC inoculation. The results suggested that inoculation with ECC caused a 1.5-fold

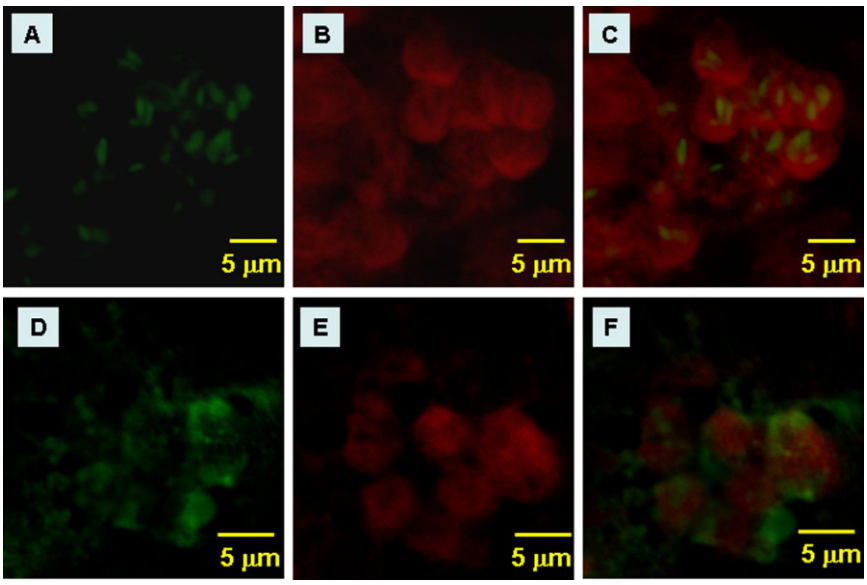

Fig. 2 Immunolocalization of plant ferredoxin-like protein (PFLP) in transgenic Arabidopsis. Images of the non-transgenic line (A–C) and transgenic line 2 (D–F) were obtained from leaf tissue of 1-month-old plants. The green pseudo-colour indicates the existence of protein recognized by the PFLP antiserum and fluorescein isothiocyanate (FITC) (A, D). The red pseudo-colour indicates chloroplast autofluorescence (B, E). The merged images show the localization of protein recognized by the PFLP antiserum (C, F). The bar represents 5 μm in length.

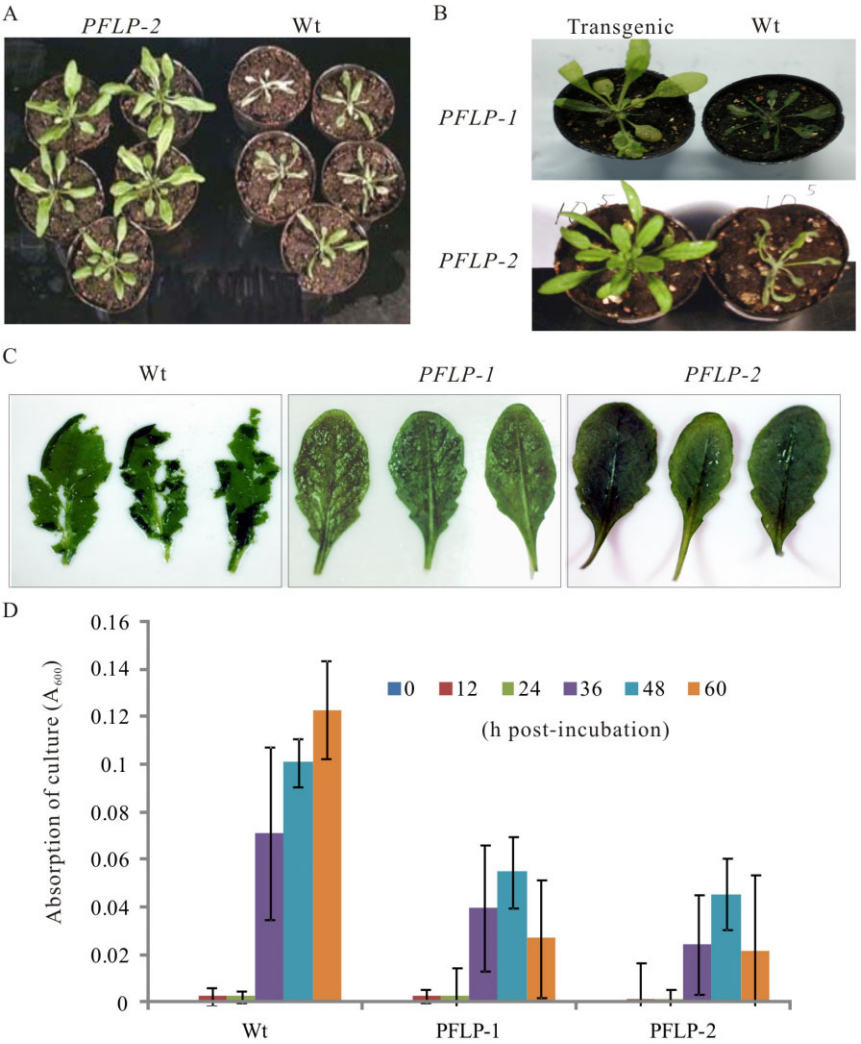

Fig. 3 Inoculation of *Erwinia carotovora* ssp. *carotovora* (ECC) in *plant ferredoxin-like protein* (*PFLP*)-transgenic Arabidopsis. The 1-month-old Arabidopsis plants were sprayed with ECC bacterial suspension [1×10^5 colony-forming units (cfu)/mL] and photographs were taken at 48 h (A) and 24 h (B) post-inoculation. The rosette leaves of 1-month-old Arabidopsis were detached and immersed in a suspension of ECC (1.0×10^3 cfu/mL) at 28 °C with shaking at 100 rpm. (C) Photograph taken at 24 h post-inoculation. The absorption of the suspension was estimated by a spectrophotometer at OD_{600} (optical density at 600 nm) at 0, 12, 24, 36, 48 and 60 h post-incubation. Data are presented as the mean ± standard error of the mean for six independent determinations (D). Wt, wild-type.

increase in electrolyte leakage in both of the *PFLP*-transgenic lines compared with the non-transgenic line between 16 and 36 hpt (Fig. 4B). H_2O_2 accumulation was estimated by titanium(II) sulphate ($TiSO_4$). The results showed that H_2O_2 accumulation inside the *PFLP*-transgenic lines increased at 8 hpt and then decreased between 8 and 24 hpt. The amount of H_2O_2 in the *PFLP*-transgenic lines increased again at 36 hpt, but then declined between 36 and 44 hpt. In non-transgenic Arabidopsis, H_2O_2 accumulation increased at 24 hpt, but decreased between 24 and 44 hpt (Fig. 4C). The H_2O_2 accumulation results were also confirmed by the detached leaf assay when the leaves were stained with polymerized 3,3'-diaminobenzidine (DAB) *in vivo* after ECC inoculation. The results showed that the brown precipitates stained by DAB were stronger in *PFLP*-transgenic Arabidopsis than in the non-transgenic line at 8 hpt. When treated with DPI, the brown precipitate produced no H_2O_2 in either transgenic or non-transgenic Arabidopsis (Fig. 4D).

Inhibitors, such as DPI, E64, z-VAD-fmk, myriocin and fumonisin, were also applied in order to investigate PFLP-mediated resistance by the detached leaf assay. The results showed that ECC caused soft-rot symptoms in non-transgenic Arabidopsis, but not in *PFLP*-transgenic Arabidopsis. When these inhibitors were present, the soft-rot symptoms caused by ECC increased in non-transgenic Arabidopsis. After treatment with DPI, E64 and z-VAD-fmk, the soft-rot symptoms caused by ECC in the *PFLP*-transgenic lines were as serious as those in the non-transgenic line, but were reduced when treated with myriocin and fumonisin at 24 hpt (Fig. 5).

Inoculation of *PFLP*-transgenic Arabidopsis with the harpin mutant strain ECC AC5082

PFLP was able to intensify the harpin-mediated HR. The harpin mutant strain, ECC AC5082, was used to infect *PFLP*-transgenic Arabidopsis. The results revealed that ECC AC5082 caused serious soft-rot symptoms in both *PFLP*-transgenic and non-transgenic Arabidopsis (Fig. 6A). Ion leakage was also compared between transgenic and non-transgenic Arabidopsis after ECC infection. The

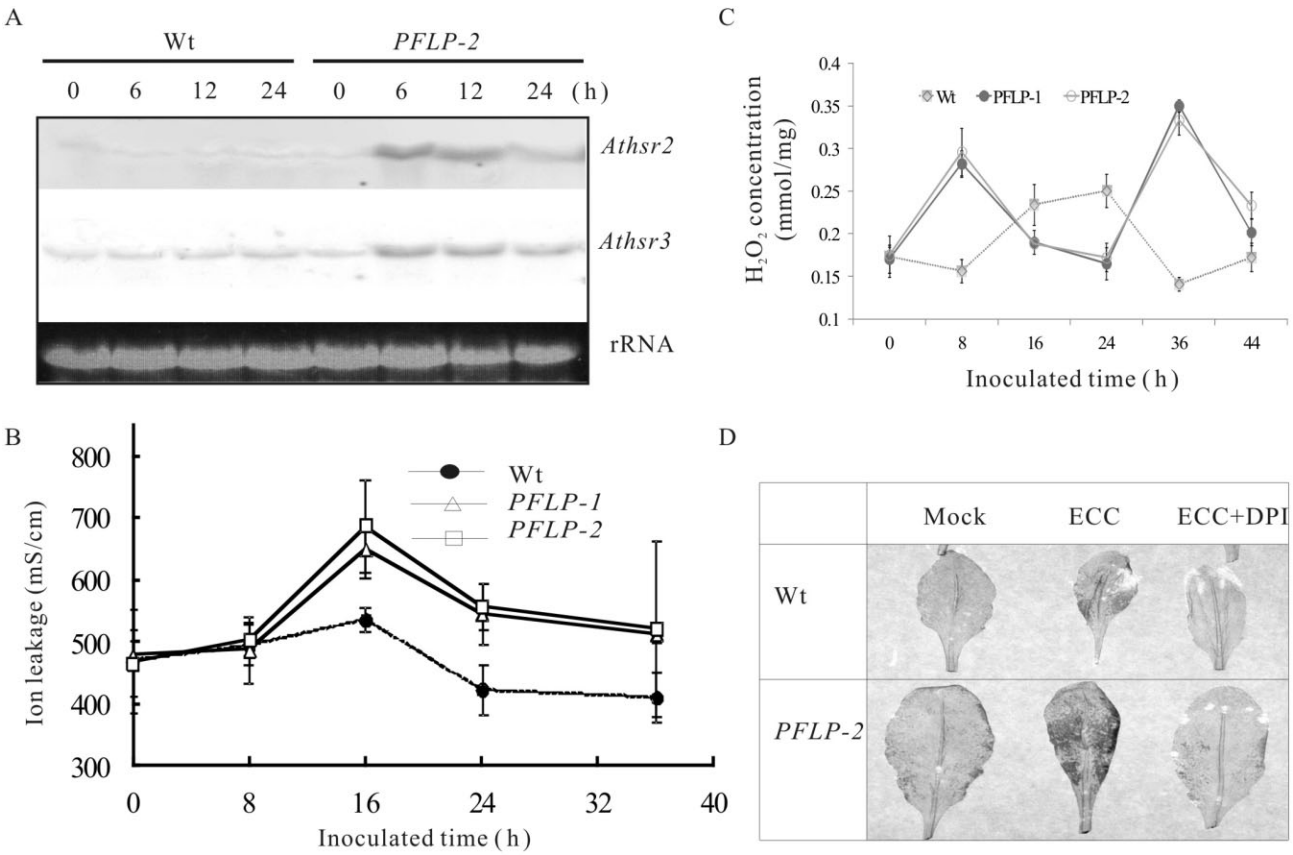

Fig. 4 The activation of the hypersensitive response (HR) in *plant ferredoxin-like protein* (*PFLP*)-transgenic Arabidopsis. RNA was extracted at 0, 6, 12 and 24 h post-inoculation of *Erwinia carotovora* ssp. *carotovora* (ECC) and investigated by Northern blot analysis with probes for the HR marker genes *ATHSR2* and *ATHSR3*. The ribosomal RNA (rRNA), stained by ethidium bromide (EtBr), was used as the loading control (A). The electrolyte leakage of plants was analysed at 0, 8, 16, 24 and 38 h post-inoculation by ECC (B). The accumulation of H_2O_2 in leaf tissue was estimated at 8, 16, 24 and 36 h post-inoculation by ECC. Data are presented as the mean ± standard error of the mean for six independent determinations (C). The accumulation of H_2O_2 in leaf tissue was assessed after staining by 3,3'-diaminobenzidine (DAB) *in vivo*. The detached leaves were immersed in phosphate buffer (Mock), ECC bacterial suspension [1.0×10^3 colony-forming units (cfu)/mL] (ECC) and bacterial suspension of ECC containing 10 μM diphenylene iodonium (ECC + DPI) for 8 h (D). Wt, wild-type.

results showed that the levels of ion leakage were similar after ECC inoculation (Fig. 6B). This suggests that inoculation with ECC AC5082 does not induce HR or PFLP-mediated resistance in *PFLP*-transgenic Arabidopsis.

PFLP-mediated resistance in defence-defective derivatives

The *PFLP* gene was transferred into defence-defective derivatives, such as *npr1*, the *eds1* mutant and *NAHG*-transgenic Arabidopsis, in order to investigate resistance to ECC by the detached leaf assay. The results revealed that soft-rot symptoms caused by ECC were reduced in the *PFLP*-transgenic lines (*PFLP-1* and *PFLP-2*) compared with the non-transgenic line. In the *npr1* mutant, ECC caused serious soft-rot symptoms, but the symptoms were reduced in the *npr1/PFLP* transgenic lines (*npr1/PFLP-1*, *npr1/PFLP-8* and *npr1/PFLP-9*) (Fig. 7A). The degree of maceration showed that absorption by the non-transgenic wild-type Arabidopsis was 0.1 at 48 hpt, but was suppressed below 0.005 in both the *PFLP-1* and *PFLP-2* transgenic lines. In the non-transgenic *npr1* mutant, the absorption was 0.15, but was suppressed below 0.075 in all three *npr1/PFLP* transgenic lines at 48 hpt (Fig. 7B). In *NAHG*-transgenic Arabidopsis, the soft-rot symptoms caused by ECC were also reduced in all three *NAHG/PFLP* double transgenic lines (*NAHG/PFLP-4*, *NAHG/PFLP-7* and *NAHG/PFLP-22*) (Fig. 7C). The maceration results revealed that the absorption of *NAHG*-transgenic Arabidopsis was 0.21 at 48 hpt, but was suppressed below 0.13 in all three *NAHG/PFLP* double transgenic lines (Fig. 7D). However, transformation of the *PFLP* gene did not reduce the soft-rot symptoms caused by ECC in the *eds1/PFLP* transgenic lines (*eds1/PFLP-3*, *eds1/PFLP-5* and *eds1/PFLP-7*) (Fig. 7E). The degree of maceration increased to 0.4–0.5 in all *eds1* samples and in the three *eds1/PFLP* transgenic lines at 48 hpt (Fig. 7F). These results revealed that overexpression of the *PFLP* gene was able to enhance disease resistance to ECC in the *npr1* mutant and *NAHG*-transgenic Arabidopsis, but not in the *eds1* mutant.

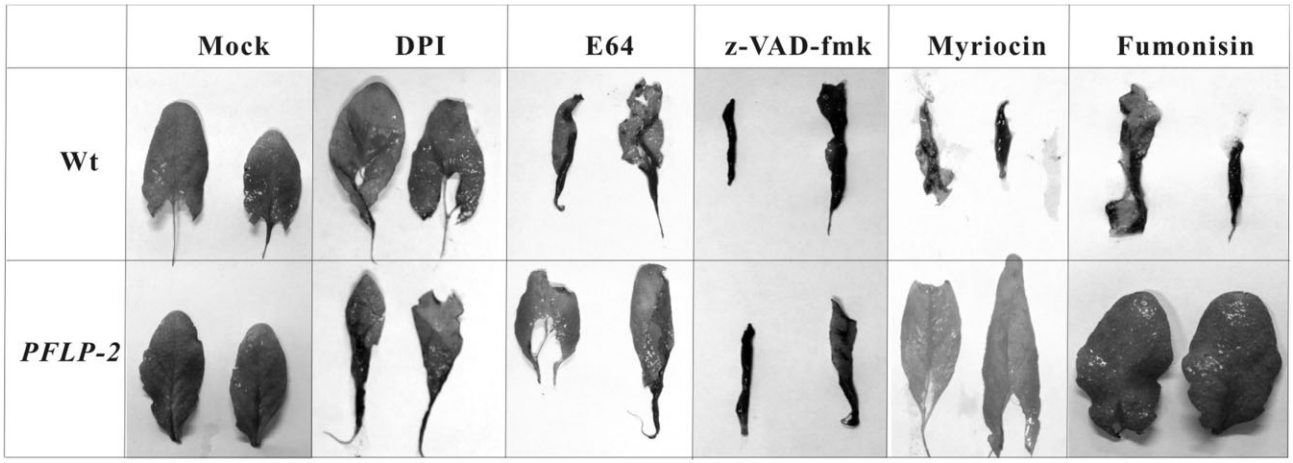

Fig. 5 The plant ferredoxin-like protein (PFLP)-mediated resistance was altered by inhibitors. The rosette leaves of non-transgenic (Wt) and transgenic (*PFLP-2*) Arabidopsis were immersed in a suspension of *Erwinia carotovora* ssp. *carotovora* (ECC) containing phosphate buffer (Mock), 10 μM diphenylene iodonium (DPI), 1-L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E64), benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), myriocin and fumonisin, and incubated at 28 °C and shaken at 100 rpm. The photograph was taken at 24 h post-incubation.

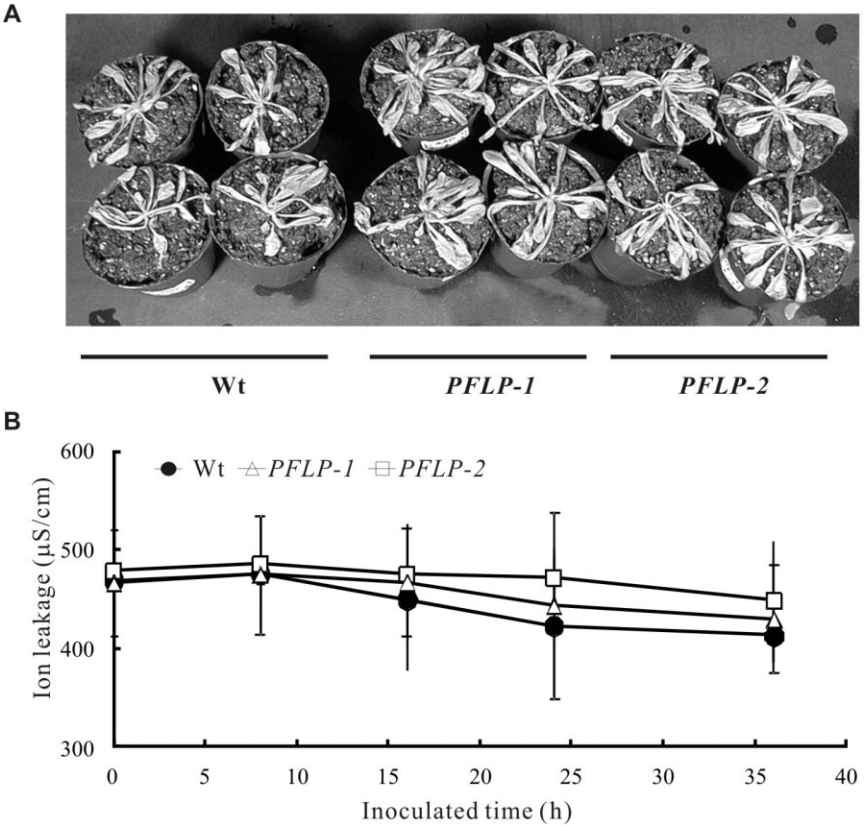

Fig. 6 Inoculation of the harpin mutant strain in the *plant ferredoxin-like protein* (*PFLP*)-transgenic plants. Non-transgenic Arabidopsis (Wt) and the transgenic lines (*PFLP-1* and *PFLP-2*) were sprayed with bacterial suspension of the harpin-defective strain, ECC AC5082 [1×10^5 colony-forming units (cfu)/mL]. The photograph was taken at 48 h post-inoculation (A). The electrolyte leakage of plants was analysed at 0, 8, 16, 24 and 36 h post-inoculation. Data are presented as the mean ± standard error of the mean for six independent determinations (B).

The levels of Fd in Arabidopsis were analysed by Western blot after treatment with SA, JA, harpin and ECC to determine whether PFLP exhibits defence properties against the pathogen. The results revealed that SA and harpin were able to increase Fd levels up to 2.8–5-fold at 24 hpt. However, Fd protein accumulation was reduced by both methyl jasmonate (MeJA) and ECC in the period 4–24 hpt (Fig 8A). To investigate the regulation of Fd in plant defence, the levels of Fd were determined in response to harpin and ECC in defence-defective derivatives. The results confirmed that ECC is able to reduce protein accumulation of Fd in both *eds1* and *pad4* mutants despite a slight delay, but not in the three *npr1* mutants and *NAHG*-transgenic Arabidopsis (Fig. 8B). Similar

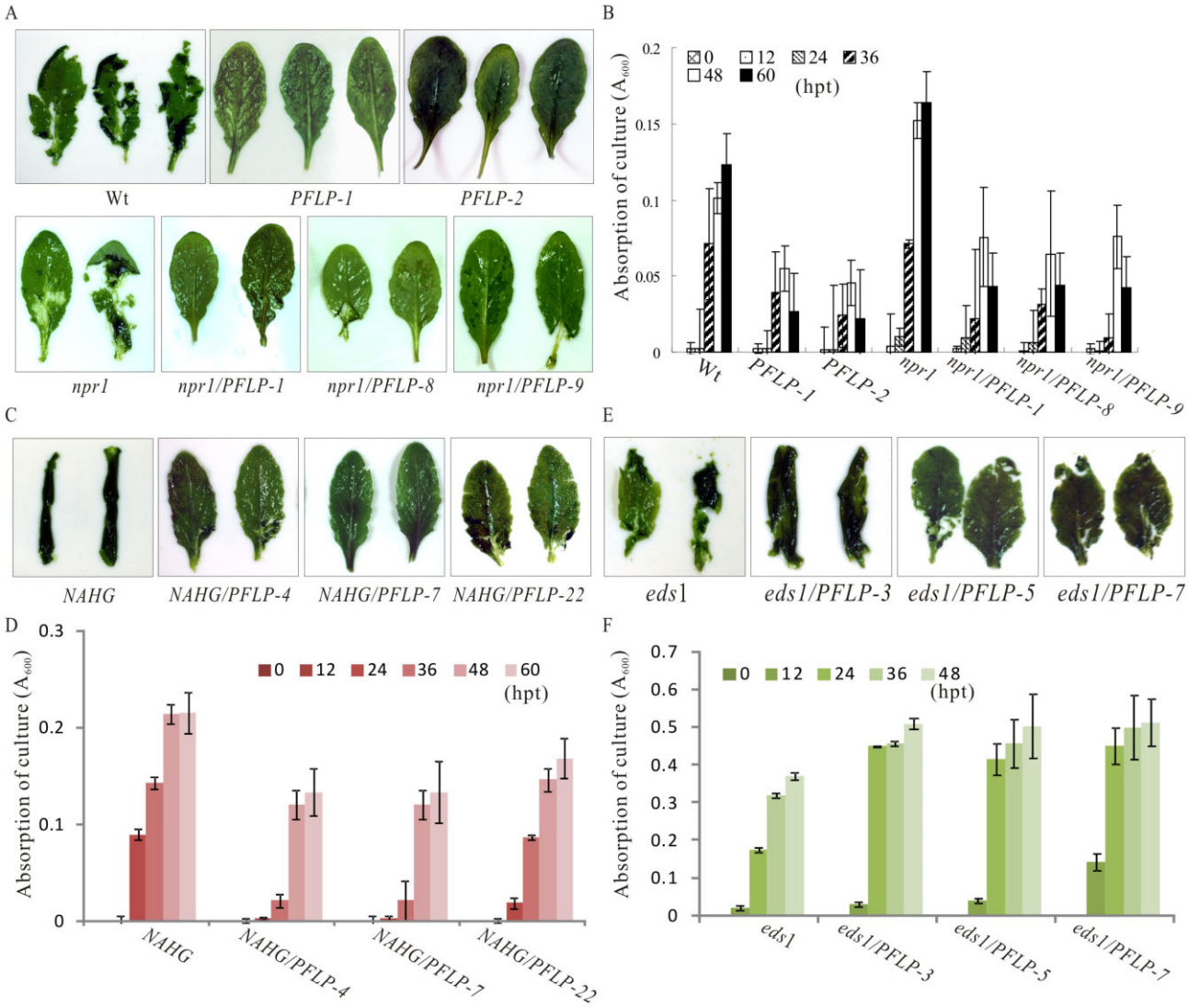

Fig. 7 Inoculation by *Erwinia carotovora* ssp. *carotovora* (ECC) of the *plant ferredoxin-like protein* (*PFLP*)-transgenic resistant-defective derivatives. The rosette leaves of non-transgenic Arabidopsis (Wt), *PFLP*-transgenic plants (*PFLP-1*, *PFLP-2*), the *npr1* (*nonexpresser of pathogenesis-related gene 1*) mutant and the *npr1/PFLP* transgenic lines (*npr1/PFLP-1*, *npr1/PFLP-8* and *npr1/PFLP-9*) were detached and immersed in a suspension of ECC. The photograph was taken at 24 h post-incubation (A). The detached leaves prepared from *NAHG*-transgenic Arabidopsis and its double transgenic lines (*NAHG/PFLP-4*, *NAHG/PFLP-7* and *NAHG/PFLP-22*) (C), or *eds1* (*enhanced disease susceptibility 1*) mutant and its *PFLP*-transgenic lines (*eds1/PFLP-3*, *eds1/PFLP-5* and *eds1/PFLP-7*) were treated as described previously (E). The degree of maceration was estimated using a spectrophotometer at OD_{600} (optical density at 600 nm). Data are presented as the mean ± standard error of the mean for six independent determinations (B, D, F). hpt, hours post-treatment.

results were also demonstrated by harpin treatment, which induced protein accumulation of Fd in both *eds1* and *pad4* mutants, but not in *npr1* mutants and *NAHG*-transgenic Arabidopsis (Fig. 8C).

DISCUSSION

Plants regulate the amount of Fd in response to environmental conditions, such as light, heavy metal levels, temperature and pathogen infection (Bhat *et al*., 2004; Caspar and Quail, 1993; Elliott *et al*., 1989; Huang *et al*., 2007a; John *et al*., 1997; Tognetti *et al*., 2006; Vorst *et al*., 1993). Recently, genome-wide analysis has also revealed that infection by pathogens changes Fd levels in Arabidopsis (Kim *et al*., 2006; Thilmony *et al*., 2006; Truman *et al*., 2006; de Vos *et al*., 2005). In this investigation, the *PFLP* gene was ectopically overexpressed in transgenic Arabidopsis when infected with the soft-rot pathogen ECC. The results revealed that the resistance of *PFLP*-transgenic Arabidopsis to ECC was accompanied by the activation of HR-associated events. However, this PFLP-mediated resistance was abolished by inhibitors, such as

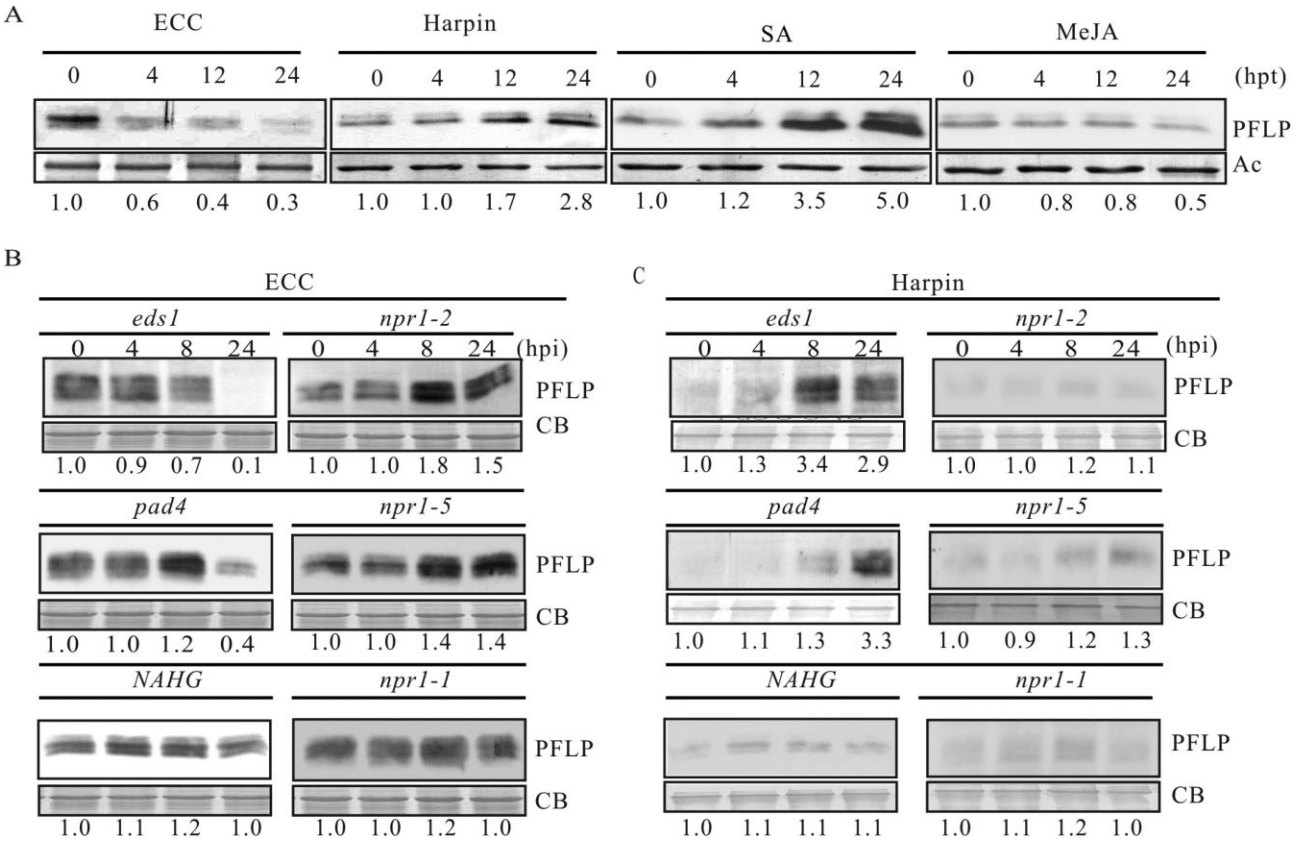

Fig. 8 The protein accumulation of ferredoxin (Fd) varied with salicylic acid (SA), methyl jasmonate (MeJA), harpin and *Erwinia carotovora* ssp. *carotovora* (ECC) treatment. The crude extract proteins (10 μg) isolated from treated plants were identified by Western blot analysis with antiserum against plant ferredoxin-like protein (PFLP) and monoclonal antiserum against actin (Ac) (A). The 10 μg of crude extracts of ECC-treated plants were identified by Western blot analysis with antiserum against PFLP (PFLP). The sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) stain with Coomassie brilliant blue (CB) was served as loading control (B). The 3 μg of crude extracts isolated from harpin-treated plants were identified as described previously (C). The number indicated is the relative ratio of the PFLP signal after normalization. hpi, hours post-inoculation; hpt, hours post-treatment.

DPI, E64 and z-VAD-fmk. The ECC strain defective in harpin lost the ability to induce resistance in *PFLP*-transgenic Arabidopsis. Furthermore, PFLP-mediated resistance only occurred in the *npr1* mutant and *NAHG*-transgenic Arabidopsis, but not in the *eds1* mutant.

The HR is an important mechanism that helps plants to resist pathogens by changing the metabolic pathways involved in the production of defence compounds. In this investigation, spontaneous HR was not observed in *PFLP*-transgenic Arabidopsis before pathogen inoculation. Similar results have also been demonstrated in previous investigations, in that transgenic plants overexpressing photosynthetic-type Fd did not cause a spontaneous HR: for example, Fd of cyanobacteria overexpressed in transgenic tobacco, Fd of pea overexpressed in transgenic tobacco (Ceccoli *et al*., 2011; Gallo-Meagher *et al*., 1992) and Fd of sweet pepper overexpressed in transgenic plants, such as Arabidopsis, tobacco, rice, calla lily, orchid and banana (Huang *et al*., 2004, 2006, 2007b; Liau *et al*., 2003; Lin *et al*., 2010; Namukwaya *et al*., 2012; Tang *et al*., 2001; Yip *et al*., 2007). These results demonstrate that the redundant Fd did not cause spontaneous HR in the transgenic plants directly.

The results from this investigation also revealed that HR events were activated in *PFLP*-transgenic Arabidopsis after infection by ECC, but did not occur in the non-transgenic line. Previous investigations have also shown that ECC or *P. syringae* pv. *tabaci* can induce the expression of the HR marker gene, *HSR203J*, and the accumulation of H_2O_2 in *PFLP*-transgenic tobacco (Huang *et al*., 2004). These results imply that certain elicitors provided by the pathogen may activate the HR in *PFLP*-transgenic plants. Many elicitors secreted by pathogens that induce plant basal immunity, via pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) or effector-triggered immunity (ETI), have been identified in previous studies (Senthil-Kumar and Mysore, 2013). Harpin, which is secreted by pathogens through the type III secretion system, has been shown to trigger ETI and the HR (Chang and Nick, 2012), and may be one of the elicitors involved in the

activation of PFLP-mediated resistance. Previous studies have revealed that recombinant PFLP is able to intensify the harpin-mediated HR in both tobacco and Arabidopsis (Dayakar *et al*., 2003; Huang *et al*., 2006; Lin *et al*., 2011). In this investigation, the results showed that only the ECC-containing intact harpin was able to induce resistance in *PFLP*-transgenic Arabidopsis. This suggested that harpin levels are regulated during the infection process, but are recognized by the overexpressed PFLP and intensify the HR-activating signal in *PFLP*-transgenic plants. However, harpin is not only an HR elicitor, but also a translocator that inputs virulent proteins into the plant cytoplasm, such as plant cell wall-degrading enzymes, necrosis-inducing protein (Terta *et al*., 2010) and an enhancer for the promotion of plant growth (Choi *et al*., 2013). The other possibility is that the activation of PFLP-mediated resistance may require certain virulent proteins that are delivered by harpin or the plant growth factor regulated by harpin.

In this investigation, inoculation of ECC caused the rapid accumulation of ROS in *PFLP*-transgenic Arabidopsis. We suggest that this phenomenon may be caused by NADPH oxidase in the plasma membrane or by an NO-dependent process in the cytoplasm, because they are both induced by harpin (Sang *et al*., 2012). This hypothesis was confirmed by the accumulation of ROS and the fact that disease resistance was abolished by DPI. DPI is able to reduce the enzyme activity of NADPH oxidase and other flavin-containing enzymes, such as NOS, xanthine oxidase, P-450 NADPH reductase, the mitochondrial respiratory chain complex I, cholinesterases and the internal Ca^{2+} pump (Tazzeo *et al*., 2009). However, the *PFLP* gene contains a putative chloroplast signal peptide for the import of protein into chloroplasts (Dayakar *et al*., 2003). To resolve this problem, the location of PFLP in transgenic Arabidopsis was traced by confocal microscopy after FITC staining. The results revealed that most of the redundant PFLP was detected outside the chloroplast in *PFLP*-transgenic Arabidopsis. Similar results have also been found in previous studies in which the overexpressed PFLP protein accumulated in the cytoplasm of transgenic tobacco (Huang *et al*., 2004). We suggest that this may have occurred because the putative chloroplast signal peptide of PFLP failed to work in these transgenic plants. The other possibility might be that certain unknown mechanisms could not transport the redundant PFLP, such as the translocon complexes at the outer (Toc) and inner (Tic) chloroplast envelope membrane, GTP-dependent receptors (Agne and Kessler, 2009; Andrès *et al*., 2010), the metabolic $NADP^+$/NADPH ratio (Stengel *et al*., 2009, 2010) or the HSP70 (heat shock protein 70) chaperones (Rial *et al*., 2003). In addition, previous studies have also shown that PFLP does not require the putative chloroplast signal peptide to intensify the harpin-mediated HR and disease resistance in *PFLP* transgenic plants (Huang *et al*., 2004; Lin *et al*., 2011). Based on these results, we suggest that the accumulation of ROS may be regulated by PFLP in the cytoplasm.

Plant innate immunity can be abolished by inhibitors. In this investigation, the inhibitors were used to estimate PFLP-mediated resistance. The results revealed that both E64 and z-VAD-fmk were able to abolish PFPL-mediated resistance in the detached leaf assay. However, myriocin and fumonisin were unable to do this. E64 is an inhibitor that can inactivate the cysteine proteinases (cathepsins B, H, L and papain) (Barrett *et al*., 1982), and z-VAD-fmk is a broad-spectrum cell-permeable caspase inhibitor (Sun *et al*., 1999; Yee *et al*., 2006). All of these are involved in protease-mediated cell death (Bonneau *et al*., 2008; Okita *et al*., 2007; Slee *et al*., 1996; Tiwari *et al*., 2002; Yang and Schnellmann, 1996). However, myriocin inhibits serine palmitoyltransferase and fumonisin is a mycotoxin produced by the fungal pathogen *Fusarium* spp., which inhibits the enzyme activity of sphinganine *N*-acyltransferase (Abbas *et al*., 1994; Takahashi *et al*., 2009). Both are inhibitors of the plant innate immunity that regulates plant resistance via sphinganine *N*-acyltransferase. Based on these results, we suggest that the activation of protease-mediated programmed cell death may be necessary for PFLP-mediated resistance, but not for sphingolipid-mediated immunity.

The results of this investigation revealed that PFLP-mediated resistance occurred in the *npr1* mutant and *NAHG*-transgenic Arabidopsis, but not in the *eds1* mutant. This suggests that PFLP is able to reverse the decrease in resistance caused by reduced SA or NPR1 protein accumulation. NPR1 is the key redox-controlled regulator of SAR in plants (Després *et al*., 2003). Previous studies have revealed that NPR1 interacts with the reduced form of TGA1, which targets the activation sequence-1 (as-1) element of the promoter region for defence proteins. Both NPR1 and TGA1 are *S*-nitrosylated after treatment with *S*-nitrosoglutathione, and the *S*-nitrosoglutathione protects TGA1 from oxygen-mediated modifications and enhances the DNA-binding activity of TGA1 in the presence of NPR1 (Lindermayr *et al*., 2010). Thus, it is reasonable to propose that the redundant PFLP is able to alter the status of TGA1-associated elements in the absence of NPR1, because the PFLP containing the iron–sulphur cluster contributes the high reductive potential needed to regulate the oxidative–reductive reaction. The other possibility is that PFLP-mediated resistance is independent of SA and the NPR1-regulated pathway, and therefore may work in the *npr1* mutant and *NAHG*-transgenic Arabidopsis. EDS1 is the other SA-signalling protein involved in the activation of basal immunity resistance and mutation in the *EDS1* gene. It usually reduces disease resistance, but not the HR (Durrant and Dong, 2004). The results of this study revealed that PFLP-mediated resistance was not activated in the *eds1* mutant. This suggests that the regulation of PFLP-mediated resistance may occur upstream of the EDS1 complex pathway. The other possibility is that PFLP-mediated resistance is independent of EDS1-mediated resistance, but is not sufficiently strong to make up for the reduced disease resistance caused by the *eds1* mutant. Further investigation into this subject is needed in the future.

EXPERIMENTAL PROCEDURES

Plant and bacterial materials

Arabidopsis thaliana var. Col-0, the Δ*eds1* mutant (SALK_057149), the Δ*npr*1-1 mutant (CS3726) and *NAHG*-transgenic Arabidopsis used in this investigation were provided by the Arabidopsis Biological Resource Center (ABRC). The *PFLP*-transgenic plants (*PFLP-1* and *PFLP-2*), the *NAHG/PFLP* double transgenic lines (*NAHG/PFLP-4*, *NAHG/PFLP-7* and *NAHG/PFLP-22*), the *eds1/PFLP* transgenic lines (*eds1/PFLP-3*, *eds1/PFLP-5* and *eds1/PFLP-7*) and the *npr1/PFLP* transgenic lines (*npr1/PFLP-1*, *npr1/PFLP-8* and *npr1/PFLP-9*) were generated by *A. tumefaciens* C58C1 (Clontech, Palo Alto, CA, USA) by the floral dip method (Clough and Bent, 1998), and contained the pBI121-SPFLP vector (Huang *et al*., 2004). These plants were cultivated in a growth chamber at 22 °C under a 16-h light/8-h dark cycle with irradiance at 48 μmol/m^2/s.

The soft-rot pathogen used to inoculate the plants was *Pe. carotovorum* ssp. *carotovorum* strain 71 (*E. carotovora* ssp. *carotovora*, ECC71) and the harpin-defective derivative was ECC AC5082 (Cui *et al*., 1996). These pathogens were cultured in a nutrient broth (NB) liquid medium (DIFCO, Le Pont de Claix, France) at 28 °C, and were shaken at 175 rpm for 1 day (OD_{600} = 0.7–1.2). The *A. tumefaciens* C58C1 incorporating the pBISPFLP plasmid (Huang *et al*., 2004) was incubated at 28 °C in YEB medium (5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl, 5 g/L sucrose and 0.5 g/L $MgSO_4.7H_2O$ at pH 7.0) containing 50 μg/mL kanamycin and 50 μg/mL rifamycin.

Generation of *PFLP*-transgenic Arabidopsis

The transformation of Arabidopsis was performed by the floral dip method (Clough and Bent, 1998) using *A. tumefaciens* C58C1 (Clontech) containing the pBI121-SPFLP vector. Arabidopsis with immature flower clusters (after 4 weeks of growth) was immersed in a bacterial suspension (OD_{600} = 0.6–1) of *A. tumefaciens* and AIM buffer [half-strength Murashige and Skoog (MS) basal medium salt, B5 vitamins, 0.01 mg/L benzylaminopurine, 500 mg/L 2-(*N*-morpholino)-ethanesulphonic acid, 5% sucrose and 0.02% Silwet-77 at pH 5.7]. The immersed plants were subjected to a vacuum pump for 15 min in a closed chamber and then kept in a high-moisture chamber for 24 h. The treated plants were then incubated in the growth chamber so that self-fertilized seeds could be collected. The subsequent transformed seeds were selected using MS medium containing 50 μg/mL kanamycin. The surviving plants were confirmed by PCR using B5-SPF5' (CGGGATCCCGATGGCTAGTGTCTCAGCTACCA) and S3-SPF3' (CGAGCTCG TTAGCCCACGAGTTCTGCTTCT) primers.

Southern blot analysis

Genomic DNA was extracted from the leaf tissue of 1-month-old Arabidopsis following the manufacturer's protocol (Qiagen, Hilden, Germany). The extracted DNA was digested with restriction enzymes *Eco*RI and *Sac*I (Roche, Mannheim, Germany) and electrophoretically separated on agarose gels. The DNA separated by the agarose gels was transferred to nylon membranes (Roche). The transferred membranes were hybridized at 65 °C using the full-length NPTII marker probe labelled by PCR using digoxigenin-11-dUTP (Roche). The membrane was washed after hybridization and the compounds were detected using a digoxigenin luminescent detection kit (Roche).

Northern blot analysis

Total RNA was isolated from the leaf tissue of 1-month-old Arabidopsis according to the manufacturer's protocol (Qiagen). Total RNA (15 μg) was electrophoresed in 1% agarose gel and then transferred to nylon membranes. The membrane was hybridized overnight at 68 °C with *PFLP*, *ATHSR2* and *ATHSR3* (Lacomme and Roby, 1999) gene probes labelled by PCR with digoxigenin-11-dUTP. After hybridization, the membrane was washed and the compounds were identified using a digoxigenin luminescent detection kit.

Semi-quantifying RT-PCR

Total RNA was isolated from the leaf tissue of 1-month-old Arabidopsis. RT-PCR was performed using 1 μg of total RNA and primers that were specific for the individual genes at an annealing temperature of 55 °C. The specific primers for the *ATFD1* gene were ATFD15' (ACACCAACTCAC TCACAAAATC) and ATFD13' (GCAACATTAGCGAAGTAACAAG). The specific primers for the *ATFD2* gene were ATFD25' (AAAATGGCTTCCAC TGCTC) and ATFD23' (TAGGTGAGGCTTAAACAATGTC). The specific primers for the *PFLP* gene were B5-SPF5' (CGGGATCCCGATGGCTA GTGTCTCAGCTACCA) and S3-PF3' (CGAGCTCGTTAGCCCACGAGTTCT GCTTCT). The primers used to amplify the gene for elongation factor 1α were ATEF1A5' (GCTGTCCTTATCATTGACTCCACC) and ATEF1A3' (TCATACCAGTCTCAACACGTCC). RT-PCR was stopped after 20 cycles, 25 cycles and 30 cycles, and was run in 1% agarose gel so that the intensity of the signal stained with ethidium bromide could be estimated.

Western blot analysis

The total protein was extracted from leaf tissue of 1-month-old Arabidopsis by homogenization (0.2 g/mL) with extraction buffer [50 mM Tris-HCl at pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 300 mM NaCl and 1 mM phenylmethylsulphonylfluoride (PMSF)]. The insoluble materials were removed by centrifugation (20 000 ***g***). The crude extracts were subjected to 15% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a poly(vinylidene difluoride) (PVDF) membrane (Perkin-Elmer, Woodbridge, ON, Canada) for Western blot analysis with antiserum against PFLP (Huang *et al*., 2004) and monoclone antiserum against the actin found in Arabidopsis (Sigma-Aldrich, Steinheim, Germany). The protein concentration was determined by Coomassie brilliant blue dye (Bio-Rad, Hercules, CA, USA).

FITC staining

The leaf tissue of 1-month-old Arabidopsis (2 mm width) was soaked in 50% ethanol at 4 °C for 24 h and washed twice with phosphate-buffered saline (PBS) solution at room temperature for 30 min. The sample was treated with 1% Cellulase R10 (Yakult, Japan) for 1 h, followed by 1% Triton X-100 in PBS at room temperature for 1 h in order to break down

the tissue. The sample was incubated with primary antibody (1:50) in PBS containing 1% bovine serum albumin (BSA), and then shaken gently overnight at room temperature. The sample was washed twice in PBS and then probed with FITC-conjugated goat anti-rabbit antibody (KPL, Gaithersburg, MD, USA; 1:80 in PBS containing 1% BSA) in the dark at room temperature for 2 h. The sample was then washed twice with PBS and placed on slides observed under a Zeiss LSM 510 confocal laser-scanning microscope.

ECC inoculation of *PFLP*-transgenic Arabidopsis

One-month-old Arabidopsis plants were sprayed with ECC bacterial suspension [1×10^6 colony-forming units (cfu)/mL] and incubated at 100% humidity for 24 h. The inoculated plants were then cultivated in a growth chamber at 22 °C under a 16-h light/8-h dark cycle with irradiance of 48 μmol/m^2/s. For the detached leaf assay, the rosette leaves of 1-month-old Arabidopsis were detached and immersed in a suspension of ECC (1.0×10^3 cfu/mL) with or without inhibitors, such as DPI (Sigma-Aldrich, Steinheim, Germany), E64 (Sigma-Aldrich, Steinheim, Germany), z-VAD-fmk (Sigma-Aldrich, Steinheim, Germany), myriocin (Sigma-Aldrich, St. Louis, MO, USA) and fumonisin (Sigma-Aldrich, St. Louis, MO, USA). The air pressure was reduced by a vacuum pump (0.5 Pa) for 15 min and then the sample was incubated at 28 °C and 100 rpm. The maceration caused by ECC was estimated using the method described previously (Huang *et al*., 2007a). The absorption of the cultured suspension was estimated using a UV-VIS spectrophotometer (U-2000, Hitachi) at OD_{600}.

Electrolyte leakage analysis

The leaves of inoculated plants were punched using a 4-mm-diameter leaf disc at different times post-inoculation. Three leaf discs from the same sample were placed in one tube with 2 mL of MilliQ-purified water and incubated at room temperature for 24 h. The conductivity was measured using a CDM230 digital conductivity meter (Radiometer Analytical SAS, Lyon, France).

Estimation of H_2O_2 by $TiSO_4$

The amount of H_2O_2 was estimated by $TiSO_4$. The leaf tissue from 1-month-old Arabidopsis (100 mg) was homogenized in 600 μL of phosphate buffer (50 mM phosphate buffer, pH 6.5; 10 mM 3-amino-1,2,4-triazole) in Eppendorf tubes. The homogenates were then centrifuged at 6000 ***g*** for 25 min to remove the residues. A $TiSO_4$ solution (0.2 mL at 0.1%) in 20% (v/v) H_2SO_4 was added to the supernatant and centrifuged at 6000 ***g*** for 15 min. The supernatant was observed using a UV-VIS spectrophotometer (U-2000, Hitachi) at a wavelength of 410 nm.

Estimation of H_2O_2 by DAB staining

DAB staining was performed as described by Torres *et al*. (2002). ECC-inoculated leaves were vacuum infiltrated with DAB staining solution (Sigma-Aldrich, Steinheim, Germany). The leaves were then placed in a plastic box under high humidity until a brown precipitate was observed (5–6 h); they were then fixed with a solution of 3:1:1 ethanol–lactic acid–glycerol.

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