Using Dynamic Changes of Chlorophyll Fluorescence in *Arabidopsis thaliana* to Evaluate Plant Immunity-Intensifying *Bacillus* spp. Strains

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ABSTRACT

The integral defense responses of plants triggered by the small molecules of plant pathogens are regarded as plant immunity. The pathogen-associated molecular pattern-triggered immunity (PTI) occurs on the recognition of a pathogen by receptors on plant cell surfaces as an infection begins. During the activation of PTI, the effectiveness of a plant's photosynthetic system may be altered. In this study, chlorophyll fluorescence was used to assay the dynamic changes of PTI. When we used flg22_{Pst} as an elicitor, we found that the photosynthetic electron transport rate (ETR) of *Arabidopsis thaliana* Col-0 was significantly decreased at 2, 4, and 24 h on treatment with a PTI-intensifying protein, plant ferredoxin-like protein (PFLP). In addition, this reduction in the photosynthetic ETR was also carried out with a PTI-intensifying *Bacillus amyloliquefaciens* strain, PMB05, on the induction of flg22_{Pst}. The disease resistance against bacterial soft rot caused

by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) was still enhanced by PMB05. Interestingly, among the eight tested *Bacillus* species strains, the PTI triggered by HrpN_{Pcc} from *P. carotovorum* subsp. *carotovorum* exhibited an ETR that was significantly decreased by PMB05. Furthermore, this decrease was consistent with rapid H₂O₂ generation and callose deposition triggered by HrpN_{Pcc} and the disease resistance against bacterial soft rot. Taken together, such results led us to conclude that the assay based on the ETR established in this study can be used as a model for evaluating the effectiveness of plant immunity-intensifying microbes for controlling plant diseases.

Keywords: *Bacillus amyloliquefaciens*, chlorophyll fluorescence, electron transport rate, plant ferredoxin-like protein, plant immunity

Protecting plants against bacterial diseases by increasing their defense responses is one strategy with potential agricultural applications. Relatedly, a response of pattern recognition receptors on the surfaces of plant cells to the small molecules of pathogens termed pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) has previously been documented. Previous studies have further shown that intensifications of PTI through ectopically plant ferredoxin-like protein (PFLP) led plants to increase their disease resistance (Hong et al. 2018; Su et al. 2014). Such increased disease resistance has been successful in controlling bacterial wilt on tomatoes and bananas, bacterial soft rot on tomatoes and orchids, bacterial blight on rice, and Fusarium wilt on bananas (Huang et al. 2007; Namukwaya et al. 2012; Tang et al. 2001; Yip et al. 2007, 2011). Similarly, PTI intensified by the *Bacillus amyloliquefaciens* strain PMB05 has been shown to provide excellent disease resistance against bacterial wilt on black rot on cabbages (Li'aini et al. 2017).

The observation of rapid reactive oxygen species (ROS) production and callose deposition or the analysis of the expression of defense-related genes can be used as indicators during the occurrence of PTI (Lu et al. 2011; Luna et al. 2011; Nie et al. 2017; Su et al. 2014); however, the examination of these parameters is destructive and cannot be used to assess dynamic changes of immune responses. Reports have shown that the occurrence of plant immune responses can be regulated by the efficacy of the

photosynthetic systems of the plants in question. In addition, the photosynthetic system of a plant is one of the systems through which the plant exhibits responsiveness in terms of ROS generation (Asada 1999; Lamb and Dixon 1997; Sierla et al. 2013). Recent studies have demonstrated that the activation of PTI signals is associated with the expression of genes and nonphotochemical quenching (NPQ) in chloroplasts (Göhre et al. 2012; Nomura et al. 2012). Therefore, the crosstalk between the dynamic changes in the chlorophyll fluorescence of a photosynthetic system and the intensification of PTI could be assessed to establish a model for evaluating possible PTI-intensifying microbes.

In this study, we used chlorophyll fluorescence analysis, as well as evaluations of rapid H₂O₂ generation and callose deposition, of *A. thaliana* in the presence of PFLP or *B. amyloliquefaciens* PMB05 on the activation of flg22_{Pst}. Meanwhile, the effect of PTI-intensifying PFLP and PMB05 on the disease resistance to bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) was also analyzed because the HrpN from *P. carotovorum* subsp. *carotovorum* is a PAMP that elicits defense responses (Kariola et al. 2003). Moreover, the effects of other *Bacillus* spp. strains in the presence of HrpN from strain Ecc17 of *P. carotovorum* subsp. *carotovorum* were also assessed. We concluded that the chlorophyll fluorescence conditions that we developed can be used as a new model for evaluating PTI-intensifying microbes that potentially could be used to control bacterial soft rot.

MATERIALS AND METHODS

Growth conditions of plants and bacteria. The *A. thaliana* ecotype Columbia (Col-0) was used in this study. Seeds of the plant were sown in peat moss. One week later, the germinated seedlings were transplanted to individual pots for 3 weeks. All the plants were grown in a growth chamber (Hipoint, Kaohsiung City, Taiwan) with a growth temperature of 22°C, a light condition of 16 h light/8 h darkness, and an illumination intensity of 200 µmol m⁻² s⁻¹. The bacterial soft rot pathogen, *P. carotovorum* subsp. *carotovorum*

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strain Ecc17, was also used in this study. This bacterial strain was cultured on agar plates in nutrient broth (Becton, Dickinson and Company, Franklin Lakes, NJ) at 28°C for 24 h for further experiments. To express the PFLP and HrpN protein, the *Escherichia coli* BL21 strains containing the pET-PFLP (OE-PFLP) (Lin et al. 2011) and pET-HrpN-Ecc17 (OE-HrpN17), respectively, were used. These strains were cultured in Luria-Bertani (LB) broth supplemented with ampicillin at 100 µg/ml at 37°C for 16 to 18 h. Eight *Bacillus* spp. strains (PMB03, PMB04, PMB05, DR81, DR237, DR243, DR244, and DR258) were assayed in this study. A single colony of each strain was cultured in nutrient broth medium at 28°C for 24 h. After the culture was centrifuged at 8,000 ×g for 10 min, each bacterial suspension was prepared and adjusted to OD₆₂₀ at 0.3 with sterilized distilled water before subsequent experiments.

Preparation of bacterial PAMP and PFLP. To induce basal PAMP-triggered immunity, the flg22_{Pst} from *Pseudomonas syringae* pv. *tabaci* (Felix et al. 1999) and HrpN from *P. carotovorum* subsp. *carotovorum* strain Ecc17 were used. The flg22_{Pst} was synthesized by LifeTien LCC (South Plainfield, NJ) and then prepared with 25 mM Tris-HCl buffer (pH 7.5) to produce a stock solution. The stock solution was applied in all experiments to 0.5 µM of final concentration in this study. To obtain the HrpN and PFLP, OE-HrpN17 and OE-PFLP cultured in LB broth were induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). Subsequently, the cells were disrupted and purified by using a Ni-NTA Spin Kit according to the manufacturer's instructions (Qiagen, Venlo, The Netherlands). Both the HrpN and PFLP were applied at 0.5 mg/ml of final concentration in all the assays.

Measurement of chlorophyll fluorescence. To investigate the spatial heterogeneity and temporal changes of photosynthetic performance in the presence of PAMPs and PFLP or the antagonistic strain PMB05, the methods of chlorophyll fluorescence imaging were applied as in previous research (Song et al. 2016; Xu and Zhou 2006) with slight modifications. Briefly, chlorophyll fluorescence parameters were measured around the inoculation site in the leaves using an imaging chlorophyll fluorometer (Imaging-PAM m-series; Walz, Effeltrich, Germany). The plants were then dark-adapted after inoculation for more than 30 min. The image of the minimum chlorophyll fluorescence value F_0 was acquired using 0.5 µmol photons m⁻² s⁻¹ light in dark adaptation and the maximum fluorescence in the dark-adapted state, F_m , was determined by applying a short saturated blue pulse light (8,000 µmol photons m⁻² s⁻¹, 0.8 s) after 30 min. Based on the F_0 and F_m values, the images of the potential photosystem II (PSII) light efficiency F_v/F_m for each image pixel were derived as $(F_m - F_0)/F_m$ (Genty et al. 1989) using the appropriate software (Imaging Win version 2.32; Walz). The decrease of F_v/F_m is used to indicate the damage of PSII reaction center. After dark adaptation, the light response curve of the chlorophyll fluorescence was determined directly. The actinic light was turned on and increased in a stepwise manner from a low light level, a photosynthetic photon flux (PPF) of 11 µmol photons m⁻² s⁻¹, to a high light level of PPF 701 µmol photons m⁻² s⁻¹, with the each of the 15 light levels maintained for 20 s. At the end of the period for each light level, a saturated pulse was applied to get the maximum fluorescence image F_m' , while the basal image at the time was F , such that the effective quantum yield ($Y_{(II)}$) of the light level was calculated as $(F_m' - F)/F_m'$. For each light level, the electron transport rate (ETR) was calculated with $0.5 \times Y_{(II)} \times \text{PPF} \times 0.84$ (Cornic and Baker 2012; Ehleringer 1981) to indicate the efficiency of photosynthetic light reaction system. The quantum yield of nonregulated energy dissipation of PSII ($Y_{(NO)}$) was calculated as F/F_m , and the quantum yield of regulated energy dissipation of PSII ($Y_{(NPQ)}$) was calculated as $F/F_m' - F/F_m$ (Klughammer and Schreiber 2008; Kramer et al. 2004). $Y_{(NPQ)}$ and $Y_{(NO)}$ comprised the ratio of light energy not into the photochemical reaction, hence the photosynthetic process. $Y_{(NPQ)}$ denoted the part plant could convert the excess energy to heat through xanthophyll or

lutein cycle, whereas $Y_{(NO)}$ denoted the part of energy potentially causing oxidative damage to PSII. The average value of all the parameters inside the area of each inoculation site was circled and calculated using the same software. Each treatment was carried out with six inoculation sites in one plant, and values from 10 plants were collected as repeats. The data for each light level were analyzed by Tukey's honestly significant difference (HSD) test at 5% significance level.

Rapid H₂O₂ generation assay. To assess whether the rapid generation of H₂O₂ was intensified by PFLP or *B. amyloliquefaciens* PMB05 during the activation of PAMP-triggered immunity (PTI), *Arabidopsis* leaves were stained and observed by fluorescence microscopy at 1 h postinfiltration (Su et al. 2014). The leaves of the *Arabidopsis* plants were stained with 20 µM 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) in PBS buffer. The leaves were reacted under dark for 15 min and were then washed with PBS buffer three times. The leaves were then observed under a fluorescence microscope with an excitation at 465 to 495 nm and emission at 515 to 555 nm filter set (Leica, Wetzlar, Germany). The strength of H₂O₂ generation was determined using the Image J software (<https://imagej.nih.gov/ij/>) to measure the fluorescent intensity under a consistent threshold (Rasband 1997-2016). Data for each treatment were collected from 10 samples of individual leaves as repeats. The data were analyzed by Tukey's HSD test at 5% significance level.

Callose deposition assay. To observe the intensification of callose deposition, the *Arabidopsis* leaves were stained and observed at 8 h postinfiltration. Before observation, the collected leaves were soaked in 95% ethanol overnight and further stained with 0.01% aniline blue (Sigma, USA) in 0.1 M phosphate buffer (pH 8) for 2 h (Hong et al. 2018). The leaves were then observed under a fluorescence microscope with an excitation at 340 to 380 nm and emission at 400 to 425 nm filter set (Leica). The strength of the 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). Data for each treatment were collected from 10 samples of individual leaves as repeats. The data were analyzed by Tukey's HSD test at 5% significance level.

Disease severity assay. A disease severity assay of bacterial soft rot on *Arabidopsis* plants was performed with the inoculation applied to detached leaves (Su et al. 2014). Briefly, bacterial suspensions of *P. carotovorum* subsp. *carotovorum* strain Ecc17 were prepared in sterilized distilled water and adjusted to 0.3 at OD₆₀₀. Then, the detached leaves of *Arabidopsis* plants were placed and vacuumed in a 100-fold dilution of bacterial suspension at 200 mm Hg for 15 min. The inoculated leaves were incubated in a growth chamber at 22°C. The disease severity was calculated based on the disease indexes of soft rot symptoms rated from 0 to 4 (0: no symptoms; 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%) at 48 h postinoculation. Data regarding the disease severity of each treatment were collected from five leaves; three replications of each treatment were performed for statistical analysis.

RESULTS

Dynamic changes of chlorophyll fluorescence in PFLP-intensified PTI triggered by flg22_{Pst}. To assess the effect of PFLP in terms of intensifying PTI responses, flg22_{Pst} was first used as a PAMP for analysis. There were no significant differences in the potential PSII light efficiency F_v/F_m of the leaves when treated with Tris, flg22_{Pst}, PFLP, or flg22_{Pst} with PFLP at 0, 2, 4, and 24 h Supplementary Fig. S1. The ETR obtained from the leaves treated with Tris, PFLP, or flg22_{Pst} alone was gradually increased with the light levels (PPF), and there were no significant differences in the ETR values for these three treatments at 0, 2, 4, and 24 h (Fig. 1).

However, the increases in ETR for treatment with flg22_{Pst} and PFLP was lower than that for the other three treatments from the second hour onward, with the difference continuing to be significant at 4 and even 24 h. Meanwhile, although the quantum yield of regulated energy dissipation of PSII ($Y_{(NPQ)}$) was increased for each of the four treatments with greater light intensities, with no significant difference between the treatments at 2 and 4 h, the $Y_{(NPQ)}$ of the treatment with a mixture of flg22_{Pst} and PFLP was significantly lower than those for the other three treatments at the light levels between 50 and 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 2). The quantum yield of nonregulated energy dissipation of PSII ($Y_{(NO)}$) showed patterns similar to those of the $Y_{(NPQ)}$ responses. Specifically, there were no significant differences in the dynamic changes between the four treatment groups at 2 and 4 h after inoculation Supplementary Fig. S2. However, the $Y_{(NO)}$ of the leaves treated with the flg22_{Pst} and PFLP mixture was significantly higher than those of the control group leaves and those treated with PFLP or flg22_{Pst} alone.

Dynamic changes of chlorophyll fluorescence in *B. amyloliquefaciens* PMB05-intensified PTI triggered by flg22_{Pst}. To analyze whether the photosynthetic system would be affected by *B. amyloliquefaciens* strain PMB05 during the

activation of PTI triggered by flg22_{Pst}, the changes in the chlorophyll fluorescence of leaves were measured. As with the results assayed for treatment with PFLP, the F_v/F_m of the leaves exhibited no significant difference among the four treatments at 0, 2, 4, and 24 h Supplementary Fig. S3. The ETR values of the leaves treated with Tris, bacterial suspension of PMB05, or flg22_{Pst} alone also showed no significant differences at 2 and 4 h postinfiltration, but the leaves inoculated with flg22_{Pst} presented a significantly lower ETR value than those treated with Tris or bacterial suspension of PMB05 alone between 200 and 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of PPF at 24 h postinfiltration. The ETR values of the leaves infiltrated with the mixture of strain PMB05 and flg22_{Pst} were significantly lower than those of the leaves treated with the other three treatments of more than 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of PPF at 2, 4, and 24 h (Fig. 3). The $Y_{(NPQ)}$ values of the leaves that received all four treatments increased with increasing light intensity. The $Y_{(NPQ)}$ values of the leaves treated with bacterial suspension of PMB05 or flg22_{Pst} alone exhibited no significant difference with those of the leaves treated with Tris at 0, 2, 4, and 24 h. However, the $Y_{(NPQ)}$ values of the leaves treated with the mixture of PMB05 and flg22_{Pst} were significantly higher than those of the other three groups at 2 h under 0 to 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of PPF, but then significantly lower at

Fig. 1. Dynamic changes in electron transport rate (ETR) on treatment with flg22_{Pst} and plant ferredoxin-like protein (PFLP) in *Arabidopsis* plants. **A to D**, Values for the treatments measured at 0, 2, 4, and 24 h postinfiltration, respectively. The open circles indicate the treatment with Tris buffer as a negative control; the gray circles indicate that with PFLP; the gray triangles indicate that with flg22_{Pst}; and the black squares indicate that with the mixture of flg22_{Pst} and PFLP. Each ETR was obtained from the mean of six inoculation sites in one plant as one repeat. Ten repeats were carried out in each treatment. The different letters indicate significant differences among the treatments at the same photosynthetic photon flux based on Tukey's honestly significant difference test ($P < 0.05$).

every light level at 4 and 24 h after inoculation (Fig. 4). On the other hand, the $Y_{(NO)}$ responses of the leaves receiving the four treatments were generally very close at 0, 2, 4, and 24 h after inoculation Supplementary Fig. S4. The only exception was that the $Y_{(NO)}$ values of the leaves treated with Tris, bacterial suspension of PMB05, or flg22_{Pst} became significantly higher than those of the leaves treated with the mixture of flg22_{Pst} and PMB05 under 0 to 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of PPF at 2 h.

Disease resistance enhanced by PFLP and *B. amyloliquefaciens* PMB05. To confirm that disease resistance was enhanced by PFLP or *B. amyloliquefaciens* PMB05, the control efficacy of bacterial soft rot was evaluated in *A. thaliana*. The results showed that the disease severity of Col-0 plants was reduced from 82.5 to 38.8% by PFLP (Fig. 5A). Further observation of the symptom development of leaves inoculated with Ecc17 alone at 0, 2, 4, and 24 h indicated that most of the leaves showed more than 3/4 of the soft rot symptoms at 24 h after inoculation. In contrast, most of the leaves treated with PFLP remained symptomless (Fig. 5B). Similarly, the disease severity of Col-0 plants was reduced from 82.2 to 12.5% by *B. amyloliquefaciens* PMB05 (Fig. 5C). Furthermore, most of the

leaves treated with PMB05 also exhibited no soft rot symptoms (Fig. 5D).

Effects of *Bacillus* spp. strains in conjunction with HrpN_{Pcc}-mediated PTI on ETRs. To assay the effects of *Bacillus* spp. strains on ETRs also affected by the activation of PTI triggered by distinct PAMP, HrpN_{Pcc} from *P. carotovorum* subsp. *carotovorum* was used as an elicitor in this assay. The results showed that the ETR was significantly reduced by HrpN_{Pcc} from 97.3 to 77.4 and from 97.3 to 78.6 under light levels of 611 and 701 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of PPF, respectively, at 2 h postinfiltration (Fig. 6). Moreover, the HrpN_{Pcc}-mediated ETRs of leaves also treated with *B. amyloliquefaciens* strains PMB03 and PMB05 were significantly reduced to 69.9 and 61.6, respectively, in comparison with those of leaves treated with HrpN_{Pcc} alone under 611 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of PPF (Fig. 6A). Meanwhile, the HrpN_{Pcc}-mediated ETRs of leaves also treated with PMB03 and PMB05 were reduced to 70.4 and 63.5, respectively, under 701 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of PPF (Fig. 6B). However, the HrpN_{Pcc}-mediated ETR results of leaves treated with *Bacillus* spp. strains PMB04, DR81, DR237, DR243, DR244, and DR258 were not altered in comparison with those of leaves treated with HrpN_{Pcc} alone at the two different light levels.

Fig. 2. Dynamic changes in $Y_{(NPQ)}$ on treatment with flg22_{Pst} and plant ferredoxin-like protein (PFLP) in *Arabidopsis* plants. **A to D,** Values for the treatments measured at 0, 2, 4, and 24 h postinfiltration, respectively. The open circles indicate the treatment with Tris buffer as a negative control; the gray triangles indicate that with PFLP; the black squares indicate that with the mixture of flg22_{Pst} and PFLP. Each $Y_{(NPQ)}$ was obtained from the mean of six inoculation sites in one plant as one repeat. Ten repeats were carried out in each treatment. The different letters indicate significant differences among the treatments at the same photosynthetic photon flux based on Tukey's honestly significant difference test ($P < 0.05$).

Effects of *Bacillus* spp. strains in conjunction with HrpN_{Pcc}-mediated PTI on rapid H₂O₂ generation and callose deposition. To further analyze the effects of *Bacillus* spp. strains on HrpN_{Pcc}-triggered plant immune signals, the rapid H₂O₂ generation and callose deposition in *Arabidopsis* leaves were observed. The results revealed that all tested *Bacillus* spp. strains did not induce the H₂O₂ generation while compared with the blank treatment. A small quantity of H₂O₂ generation was induced by HrpN_{Pcc} alone. More abundant amounts of H₂O₂ were generated by HrpN_{Pcc}-treatments with *B. amyloliquefaciens* strain PMB03 or PMB05 than by those with other *Bacillus* spp. strains (Fig. 7A). The HrpN_{Pcc}-mediated fluorescent intensities of H₂O₂ signals were intensified by 3.7- and 8.6-fold by *B. amyloliquefaciens* strains PMB03 and PMB05, respectively. In contrast, the intensities obtained from the treatments with other *Bacillus* spp. strains did not show significant differences in comparison with that of treatment with HrpN_{Pcc} alone (Fig. 7B). The callose deposition induced by HrpN_{Pcc} was observed at 8 h postinfiltration. Similarly, all tested *Bacillus* spp. strains did not induce callose deposition while compared with the blank treatment in the infiltration of *Arabidopsis* leaves. A small amount of fluorescent callose signals were induced

by HrpN_{Pcc}. In addition, more abundant HrpN_{Pcc}-mediated callose deposition was observed in the treatments with *B. amyloliquefaciens* strain PMB03 or strain PMB05 than in the treatments with the other *Bacillus* spp. strains (Fig. 8A). Compared with the treatment with HrpN_{Pcc} alone, the HrpN_{Pcc}-triggered fluorescent intensity was increased by 4.6- and 8.2-fold in the treatment with *B. amyloliquefaciens* strains PMB03 and PMB05, respectively. In contrast, the intensities of HrpN_{Pcc}-mediated callose deposition in the treatments with the other *Bacillus* spp. strains did not show significant increases in comparison with that of treatment HrpN_{Pcc} alone (Fig. 8B).

Effects of *Bacillus* spp. strains on disease resistance to bacterial soft rot. To assess whether the *B. amyloliquefaciens* strains PMB03 and PMB05 intensified the resistance to bacterial soft rot disease, the disease severities caused by *P. carotovorum* subsp. *carotovorum* were evaluated in *Arabidopsis* plants. The results showed that the disease severity was reduced from 82.2 to 27.9 and 35.5% by *B. amyloliquefaciens* strains PMB03 and PMB05, respectively (Fig. 9). In contrast, the disease severities of bacterial soft rot were not reduced by the treatments with *Bacillus* spp. strains PMB04, DR81, DR237, DR243, DR244, or DR258.

Fig. 3. Effects of *Bacillus amyloliquefaciens* PMB05 on dynamic changes in electron transport rate (ETR) on treatment with flg22_{Pst} in *Arabidopsis* plants. **A to D.** Values of the treatments measured at 0, 2, 4, and 24 h postinfiltration, respectively. The open circles indicate the treatment with Tris buffer as a negative control; the gray triangles indicate that with *B. amyloliquefaciens* PMB05; and the black squares indicate that with the mixture of flg22_{Pst} and strain PMB05. Each ETR was obtained from the mean of six inoculation sites in one plant as one repeat. Ten repeats were carried out in each treatment. The different letters indicate significant differences among the treatments at the same photosynthetic photon flux based on Tukey's honestly significant difference test ($P < 0.05$).

DISCUSSION

Given the need to protect plants against various diseases, the development of effective and safe control strategies has become increasingly important. A number of control strategies based on the intensification of innate plant immunity by PFLP or beneficial microorganisms aimed at controlling various plant diseases have been demonstrated recently (Ger et al. 2014; Huang et al. 2004, 2006; Liau et al. 2003; Lin et al. 2011; Namukwaya et al. 2012; Su et al. 2014).

Previous studies have reported that PFLP intensifies signal transduction in the mitogen-associated protein kinase (MAPK) pathways of PTI to further enhance disease resistance to bacterial soft rot (Hong et al. 2018). Our own previous studies have further demonstrated that *B. amyloliquefaciens* strain PMB05 intensifies PTI such that cabbage becomes resistant to bacterial black rot disease (Li'aini et al. 2017). In the past, it was not possible to demonstrate the effect of such strains on PTI by analyzing the signals for the initiation of the defense response, such as rapid ROS generation and callose deposition, in a manner that was not invasive and destructive of the leaves (Hong et al. 2018; Lu et al. 2011; Luna et al. 2011). Recently, however, a nondestructive analytic method based on chlorophyll fluorescence found that such fluorescence

was associated with the activation of PTI triggered by flg22. Specifically, treatment with flg22 causes a reduction in non-photochemical quenching ($Y_{(NPQ)}$) values at 2 h but not 4 h postinfiltration (Göhre et al. 2012). Although the results of the present study did not indicate a significant reduction in $Y_{(NPQ)}$ caused by flg22 alone, $Y_{(NPQ)}$ was strongly reduced by treatment with a mixture of flg22_{Pst} and PFLP (Fig. 2) or by treatment with *B. amyloliquefaciens* strain PMB05 at 24 h postinfiltration (Fig. 4). We also observed that PMB05 increased the $Y_{(NPQ)}$ on treatment with flg22_{Pst} at 2 h postinfiltration, a result that suggested the application of PMB05 reduced the light inhibition and increased the light protection mechanisms of the photosynthetic system at the beginning of PTI activation. In the analysis of ETR values, our results showed that treatment with flg22_{Pst} alone did not result in a significant reduction within 24 h postinfiltration. However, PFLP or strain PMB05 caused dramatic reductions in the ETR under higher light levels on flg22-triggered responses at 2, 4, and 24 h postinfiltration. These results suggested that the reduction in the light protection and electron transfer efficiency of the photosynthetic system may occur with the initial steps of PTI activation. Such a dramatic reduction in the ETR also has been demonstrated in the infiltration of *Pseudomonas syringae* avirulent strain rather than virulent strain on *Arabidopsis* leaves at 3 h (Bonfig et al. 2006). This

Fig. 4. Effects of *Bacillus amyloliquefaciens* PMB05 on dynamic changes in $Y_{(NPQ)}$ on treatment with flg22_{Pst} in *Arabidopsis* plants. **A to D,** Values for the treatments measured at 0, 2, 4, and 24 h postinfiltration, respectively. The open circles indicate the treatment with Tris buffer as a negative control; the gray circles indicate that with *B. amyloliquefaciens* PMB05; the gray triangles indicate that with flg22_{Pst}; and the black squares indicate that with the mixture of flg22_{Pst} and strain PMB05. Each $Y_{(NPQ)}$ was obtained from the mean of six inoculation sites in one plant as one repeat. Ten repeats were carried out in each treatment. The different letters indicate significant differences among the different treatments at the same photosynthetic photon flux based on Tukey's honestly significant difference test ($P < 0.05$).

Fig. 5. Effects of plant ferredoxin-like protein (PFLP) or *Bacillus amyloliquefaciens* PMB05 on disease resistance against bacterial soft rot in *Arabidopsis* plants. **A and C,** The disease severity observed with the coinfiltration of *Pectobacterium carotovorum* subsp. *carotovorum* strain Ecc17 at 10^6 CFU/ml and PFLP recombinant protein at 1 mg/ml or *B. amyloliquefaciens* PMB05 at 10^8 CFU/ml in 4-week-old leaves. The disease severities were calculated from the index of five leaves at 24 h postinfiltration as one repeat. Three repeats were carried out in each treatment. The asterisks indicate significant differences compared with the inoculation performed with strain Ecc17 alone based on the *t* test ($P < 0.05$). **B and D,** Symptoms observed at 0, 2, 4, and 24 h postinfiltration.

Fig. 6. Effects of *Bacillus* spp. on HrpN_{Pcc}-mediated electron transport rate (ETR) in *Arabidopsis* plants. **A and B,** Treatments with distinct *Bacillus* spp. strains measured at 2 h postinfiltration under 611 and 701 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetic photon flux (PPF), respectively. Blank (B) indicates the treatment with Tris buffer as negative control. Each ETR was obtained from the mean of six inoculation sites in one plant as one repeat. Ten repeats were carried out in each treatment. The different letters indicate significant differences among the treatments at the same PPF based on Tukey's honestly significant difference test ($P < 0.05$).

result indicates the ETR reduction occurs during the activation of effector-triggered immunity. And, their results may suggest the initial activation of PTI by a virulent strain does not achieve the threshold of defense amplitude to lead to ETR alteration. It is also suggested that the occurrence of PTI does affect the ETR of photosynthesis and that the ETR can be used as an index to evaluate

the intensification of PTI by recombinant proteins or microorganisms within 2 h. The reductions in the ETR of *A. thaliana* are similar to the intensification of PTI induced by PFLP in the presence of flg22 (Hong et al. 2018; Su et al. 2014). Compared with values of $Y_{(NPQ)}$, $Y_{(NO)}$, and ETR, ETR is best echoed to the intensifying signals on PTI. It is because the calculation of ETR is based on the

Fig. 7. Effects of *Bacillus* spp. strains on rapid H_2O_2 generation on treatment with $HrpN_{Pcc}$ in *Arabidopsis* plants. **A**, The images of infiltrated leaves stained with $20 \mu M$ H_2DCFDA at 1 h postinfiltration to observe rapid H_2O_2 generation. Each bar indicates $20 \mu m$ in length. **B**, The fluorescent intensity of H_2O_2 generated by the treatments in infiltrated leaves measured by Image J. The different letters indicate significant differences between the treatments based on Tukey's honestly significant difference test ($P < 0.05$).

PPF, the higher PPF would expand more differences to observe the changes on photosynthetic efficiency. We may further suggest this amplification effect of ETR helps to evaluate the PTI intensification during the initiation of HR cell death. Moreover, the disease severities of bacterial soft rot were found to be significantly reduced by PFLP or *B. amyloliquefaciens* strain PMB05 in our assays. Therefore, we suggest that the reductions in the ETR were

consistent with the intensification of PTI triggered by flg22_{Pst} and the disease resistance to bacterial soft rot triggered by PFLP or *B. amyloliquefaciens* strain PMB05. PTI is an innate plant defense response triggered by the recognition of PAMPs on the plasma membrane of a plant (Dodds and Rathjen 2010). Bacterial PAMPs, such as harpin, flagellin, peptidoglycan, and elongation factor Tu, are identified by distinct

Fig. 8. Effects of *Bacillus* spp. strains on callose deposition on treatment with HrpnPcc in *Arabidopsis* plants. **A**, Images of infiltrated leaves stained with 0.01% aniline blue at 8 h postinfiltration to observe callose deposition. Each bar indicates 20 μ m in length. **B**, Fluorescent intensity of callose signals in infiltrated leaves measured by Image J. The different letters indicate significant differences between the treatments based on Tukey's honestly significant difference test ($P < 0.05$).

specific receptors that activate defense signals (Gust et al. 2007; Postel and Kemmerling 2009). In this study, to further validate the interaction of PTI activation and ETR reduction in the presence of *Bacillus* spp. strains, HrpN_{Pcc} from *P. carotovorum* subsp. *carotovorum* and *Bacillus* spp. strains were used to assay the effects of those strains on the ETR, rapid H₂O₂ generation, callose deposition, and disease resistance. The results showed that the HrpN_{Pcc}-mediated ETR was significantly reduced under light levels of 611 and 701 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of PPF at 2 h postinfiltration. This result not only confirms that the activation of PTI and ETR reductions occurred simultaneously but also exhibits that HrpN_{Pcc} triggers stronger ETR reductions than flg22_{Pst}. Among eight *Bacillus* spp. strains, we found that the *B. subtilis* strain PMB03 and the *B. amyloliquefaciens* strain PMB05 strain reduced the HrpN_{Pcc}-mediated ETR under two different light levels. Meanwhile, the HrpN_{Pcc}-mediated PTI signals and disease resistance to bacterial soft rot were intensified by these two strains. In addition, the other strains, which did not reduce the HrpN_{Pcc}-mediated ETR, also did not increase the PTI signals or disease resistance. Therefore, we suggest that the HrpN_{Pcc}-mediated ETR under the light levels of 611 and 701 $\mu\text{mol m}^{-2} \text{s}^{-1}$ would provide an effective means of evaluating the effects of PTI-intensifying microorganisms at 2 h postinfiltration. In addition to the finding that the HrpN_{Pcc}-mediated ETR reduction, rapid ROS generation and callose deposition caused by PMB05 was stronger than that caused by PMB03, it also should be noted these two strains exhibited equal effects in terms of enhancing the disease resistance to bacterial soft rot. The inhibitory effect assay revealed that PMB03 had a stronger antagonistic effect than PMB05. Based on this result, we speculated that the selection of microorganisms based on their antagonistic effects would provide PTI-intensifying microorganisms with superior effectiveness for plant disease control.

Taken together, the results of the PAMP-mediated ETR evaluation system based on chlorophyll fluorescence established in this study indicate that the system could serve as a model for determining whether a microbe is able to intensify PTI. This system

could be used to measure PTI signals directly without requiring the dark adaption or destruction of leaves. The system thus offers a promising approach for screening PTI-intensifying microbes for plant disease control in the future.

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Fig. 9. Effects of *Bacillus* spp. strains on disease resistance to bacterial soft rot in *Arabidopsis* plants. **A**, Disease severities elicited by the coinfiltration of *Pectobacterium carotovorum* subsp. *carotovorum* strain Ecc17 at 10^6 CFU/ml and distinct *Bacillus* spp. strains at 10^8 CFU/ml in 4-week-old leaves. The disease severities were calculated from the index of five leaves at 24 h postinfiltration as one repeat. Three repeats were carried out in each treatment. The different letters indicate significant differences between the treatments based on Tukey's honestly significant difference test ($P < 0.05$). **B**, Symptoms appearance of infiltrated leaves observed at 24 h postinfiltration.

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