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Plant ferredoxin-like protein enhances resistance to bacterial soft rot disease through PAMP-triggered immunity in *Arabidopsis thaliana*

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Abstract The protection of plants against bacterial disease is one of the important issues that need to be studied in agricultural applications. The application of a transgene, such as a gene that encodes plant ferredoxin-like protein (PFLP), to generate resistant plants is one possible strategy. Our previous reports have demonstrated that transgenic plants that express extracellular PFLP (ESF plants) are more resistant to bacterial pathogens. This protein intensifies the hypersensitive response (HR) in plants when they are infiltrated by a pathogen-associated molecular pattern (PAMP), harpin (HrpZ), from *Pseudomonas syringae*. In addition, this intensified HR is associated with the expression of membrane-bound NADPH oxidase. Thus, we attempted to determine the involvement of PFLP in intensifying PAMP-triggered immunity (PTI) to enhance disease resistance. First, we showed that transgenic *Arabidopsis* plants with the *pflp* gene were resistant to bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc). Then, the *fliC* gene which encoded flagellin from Pcc was cloned and expressed. The FliC protein was used in the functional study with PFLP in *Arabidopsis* Col-0 plants. The reactive oxygen species (ROS) generation and HR ratio were induced by the treatment with both PFLP and FliC together, but they were not induced by treatment with PFLP or FliC alone. Similar results were confirmed in ESF plants, where FliC elicited rapid ROS accumulation and callose deposition. Moreover, we demonstrated that the PFLP-intensified ROS generation and HR were related to Ca^{2+} influx and activation of NADPH oxidase. We concluded that the PFLP-intensified disease resistance is associated with the intensification of PAMP-triggered immunity.

Keywords Disease resistance · Flagellin · Plant ferredoxin-like protein (PFLP) · Plant immunity · Hypersensitive response (HR) · Reactive oxygen species (ROS)

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Introduction

Research on reducing crop loss due to bacterial infection is an important effort in agriculture. One of the strategies for protecting plants from pathogen attack is the application of a transgene such as *pflp* (Huang et al. 2006, 2007; Liau et al. 2003; Lin et al. 2010; Tripathi et al. 2010; Yip et al. 2007, 2011). Transgenic *Arabidopsis* plants with extracellular plant ferredoxin-like protein (PFLP) show strong resistance to *Ralstonia solanacearum*, and this resistance is associated with the expression of *AtRbohD* (Lin et al. 2010, 2011). RBOHD is a membrane bound NADPH oxidase that can produce reactive oxygen species (ROS) as a defence signal. One of the pathways to initialize this defence response can be achieved by the recognition of pathogen-associated molecular patterns (PAMPs) (Torres 2010; Torres et al. 2002). Previous studies have demonstrated that the HR induced by a PAMP, harpin

from *Pseudomonas syringae* pv. *syringae* (HrpZ), is intensified by PFLP (Dayakar et al. 2003; Lin et al. 2011). However, the role of harpin in plant immune systems is still controversial. Harpin is a PAMP (or an elicitor protein) which is secreted from type three secretion systems by many pathogenic bacteria, including *Erwinia*, *Pseudomonas*, *Ralstonia* spp., and *Xanthomonas* spp. (Chen et al. 2008; Galán and Collmer 1999; Galán and Wolf-Watz 2006; Postel and Kemmerling 2009; Wei et al. 1992). When bacterial harpin is delivered to the surface of plant cells, it eventually results in effector-triggered immunity (ETI)-like HR (Guan et al. 2013). Therefore, whether PFLP participates in PAMP-triggered immunity requires further research and discussion.

The PAMP-triggered immunity occurs when plants sense bacteria via recognition of bacterial PAMPs by pattern-recognition receptors (PRRs) located on the cell surface (Abramovitch et al. 2006; Zhang and Zhou 2010; Zipfel 2008). The recognition of one bacterial PAMP, flagellin, in *Arabidopsis* and *Solanaceae* plants has been well demonstrated by the binding of flagellin and a PRR, FLAGELLIN-SENSING 2 (FLS2) (Chinchilla et al. 2006; Felix et al. 1999; Robatzek et al. 2007). The FLS2 recognizes bacterial flagellin to initiate immune responses, including calcium influx, ROS production, callose deposition, and activation of mitogen-associated protein kinase (MAPK) pathway (Lu et al. 2011; Luna et al. 2011; Robatzek and Wirthuneller 2012).

In this study, we demonstrated that the extracellular PFLP enhances disease resistance against bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc). In order to develop greater insight regarding how the PFLP participates in PAMP-triggered immunity to confer disease resistance, the flagellin encoded by the *fliC* gene from Pcc was cloned and expressed in this study. The ROS generation, callose deposition and defence signals induced by $flagellin_{Pcc}$ were further analyzed in *Arabidopsis* plants with extracellular PFLP. We demonstrated that the disease resistance enhanced by PFLP was associated with PAMP-triggered immunity in *Arabidopsis thaliana*.

Materials and methods

Plants and bacteria growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) and transgenic *Arabidopsis* plants with extracellular PFLP (ESF) (Lin et al. 2010) were used in this study. To prepare all plant materials, seeds were sown in sterilized peat moss for 1 . The seedlings were then transferred to individual pots for another 3 weeks. The 4-week-old plants were used to analyze the plants' immune responses and pathogen challenge. The plants were grown in a growth chamber at 22 °C (16 h light/8 h dark).

A strain of *Pectobacterium carotovorum* subsp. *carotovorum* Ecc17, which causes bacterial soft rot in vegetables and *Arabidopsis thaliana* Col-0, was used in this study. The fresh single colony of Ecc17 was transferred on a nutrient broth agar plate (NA) and further incubated at 28 °C for 48 h before the experiments were conducted. *Escherichia coli* strains DH5α and BL21 (DE3) were grown at 37 °C in Luria-Bertani (LB) broth or on LB agar plates containing 100 μg/ml of ampicillin.

Disease severity assay

Disease severity assay of soft rot on *Arabidopsis* plants was performed with detached leaves. Briefly, cells of *Pectobacterium carotovorum* subsp. *carotovorum* Ecc17 were washed from NA with sterilized distilled water and adjusted to 0.3 at OD_{600} as a stock bacterial suspension. The 100-fold diluted stock bacterial suspension was prepared as inoculum. Detached leaves of *Arabidopsis* Col-0 and ESF plants were placed in inoculation suspension and vacuumed at 200 mmHg for 15 min. The inoculated leaves were then incubated in a growth chamber at 22 °C for 48 h. The indexes of soft rot symptoms were rated from 0 to 4 (0: no symptom; 1: watery or soft rot area ratio of 0 to 25 %; 2: soft rot area ratio of 25 to 50 %; 3: soft rot area ratio of 50 to 75 %; 4: soft rot area ratio of 75 to 100 %). The total number (N) of leaves was then counted with the rated indexes. The disease severity for each trial was calculated with the following formula: $[(0\times N0 + 1\times N1 + 2\times N2 + 3\times N3 + 4\times N4)/(4\times N)] \times 100\%$. Each disease severity was collected from five leaves as one repeat; a total of three repeats were performed for statistical analysis in this assay.

Plasmid construction for FliC expression

To express the FliC of *Pectobacterium carotovorum* subsp. *carotovorum* Ecc17, an expression vector, pET16b (Novagen, USA), which can express the recombinant protein with a 6× His tag at N-terminal, was used in this study. The coding sequence of the *fliC*

gene was amplified with specific primers designed from the sequence of the *fliC* gene (AB205025) from Pcc EC1 (Hossain et al. 2005). Then, the coding sequence of the *fliC* gene was subcloned in pET16b to generate pET16b-FliC. After the pET16b-FliC was transformed into *Escherichia coli* BL21 (DE3), a transformant (ExFliC) with pET16b-FliC was confirmed by sequencing. The confirmed ExFliC was used to express FliC, and a transformant (Ex16B) with pET16b vector alone was used as control. The expression of FliC in ExFliC1 was performed in LB broth with 1 mM of isopropylthio-β-D-galactoside at 37 °C for 8 h. The cells of ExFliC1 were then collected and sonicated in 25 mM Tris–HCl (pH7.0) for 1 h to obtain the protein extract. The protein extracts with 0.5 μg of protein were fractionated in 15 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to an Immun-Blot® PVDF Membrane (Bio-Rad, USA). The western blot was performed by using Anti-6x-His rabbit polyclonal antiserum (1:3,000) specific for 6x-His tag and HRP-conjugated goat anti-rabbit IgG (1:3000). The immuno-signals were then colored with a Metal Enhanced DAB Substrate Kit (Thermo, USA).

Hypersensitive response and H_2O_2 accumulation assay

To assess whether the hypersensitive response (HR) could be induced by co-infiltrating PFLP and flagellin, PFLP protein purification and leaf infiltration were carried out following the previously described protocol (Lin et al. 2011). Briefly, the protein extraction of FliC and purified PFLP were mixed at 0.5 mg/ml of the final concentration. The mixture was then punctured with a syringe into six inoculation sites in one plant as one repeat. The HR ratio was calculated as (Nn/6)×100 % at 24 h post-inoculation, where Nn is the number of inoculation sites with HR necrosis. Thirty plants were analyzed as replications in this assay. In order to observe H_2O_2 accumulation, the 16 h post-inoculated leaves were detached and stained with 5 mM of 3,3'-diaminobenzindine (DAB) at pH 3.8 (Sigma, USA) for 4 h under dark. Then the stained leaves were washed in 95 % ethanol, after which the appearance of brown precipitates was used as the indication of H_2O_2 accumulation. In order to detect rapid H_2O_2 generation, the observation was performed by fluorescence microscopy. Leaves of *Arabidopsis* plants were stained with 20 μM 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, USA) in PBS buffer after the FliC protein was infiltrated for 1 h. The leaves were stained under dark for 15 min and were further washed three times with PBS buffer. The leaves were observed under a fluorescence microscopy with an excitation at 465–495 nm and emission at 515–555 nm filter set (Leica, Germany).

Observation of callose accumulation

Callose deposition is an indicator that demonstrates the activation of immune response triggered by PAMPs (Luna et al. 2011). To observe callose deposition, the *Arabidopsis* leaves were collected at 8 h post-infiltration. First, the leaves were washed with 95 % ethanol until all tissues became transparent. The transparent leaves were then washed in 0.1 M phosphate buffer (pH8.0). Next, the leaves were incubated with 0.01 % aniline-blue (sigma, USA) in 0.1 M phosphate buffer for 2 h prior to microscopic analysis. The leaves were observed under a fluorescence microscope with an excitation at 340–380 nm and emission at 400–425 nm filter set (Leica, Germany).

Inhibition of plant defense pathway

In the plant immune system, calcium influx and activation of NADPH oxidase are early hallmarks for reactive oxygen species formation and activation of defence response (Torres 2010). In order to determine whether the PFLP-intensified HR was associated with the PAMP-triggered immune signals, inhibition of calcium influx and NADPH oxidase in the HR occurrence were assayed. Inhibition of calcium influx and NADPH oxidase was performed by using lanthanum chloride ($LaCl_3$) and diphenyleneiodonium chloride (DPI), respectively (Dayakar et al. 2003). The infiltration was carried out with 0.5 mg/ml of FliC and PFLP mixture with 80 μM of $LaCl_3$ or DPI. The HR appearance at the inoculation sites was then calculated to evaluate the HR ratio at 24 h post-infiltration. Thirty plants were assayed in each treatment as replicates in this assay.

Results

Appearance of soft rot on *Arabidopsis* plants

To assess whether the transgenic *Arabidopsis* ESF plants with extracellular PFLP were resistant to bacterial

soft rot, the disease severities caused by *Pectobacterium carotovorum* subsp. *carotovorum* Ecc17 were used. Lower disease severities would indicate that these plants have higher disease resistance. The results showed that the disease severity of ESF plants (31.7 %) was lower than that of Col-0 plants (76.7 %) at 3-day post-inoculation (Fig. 1a). Compared to the severe soft rot symptoms which appeared on inoculated leaves of Col-0 plant, most leaves of ESF plants were symptomless at 3-day post-inoculation (Fig. 1b).

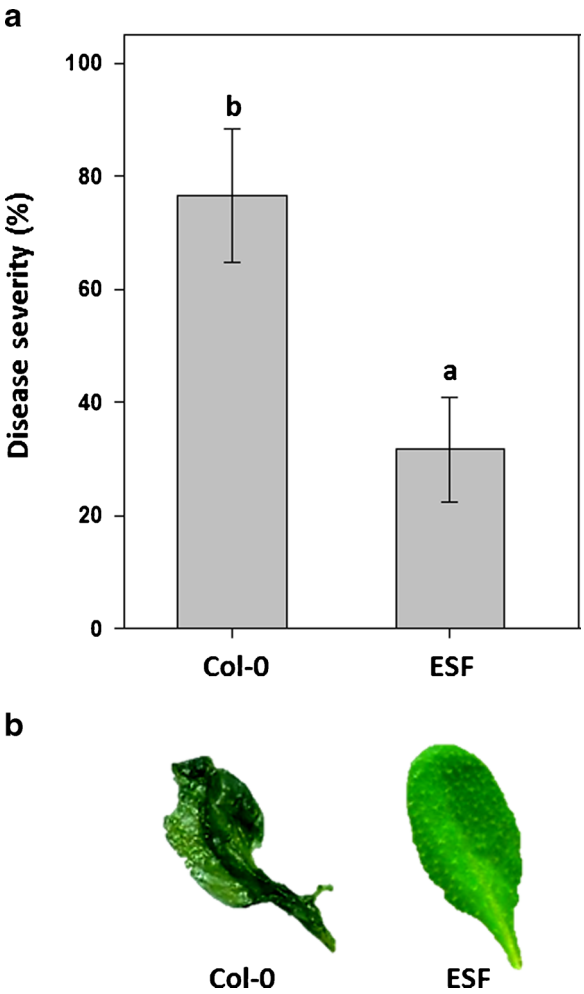

Fig. 1 Disease severities and soft rot symptoms of *Arabidopsis* plant caused by *Pectobacterium carotovorum* subsp. *carotovorum*. Leaves of four-week-old plants were inoculated with *P. carotovorum* subsp. *carotovorum* Ecc17. Panel a illustrates the disease severities exhibited in the appearance of soft rotting leaves at 48 h post inoculation, while Panel b illustrates the bacterial soft rot symptoms caused by Ecc17 on Col-0 and ESF plants. The different letters above the columns indicate significant differences based on Tukey-Kramer test ($P<0.05$)

FliC-mediated HR with PFLP in *Arabidopsis* plants

In order to clarify the role that the PAMP of *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) plays in PFLP-intensified disease resistance, the *fliC* gene which encodes a flagellin was amplified from Ecc17. The DNA sequence of the *fliC* gene from Ecc17

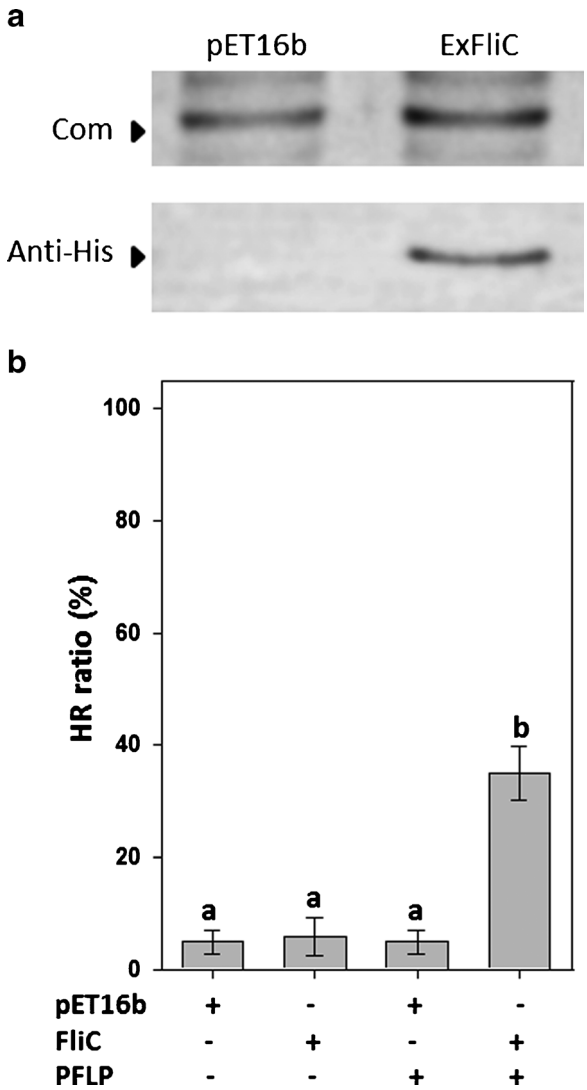

Fig. 2 Hypersensitive response (HR) assay of FliC in *Arabidopsis* Col-0 plants. Panel a reveals the expression of FliC in *Escherichia coli* BL21. One microgram of total protein was separated and stained with coomassie blue (Com). The separated proteins were detected by anti-histidine antibody (Anti-His). Panel b shows that the FliC-mediated HR was induced by PFLP. Protein mixtures were infiltrated into the leaves, and the ratio of induced HR was calculated at 24 h after infiltration. The different letters above the columns indicate significant differences based on Tukey-Kramer test ($P<0.05$)

(JX261932) was identical (99 %) to that from EC1 (AB205025). The coding sequence of the *fliC* gene was first constructed into pET16b and then further transformed into *E. coli* BL21 (DE3) in order to obtain ExFliC. The ExFliC transformant was used to express FliC. Compared to the crude protein extract from Ex16B, N-terminal His tagged FliC was detectable with anti-6×His antiserum in western blot analysis. In addition, the signal of FliC was visualized at 30 kDa (Fig. 2a). The protein extract was then used in FliC-induced HR assay. The results revealed that the FliC did not induce HR while PFLP protein was absent. However, the HR appeared noticeably in the infiltration of FliC while PFLP protein was present (Fig. 2b).

FliC-mediated HR in transgenic *Arabidopsis* plants

Because the PFLP protein is required for intensifying FliC-mediated HR, its effect was confirmed by evaluating the H_2O_2 accumulation and degree of HR in transgenic *Arabidopsis* plants with extracellular PFLP (ESF plants). Compared to the infiltration of pET16b, FliC did not induce H_2O_2 accumulation in *Arabidopsis* Col-0 plants at 16 h post-infiltration. However, the dark brown precipitate was clearly detected by infiltrating FliC in ESF plants (Fig. 3a). This result indicated that the H_2O_2 accumulation could be induced by FliC in ESF plants. In the HR assay, the results showed that the HR was not induced by FliC in Col-0 plant. Compared to the results obtained with the ESF plants, the HR was induced more significantly by FliC than by crude protein extract from Ex16B (Fig. 3b).

Reactive oxygen species generation and callose deposition in transgenic *Arabidopsis* plants

The ROS generation induced by FliC was observed at 1 h post-infiltration. The result demonstrated that the ROS signals were induced by FliC around the cells in Col-0 and ESF plants. In addition, FliC induced more abundant ROS in ESF plants than it did in Col-0 plants (Fig. 4a). The callose deposition induced by FliC was observed. The results revealed that a small quantity of callose deposition was induced by FliC in Col-0 plants. In addition, more abundant callose deposition was observed in ESF plants (Fig. 4b).

Inhibition of FliC-mediated HR by $LaCl_3$ and DPI

In order to provide details regarding the role PFLP plays in FliC-mediated HR, the roles of calcium channel and NADPH oxidase were discussed in transgenic ESF plants. Results revealed that the intensification of PFLP in FliC-mediated HR was abolished by calcium channel blocker, $LaCl_3$. Similarly, this HR was inhibited by the inhibitor of NADPH oxidase, DPI (Fig. 5).

Discussion

Improving the disease resistance of plants can be achieved by generating transgenic plants with the *pflp*

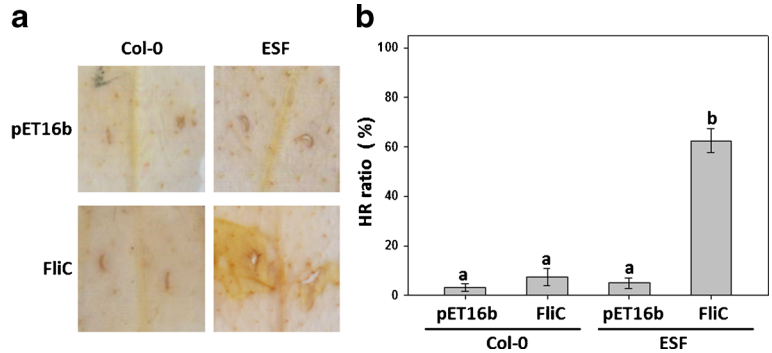

Fig. 3 FliC-mediated reactive oxygen species accumulation and hypersensitive response in *Arabidopsis* plants. The FliC protein was infiltrated into the leaves of transgenic ESF plants with extracellular PFLP and non-transgenic Col-0 plants. Reactive oxygen species accumulation was observed at 16 h post-infiltration. HR necrosis was observed to calculate the HR ratio at 24 h post-infiltration. Panel a shows the H_2O_2 accumulation revealed by DAB staining. Panel b indicates that the HR ratios of Col-0 and ESF plants responded to FliC. The different letters indicate significant differences based on Tukey-Kramer test ($P<0.05$)

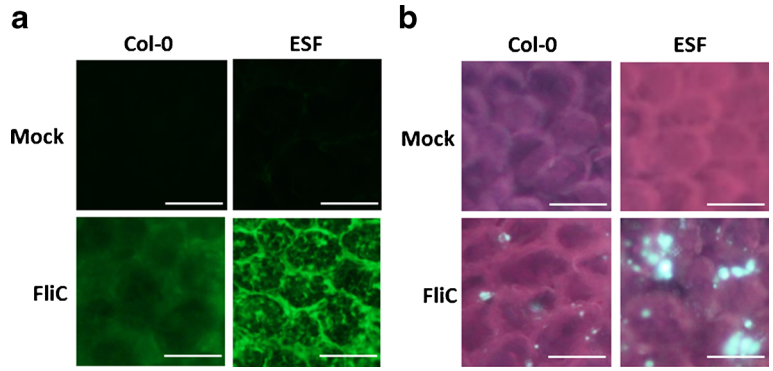

Fig. 4 FliC-mediated reactive oxygen generation and callose deposition in *Arabidopsis* plants. The FliC protein at 0.5 mg/ml was infiltrated into the leaves of transgenic ESF and non-transgenic Col-0 plants. Mock indicates the infiltration of 25 mM Tris–HCl as a negative control. Rapid ROS generation and callose deposition were observed at 1 and 8 h post-infiltration, respectively. Panel a shows the observation of rapid ROS generation. The infiltrated leaves were collected and stained with 20 μM of H_2DCFDA. Panel b reveals the observation of callose deposition. The leaves were stained with 0.01 % aniline-blue. The bars indicate 50 μm in length

gene (Huang et al. 2006, 2007; Liau et al. 2003; Lin et al. 2010; Tripathi et al. 2010; Yip et al. 2007, 2011). Transgenic *Arabidopsis* ESF plants with extracellular PFLP exhibit strong resistance against bacterial wilt caused by *Ralstonia solanacearum* (Lin et al. 2010). In this study, we also demonstrated that the ESF plants were resistant against *Pectobacterium carotovorum* subsp. *carotovorum*. These results suggested that efficient resistance against bacterial pathogens could be provided by the extracellular PFLP in transgenic plants. Therefore, possible mechanisms of this protein involved in intensifying disease resistance need to be further investigated. Recent studies reveal an excellent model to describe disease resistance by activating plant immunity (Boller and Felix 2009; Davidsson et al. 2013; Jones and Dangl 2006; Postel and Kemmerling 2009; Zipfel 2008). Previous studies have shown that the disease resistance intensified by PFLP is associated with a pathogen-associated molecular pattern (PAMP), harpin (Lin et al. 2010, 2011). Although a harpin binding site was found on tobacco plasma membrane, the receptors and pathways involved in the defence response still remain unclear (Lee et al. 2001). In addition, a recent report demonstrated that HR cell death induced by harpin is more similar to the manner of effector-trigged immunity (Chang and Nick 2012). In order to develop greater insight into the PFLP-intensified disease resistance, a clear model can be used to study the possible mechanisms involved in PFLP. The defence responses of flagellin/FLS2 system is a well-established model in PAMP-triggered immunity (Gómez-Gómez and Boller 2002; Zipfel et al. 2004).

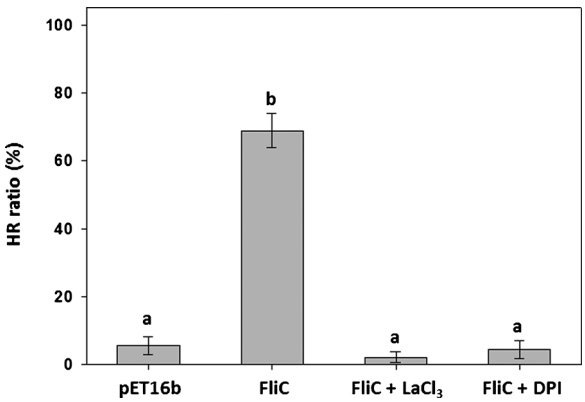

Fig. 5 The effects of Ca^{2+} channel blocker and NADPH oxidase inhibitor on FliC-mediated HR in transgenic *Arabidopsis* ESF plants. Inhibition of Ca^{2+} channel and NADPH oxidase was performed with lanthanum chloride ($LaCl_3$) and diphenyleneiodonium chloride (DPI), respectively. HR necrosis was observed to calculate the HR ratio at 24 h post-infiltration. The different letters above the columns indicate significant differences based on Tukey-Kramer test ($P<0.05$)

Flagellin encoded by *fliC* is assembled in bacterial flagella, and it is a protein which has the potential to elicit hypersensitive cell death in incompatible plants (Che et al. 2000; Shimizu et al. 2003; Taguchi et al. 2003). Nevertheless, the flagellin of *Erwinia carotovora* subsp. *carotovora* may not play a significant role in inducing HR in tobacco leaves (Hossain et al. 2005). Our results showed that neither HR nor ROS accumulation were significantly induced by FliC from *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) in *Arabidopsis* Col-0 plants. However, the HR

and ROS accumulation was significantly induced by FliC in the existence of PFLP. The contribution of PFLP in intensifying FliC-mediated HR was demonstrated with recombinant protein and in transgenic *Arabidopsis* plants. Since the occurrence of HR is an effective mechanism associated with plant disease resistance during pathogens infection (Mehdy 1994), we therefore suggested that the PFLP-mediated disease resistance against Pcc was associated with the PTI. We observed rapid ROS generation; furthermore, callose deposition induced by FliC from Pcc was significantly intensified in transgenic plants with extracellular PFLP. In flg22-triggered response, rapid ROS generation and callose deposition are indicators of activating a defense response (Göhre et al. 2012; Luna et al. 2011; Muthamilarasan and Prasad 2013; Robatzek and Wirthuneller 2012). Therefore, these results support the correlation between PFLP and PAMP-triggered immunity. In addition, the signal transduction of the defence response elicited by elicitors relies on ion flux across plasma membranes, such as calcium influx (Ebel and Mithöfer 1998; Umemura et al. 2002). Our results demonstrated that Ca^{2+} was involved in PFLP-intensified defence reactions triggered by flagellin. An increase in cytosolic Ca^{2+} is required for Rboh protein (NADPH oxidase) activation to produce ROS for further HR occurrence (Lebrun-Garcia et al. 1999; Torres 2010). The results revealed that the HR triggered by FliC was abolished by DPI, NADPH oxidase inhibitor, in transgenic plants with PLFP. Our previous studies also demonstrate that PFLP-mediated HR triggered by harpin is associated with the expression of an NAPDH oxidase gene (*AtRbohD*) (Lin et al. 2010, 2011). Therefore, we suggested that PFLP may act upstream in calcium influx to enhance ROS generation and HR occurrence in flagellin-triggered defence responses. In order to identify the possible mechanisms of PFLP-intensified disease resistance against other bacterial pathogens, it would be worthwhile to conduct further investigations regarding the defense responses elicited by flg22 from other bacterial pathogens. Taken together, we concluded that PFLP-mediated disease resistance against bacterial soft rot pathogen was associated with the intensification of PAMP-triggered immunity.

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