PFLP-Intensified Disease Resistance Against Bacterial Soft Rot Through the MAPK Pathway in PAMP-Triggered Immunity

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Accepted for publication 2 July 2018.

ABSTRACT

Bacterial soft rot is a devastating disease affecting a variety of vegetable crops worldwide. One strategy for controlling this disease could be the ectopic expression of the plant ferredoxin-like protein (*pflp*) gene. PFLP was previously shown to intensify pathogen-associated molecular pattern-triggered immunity (PTI), an immune response triggered, for example, by the flagellin epitope flg22. To gain further insight into how PFLP intensifies PTI, flg22 was used as an elicitor in *Arabidopsis thaliana*. First, PFLP was confirmed to intensify the rapid generation of H₂O₂, callose deposition, and the hypersensitive response when coinfiltrated with flg22. This response correlated with increased

expression of the FLG22-induced receptor kinase 1 gene, which is part of the mitogen-activated protein kinase (MAPK) pathway. Although the increased response to flg22 alone did not depend on the MAPK pathway genes *MEKK1*, *MKK5*, and *MPK6*, the protective effect of PFLP decreased when plants mutated in these genes were inoculated with *Pectobacterium carotovorum* subsp. *carotovorum*. Furthermore, expression of *PR1* and *PDF1.2* also increased upon treatment with flg22 in the presence of PFLP. Taken together, these results suggest that activation of the MAPK pathway contributes to the increased resistance to bacterial soft rot observed in plants treated with PFLP.

Bacterial soft rot disease caused by *Pectobacterium carotovorum* subsp. *carotovorum* is a limiting factor in vegetable production worldwide (Pérombelon and Kelman 1980). This bacterium produces large quantities of pectic enzymes to macerate the tissues of vegetables and ornamental plants (Hauben et al. 1998; Pérombelon and Kelman 1980). To control bacterial soft rot, transgenic plants that express a plant ferredoxin-like protein (*pflp*) gene are resistant to this disease. The PFLP-mediated resistance has been demonstrated in tobacco (*Nicotiana tabacum*), *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), calla lily (*Zantedeschia elliptica*), and *Oncidium* orchids (Huang et al. 2004, 2007; Liao et al. 2003; Su et al. 2014; Yip et al. 2007). The resistance associated with the reactive oxygen species (ROS) generation and hypersensitive response (HR) triggered by pathogen elicitors such as harpin from *Pseudomonas syringae* pv. *syringae* is intensified by PFLP (Dayakar et al. 2003; Lin et al. 2010, 2011). Harpin, flagellin, peptidoglycan, and elongation factor Tu are pathogen-associated molecular patterns (PAMP) found in plant-pathogenic bacteria that are recognized by distinct specific receptors outside plant cells to trigger PAMP-triggered immunity (PTI) (Gust et al. 2007; Oh and Beer 2005; Postel and Kemmerling 2009). However, a report indicates that HR responses induced by harpin are similar to the responses of effector-triggered immunity based on the observation of cytoskeleton disruption (Guan et al. 2013). To clarify whether the PFLP-mediated disease resistance is associated with the enhancement of PTI, the flagellin from *Pectobacterium carotovorum* subsp. *carotovorum* was used to demonstrate that PFLP confers disease resistance through intensifying PTI responses (Su et al. 2014).

PTI is a plant innate defense response triggered by the recognition of PAMP recognized by pattern recognition receptors (PRR) on the plasma membrane of host plant cells to further activate defense pathways (Dodds and Rathjen 2010). In the example of flagellin, this protein is the bacterial surface structure for motility. On flagellin, flg22 is the fragment which consisted of the most conserved 22 amino acid residues at the N-terminal region (Felix et al. 1999). The flg22 specifically binds to the leucine-rich repeat domain of a PRR on plant cell membranes such as flagellin-sensing 2 (FLS2) found in *Arabidopsis thaliana* (Chinchilla et al. 2006; Gómez-Gómez and Boller 2002). This recognition further induces defense pathways such as Ca²⁺ influx, rapid ROS generation, and the activation of calcium-dependent protein kinase and mitogen-activated protein kinase (MAPK) pathways (Ali and Reddy 2008; Boudsocq et al. 2010; Monaghan and Zipfel 2012; Segonzac and Zipfel 2011). Moreover, the activation of the MAPK cascade (MEKK1, MKK4/MKK5, and MPK3/MPK6) induced by flg22 is regarded as a key factor for further conferring resistance against plant pathogens (Asai et al. 2002). Therefore, the intensification of PTI to enhance disease resistance may be associated with the activation of the MAPK cascade.

In this study, we sought to clarify whether PFLP-intensified disease resistance was due to the activation of a PTI-mediated MAPK pathway. The flg22_{Pst} from *Pseudomonas syringae* pv. *tabaci* was used as an elicitor (Felix et al. 1999). In the flg22-triggering defense response, induction of the FLG22-induced receptor kinase 1 (*FRK1*) gene is specific to the activation of MAPK pathways (Asai et al. 2002; Boudsocq et al. 2010). Thus, the expression of *FRK1* induced by flg22_{Pst} upon treatment with PFLP was assayed. To further realize the role of MAPK pathways in PFLP-mediated disease resistance, resistance to bacterial soft rot disease intensified by PFLP on MAPK mutants of *A. thaliana* was evaluated.

MATERIALS AND METHODS

Growth conditions of plants and bacteria. *A. thaliana* ecotype Columbia (Col-0), a transgenic *Arabidopsis* ESF plant which overexpressed the extracellular PFLP (Lin et al. 2010), the *mekk1* mutant (SALK_069473C), the *mkk5* mutant (SALK_047797C),

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Funding: This work was supported by grants to Dr. Yi-Hsien Lin from the Ministry of Science and Technology, Taiwan (MOST-104-2311-B-020-001 and MOST 105-2628-B-020-002-MY3).

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and the *mpk6* mutant (SALK_062471C) were used in this study. The plants were grown in a growth chamber (Hipoint) at 22°C with 16 h of light and 8 h of darkness. The 4-week-old plants were used as materials to analyze plant immune responses, gene expression, and pathogen challenge. The soft rot bacterium *Pectobacterium carotovorum* subsp. *carotovorum* Ecc17 was used in the inoculation

TABLE 1. Quantitative real-time polymerase chain reaction primers used in this study

Gene	Sequence (5'–3')	Reference
<i>FRK1</i>	F: CGGTGAGATTTCAACAGTTGTC	Boudsocq et al. 2010
	R: AATAGCAGGTTGGCCTGTAATC	
<i>MPK6</i>	F: GCGGCTCCATCGCCTCAGAT	Kannan et al. 2012
	R: ACGATGCCATAAGCACCTTGC	
<i>PR1</i>	F: TTCTTCCCTCGAAGCTCAA	Göhre et al. 2012
	R: AAGGCCACACAGAGTGTATG	
<i>PDF1.2</i>	F: TTGCTGCTTTTCGACGCA	Brown et al. 2003
	R: TGTCCCACTTGGCTTCTCG	
<i>TUB4</i>	F: AGGGAAACGAAGACAGCAAG	Boudsocq et al. 2010
	R: GCTCGTAATCTACCTTTGG	

assay. Before inoculation, Ecc17 was transferred on a nutrient broth agar plate and incubated at 28°C for 48 h.

Preparation of flg22_{Pst} and PFLP. In this study, flg22_{Pst} (TRLSSGLKINSKDDAAGLQIA) from *Pseudomonas syringae* pv. *tabaci* was synthesized by LifeTein LLC. The synthesized flg22_{Pst} was prepared in 25 mM Tris-HCl buffer (pH 7.5) at 5 mM before assay. PFLP was obtained from *Escherichia coli* (Lin et al. 2011). Briefly, the crude extract of recombinant protein was purified in a column containing Ni-NTA (GE Healthcare). Subsequently, the eluted protein was desalted with a centrifugal filter tube (Amicon Ultra). Finally, the purified PFLP was dissolved in 25 mM Tris-HCl buffer.

HR assay. Our previous studies showed that the strength of weak defense responses triggered by PAMP was intensified by PFLP to achieve the occurrence of HR postinfiltration (Dayakar et al. 2003; Lin et al. 2010, 2011; Su et al. 2014). To assess whether the HR could be induced by coinfiltrating of PFLP and flg22_{Pst}, mixtures of PFLP and flg22_{Pst} were prepared at 0.5 mg/ml and 0.5 μM, respectively. The mixture was then assayed on *Arabidopsis* plants following the previously described protocol (Lin et al. 2010).

Fig. 1. Effects of protein extract of plant ferredoxin-like protein (PFLP) on callose deposition upon flg22_{Pst} treatment in *Arabidopsis thaliana* plants. **A** and **B**, Images and fluorescent intensities of callose deposition with the infiltration of flg22_{Pst}, PFLP, or a mixture of flg22_{Pst} and PFLP in *Arabidopsis thaliana* ecotype Columbia (Col-0) plants. Final concentrations of flg22_{Pst} and PFLP in treatments were 50 nM and 0.05 mg/ml, respectively. **C** and **D**, Images and fluorescent intensities of callose deposition by infiltration of 50 nM of flg22_{Pst} in Col-0 and ESF (overexpresses the extracellular PFLP) plants. Infiltrated leaves were collected at 8 h postinfiltration and stained with 0.01% aniline blue to observe callose deposition. Bar indicates 50 μm in length. Fluorescent intensities of callose deposition were counted by ImageJ. Different letters above the bars indicate significant differences according to Tukey's honestly significant difference test ($P < 0.05$).

Similarly, infiltration carried out with six inoculation sites in one plant was regarded as one repeat. The HR ratio was calculated as $(Nn/6) \times 100\%$ at 24 h postinfiltration, where Nn is the number of inoculation sites with HR necrosis. Thirty plants were assayed as replications in one assay.

Callose staining. To observe callose deposition, the infiltrated *Arabidopsis* leaves were collected at 8 h postinfiltration. Leaf infiltration was performed with flg22_{Pst} and PFLP at 50 nM and 0.05 mg/ml, respectively. Control plants were infiltrated with 25 mM Tris-HCl (pH 7.5) or PFLP at 0.05 mg/ml alone. The collected leaves were soaked in 95% ethanol overnight and further stained with 0.01% aniline blue (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 8) for 2 h. Callose deposition was observed under a fluorescence microscope filter set (Leica) with an excitation at 340 to 380 nm and an emission at 400 to 425 nm. The strength of callose deposition was determined using the Image J software (<https://imagej.nih.gov/ij/>) to measure the fluorescent intensity under a consistent threshold (Rasband 1997-2016). Each value was collected from 10 samples of individual leaves in each treatment as repeated. The data were analyzed by Tukey's honestly significant difference test at a 5% significance level.

Quantitative real-time polymerase chain reaction. To monitor the changes in gene expression levels, the infiltrated *Arabidopsis* leaves were collected at 0, 0.5, 4, and 24 h postinfiltration. The infiltration of flg22_{Pst} and PFLP was carried out at 0.5 μ M and 0.5 mg/ml, respectively. Total RNA was extracted from collected infiltrated leaves using a Total RNA Mini Kit (Geneaid). Total RNA (1 μ g) was used to synthesize the cDNA with the Transcriptor First-Strand cDNA Synthesis Kit (Roche). Quantitative real-time polymerase chain reaction (PCR) was performed with 200 ng of cDNA and 500 nM each gene-specific primer in iQ SYBR Green supermix reagent (Bio-Rad). Specific primers for quantitative real-time PCR shown in Table 1 were used, and the program was performed as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 10 s and 50°C for 30 s. Analysis of the melting curve was conducted to identify the specificity of products. The expression of *TUB4* was used as a reference gene, and data were analyzed using CFX Manager 3.0 (Bio-Rad). The fold induction of each treatment was normalized by mock treatment at 0 h postinfiltration. At least nine samples of each treatment were analyzed as replicates in this assay.

Disease severity assay. Bacterial soft rot disease was assayed in *Arabidopsis* plants. Before inoculation, bacterial suspension of Ecc17 was prepared to an optical density at 600 nm of 0.3 (10^8 CFU/ml). The 100-fold dilution of bacterial suspension (10^6 CFU/ml) was prepared as an inoculating suspension. Inoculation was performed by infiltrating with a mixture of the inoculating suspension and PFLP at 1 mg/ml at equal volume. The infiltrated plants were incubated in a growth chamber at 25°C for 24 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%, and 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: $[(0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4) / (4 \times N)] \times 100\%$. Each disease severity was collected from one plant as one repeat, and nine repeats were performed for statistical analysis in this assay.

RESULTS

Callose deposition induced by flg22_{Pst} in *Arabidopsis* plants. The callose deposition induced by flg22_{Pst} was observed at 8 h postinfiltration. Among the results, fluorescent signals indicating callose deposition were induced by flg22_{Pst} surrounding the cells in Col-0 plants. Furthermore, more abundant callose deposition was observed in the infiltration with the mixture of flg22_{Pst} and PFLP recombinant protein (Fig. 1A). Compared with the treatment with flg22_{Pst} alone, the fluorescent intensity was increased by 8.3-fold in the treatment with flg22_{Pst} and PFLP (Fig.

1B). No signals were observed in the blank treatment or that with PFLP recombinant protein alone. To observe the callose signals induced by flg22_{Pst} in transgenic plants with PFLP, ESF plants were assayed. The results indicated that abundant callose deposition was induced by flg22_{Pst} in ESF plants (Fig. 1C). The fluorescent intensity triggered by flg22_{Pst} in the ESF plant was 7.1-fold higher than that in the Col-0 plant (Fig. 1D). However, in ESF plants (similar to what occurred in Col-0 plants), no callose deposition was observed in the infiltrations without flg22_{Pst}.

HR induced by flg22_{Pst} in *Arabidopsis* plants. To clarify whether or not the PTI was intensified by PFLP in *A. thaliana*, the HR was evaluated by infiltrating flg22_{Pst} as a PAMP. Compared with the negative control, PFLP or flg22_{Pst} alone did not induce the HR response (Fig. 2A). However, an HR ratio was significantly induced by the mixture of PFLP and flg22_{Pst}. Compared with the results in ESF plants, the HR ratio was induced more strongly by flg22_{Pst} than that by the blank treatment (Fig. 2B).

Expression of *FRK1* gene in flg22_{Pst}-induced response intensified by PFLP. In order to clarify the role of PFLP in the activation of MAPK pathways induced by flg22_{Pst}, the expression of the *FRK1* gene was monitored in Col-0 plants after infiltration. Compared with the blank treatment, *FRK1* was not induced by

Fig. 2. Effects of protein extract of plant ferredoxin-like protein (PFLP) on flg22_{Pst}-induced hypersensitive response (HR) in *Arabidopsis* plants. **A**, HR induced by infiltration with flg22_{Pst}, PFLP, or mixture of flg22_{Pst} and PFLP in Col-0 plants. **B**, HR induced by infiltration with 0.5 μ M flg22_{Pst} in *Arabidopsis* Col-0 and ESF plants. Final concentrations of flg22_{Pst} and PFLP in treatments were 0.5 μ M and 0.5 mg/ml, respectively. HR ratios in *Arabidopsis* plants were recorded at 24 h postinfiltration. More than 30 plants of each treatment were analyzed as replicates in this assay. Different letters indicated significant differences between treatments based on Tukey's honestly significant difference test ($P < 0.05$), and an asterisk indicates significant differences based on a *t* test ($P < 0.05$).

flg22_{Pst} or by the treatment with flg22_{Pst} combined with PFLP (i.e., the mixture) at 0.5 h postinfiltration. At 4 and 24 h postinfiltration, the relative expression levels of *FRK1* induced by flg22_{Pst} were 19.7- and 26.2-fold, respectively. This induction at 4 and 24 h postinfiltration was significantly increased by PFLP to 42.1- and 68.6-fold, respectively (Fig. 3A). Compared with the blank treatment, *MPK6* was not induced by flg22_{Pst} or by flg22_{Pst} combined with PFLP at 0.5 and 4 h postinfiltration. At 24 h postinfiltration, however, the relative expression levels of *MPK6* were significantly induced by the flg22_{Pst} and the mixture to 2.7- and 2.9-fold, respectively. In addition, PFLP did not increase the expression level of *MPK6* at 24 h postinfiltration (Fig. 3B).

PFLP-intensified disease resistance in *Arabidopsis* mutants. To assess whether the PFLP still intensified resistance to bacterial soft rot in *Arabidopsis* mutants via the MAPK pathway, the disease severities caused by *Pectobacterium carotovorum* subsp. *carotovorum* Ecc17 were evaluated on *mekk1*, *mkk5*, and *mpk6* plants. The results indicated that the disease severity on Col-0 plants was reduced from 56.6 to 16.7% by PFLP. Compared with the severe soft rot symptoms on inoculated leaves, most leaves were symptomless in the treatment with PFLP at 24 h postinoculation. However, the intensification of disease resistance against bacterial soft rot by PFLP was abolished in *mekk1* (49.6%), *mkk5* (53.1%), and *mpk6* (48.9%) plants (Fig. 4).

Fig. 3. Effects of recombinant protein extract of plant ferredoxin-like protein (PFLP) on the expression of FLG22-induced receptor kinase 1 (*FRK1*) and *MPK6* transcripts upon flg22_{Pst} treatment in *Arabidopsis thaliana* Col-0. **A**, Expression of *FRK1*. **B**, Expression of *MPK6*. Four-week-old leaves were infiltrated with flg22_{Pst} or a mixture of flg22_{Pst} and PFLP. Infiltrated leaves were collected at 0, 0.5, 4, and 24 h postinfiltration to analyze gene expression of mRNA. Fold induction of each treatment was normalized by mock treatment at 0 h. At least three plants of each treatment were analyzed as replicates in this assay. An asterisk indicates significant differences compared with mock treatment based on a *t* test ($P < 0.05$). Double asterisks indicate significant differences compared with flg22_{Pst} treatments based on a *t* test ($P < 0.05$).

Fig. 4. Effects of protein extract of plant ferredoxin-like protein (PFLP) against bacterial soft rot in *Arabidopsis* mutants. Leaves of 4-week-old *mekk1*, *mkk5*, and *mpk6* plants were infiltrated with *Pectobacterium carotovorum* subsp. *carotovorum* Ecc17 at 10^6 CFU/ml as a control. Experiments were carried out with the mixture of Ecc17 and PFLP at 10^6 CFU/ml and 1 mg/ml, respectively. Infiltrated leaves were observed at 24 h postinfiltration to analyze disease severity. At least nine infiltrated leaves of each treatment were analyzed as replicates in this assay. An asterisk indicates significant differences based on a *t* test ($P < 0.05$).

PFLP-intensified callose deposition in *Arabidopsis* mutants. To ascertain whether the PFLP-intensified callose deposition was affected by the MAPK pathway, it was also observed in *mekk1*, *mkk5*, and *mpk6* mutants. The results indicated that the callose signals were induced by the infiltration of flg22_{Pst} around the cells of these mutants (Fig. 5A). The fluorescent intensities of callose deposition induced by flg22_{Pst} were intensified by PFLP to 5.5-, 6.5-, and 6.6-fold in *mekk1*, *mkk5*, and *mpk6* mutants, respectively (Fig. 5B).

PFLP-intensified defense signals in salicylic acid and ethylene/jasmonate pathways. In order to further understand the association between PFLP-mediated disease resistance and signals in induced resistance, the expression of the pathogenesis-related 1 (*PR1*) gene in the salicylic acid pathway and *PDF1.2* gene in the ethylene/jasmonate pathway were evaluated. In the salicylic acid pathway, *PR1* was not induced by the treatment with flg22_{Pst} or by the treatment with flg22_{Pst} combined with PFLP (i.e., the mixture) at 0.5 and 4 h postinfiltration. However, the relative expression level of *PR1* was induced by flg22_{Pst} to 104.5-fold and was strongly intensified by PFLP to 1,066.5-fold at 24 h postinfiltration (Fig. 6A). In the ethylene/jasmonate pathway, the relative expression levels of the *PDF1.2* gene were significantly induced by flg22_{Pst} at 0.5, 4, and 24 h postinfiltration. Similarly, it was significantly intensified by PFLP to 10.1-fold at 24 h postinfiltration (Fig. 6B).

DISCUSSION

The fact that PFLP enhances disease resistance against bacterial diseases was well documented (Ger et al. 2014; Huang et al. 2007; Liao et al. 2003; Lin et al. 2010, 2011; Namukwaya et al. 2012; Tang et al. 2001; Yip et al. 2007). Our previous study showed that the

Fig. 5. Effects of recombinant protein extract of plant ferredoxin-like protein (PFLP) on callose deposition upon flg22_{Pst} treatment in *Arabidopsis* mutants. **A**, Callose deposition was performed with the infiltration of flg22_{Pst}, PFLP, or the mixture of flg22_{Pst} and PFLP in *mekk1*, *mkk5*, and *mpk6* plants. Infiltrated leaves were collected at 8 h postinfiltration and further stained with 0.01% aniline blue to observe callose deposition. Bar indicates 50 μ m in length. **B**, Fluorescent intensities of callose deposition count by ImageJ. Different letters above the bars indicate significantly different according to Tukey's honestly significant difference test ($P < 0.05$).

enhanced disease resistance against bacterial soft rot may be associated with the PTI activated by flagellin (Su et al. 2014). Such activation of plant innate immune responses by flagellin is due to the fact that its conserved region, flg22, is recognized by FLS2 on the plasma membrane of plant cells (Chinchilla et al. 2006; Denoux et al. 2008; Monaghan and Zipfel 2012; Zipfel 2008). In this study, the callose deposition and HR ratio were increased by PFLP upon the infiltration of flg22_{Pst}. The results induced by flg22_{Pst} are similar to those induced with the flagellin from *P. carotovorum* subsp. *carotovorum* (Su et al. 2014). Compared with the amino acid sequences of flg22 from various bacterial pathogens, the flg22s found in different genera of plant-pathogenic bacteria exhibit conservation of their sequences. In addition, the flg22_{Pcc} from

P. carotovorum subsp. *carotovorum* was shown to be 81.8% identical to the flg22_{Pst} from *Pseudomonas syringae* pv. *tabaci* (Fig. 7). Therefore, we suggest that the PTI triggered by flagellin could be intensified by PFLP.

The MAPK pathway is one of the main routes by which plant defense responses are activated during PTI (Ali and Reddy 2008; Asai et al. 2002; Denoux et al. 2008; Jones and Dangl 2006; Nicaise et al. 2009; Zhang and Zhou 2010). To demonstrate in greater detail how PFLP acts upon the MAPK pathway, the expression level of *FRK1* was assayed as an indicator (Asai et al. 2002; Boudsocq et al. 2010). Here, we demonstrated that the expression of *FRK1* was induced by flg22_{Pst} and that this induction could be further intensified by PFLP. This increase in the expression level of *FRK1* is

Fig. 6. Effects of recombinant protein extract of plant ferredoxin-like protein (PFLP) on the expression of *PRI* and *PDF1.2* transcripts upon flg22_{Pst} treatment in *Arabidopsis thaliana* Col-0. **A**, Expression of *PRI*. **B**, Expression of *PDF1.2*. Four-week-old leaves were infiltrated with flg22_{Pst} or a mixture of flg22_{Pst} and PFLP. Infiltrated leaves were collected at 0, 0.5, 4, and 24 h postinfiltration to analyze gene expression of mRNA. Fold induction of each treatment was normalized by mock treatment at 0 h. At least three plants of each treatment were analyzed as replicates in this assay. An asterisk indicates significant differences compared with mock treatment based on a *t* test ($P < 0.05$). Double asterisks indicate significant differences compared with flg22_{Pst} treatments based on a *t* test ($P < 0.05$).

Fig. 7. Amino acid sequences of flg22 in flagellin of phytopathogenic bacteria. *Pcc*, *Pst*, *Xcc*, and *Rs* indicate *Pectobacterium carotovorum* subsp. *carotovorum* Ecc17 (JX261932), *Pseudomonas syringae* pv. *tabaci* 6605 (AB061230), *Xanthomonas campestris* pv. *campestris* B305 (DQ356465), and *Ralstonia solanacearum* (AF283285), respectively. Asterisks indicate the conserved sequences in these bacteria.

accompanied by the phosphorylation of MPK3/MPK6 by flg22_{Pst} (Asai et al. 2002). However, PFLP did not intensify the expression level of MPK6 induced by flg22_{Pst}. Therefore, we suggest that PFLP may contribute to the intensification of signal transduction rather than to that of gene induction in the MAPK pathway.

The activation of the flg22-mediated MAPK pathway through the signaling of MEKK1-MKK4/MKK5-MPK3/MPK6 leads to the expression of defense genes (Ali and Reddy 2008; Asai et al. 2002; Muthamilarasan and Prasad 2013). In this study, the results indicated that the PFLP-mediated disease resistance against bacterial soft rot induced via the MAPK pathway was abolished in all *Arabidopsis* mutants. However, the flg22_{Pst}-induced callose deposition was still intensified by PFLP in these mutants. Callose is accumulated during pathogen penetration on cell walls, and overexpression of its synthase in transgenic *Arabidopsis* results in successful suppression of the penetration of powdery mildew (Ellinger et al. 2013). However, the abundant callose deposition in *mekk1*, *mkk5*, and *mpk6* mutants intensified by PFLP shown in this study did not evoke equivalent disease resistance against bacterial soft rot disease. This might be due to callose deposition acting as one of the independent defense responses activated in PTI (Eggert et al. 2014; Luna et al. 2011; Ma et al. 2016; Panstruga et al. 2009; Zipfel and Robatzek 2010). Thus, we further suggest that PFLP was able to intensify defense routes other than signal transduction through the MAPK pathway. Nevertheless, the degree to which disease resistance against fungal pathogens intensified by PFLP is still unknown and needs to be further investigated.

Plant immune responses are also regulated by small-hormone networks such as the salicylic acid and jasmonate/ethylene pathways (Eulgem and Somssich 2007; Pieterse et al. 2009; Torres 2010). Our results indicated that the expression of both *PR1* and *PDF1.2* was induced by more than twofold by flg22_{Pst} at 24 h postinfiltration, and this induction was strongly intensified by PFLP. Relatedly, our previous study showed that PFLP still intensified disease resistance in *npr1* and *NAHG* plants (Ger et al. 2014). Compared with our inoculation results for MAPK mutants against bacterial soft rot, we suggest that the signals of the small hormones might be of secondary importance in this PFLP-mediated disease resistance.

Taken together, the results of this study lead us to conclude that the multiple PTI-triggered defense responses are intensified by PFLP. Most importantly, the results of this study demonstrate that the PFLP-intensified disease resistance against bacterial soft rot requires signal transduction through the MAPK pathway.

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