Colonization of *Piriformospora indica* enhances insect herbivore resistance of rice plants through jasmonic acid- and antioxidant-mediated defense mechanisms

Chih-Yun Chen\(^a\), Po-Hsun Huang\(^a\), Kai-Wun Yeh\(^{b,c}\) and Shu-Jen Wang \(^a\)

\(^a\)Department of Agronomy, National Taiwan University, Taipei, Taiwan
\(^b\)Institute of Plant Biology, National Taiwan University, Taipei, Taiwan
\(^c\)Research Center for Climate Change and Sustainable Development, National Taiwan University, Taipei, Taiwan

**ABSTRACT**

*Piriformospora indica* (*P. indica*) is a mutualistic endophyte that colonizes plant roots. In this study, effects of *P. indica* on rice resistance against rice leaffolder (*Cnaphalocrocis medinalis* Guenée) were investigated. Growth inhibition and leaf injury caused by leaffolder larvae infestation in *P. indica*-colonized rice was significantly alleviated. Moreover, growth retardation was observed in larvae which fed on *P. indica*-colonized plants. JA-Ile levels and trypsin inhibitor activities in leaf tissues of *P. indica*-colonized plants were higher than those in noncolonized plants under leaffolder-infested conditions. *P. indica* effects on increasing trypsin inhibitor expression and reducing larval growth were repressed by aspirin. JA signaling drives *P. indica*-enhanced insect resistance in rice, and trypsin inhibitors might be candidates involved in this mechanism. Changes in antioxidant enzyme activities and malondialdehyde levels in *P. indica*-inoculated plants were observed, and it was demonstrated that oxidative stress induced by insect infestation could be reduced in *P. indica*-colonized plants.

**Introduction**

*Piriformospora indica* (*P. indica*) is a root endophytic fungus mutually beneficial to associated plants, and it has a broad range of hosts including monocots and dicots (Verma et al. 1998). The effects of *P. indica* colonization on increasing biomass have been proven in several plant species such as rice, Chinese cabbage and barley (Lee et al. 2011; Hilbert et al. 2012; Tsai et al. 2020). The capability of *P. indica* to accelerate plant growth and to enhance crop yield could result from promoting root development and nutrient uptake (Achatz et al. 2010; Ansari et al. 2013). *P. indica* colonization not only benefits plant growth but also improves environmental stress tolerance (Sherameti et al. 2008; Jiang et al. 2020). Several studies have indicated that the contributions of beneficial endophytes to improving tolerance against abiotic stresses are mediated enhancing the activity of reactive oxygen species (ROS) scavenging (Gill and Tuteja 2010; Lata et al. 2018; White et al. 2019). Under drought stresses, a reduction in malondialdehyde (MDA) and increased activities of antioxidative enzymes were found in *P. indica*-colonized Chinese cabbage and rice (Sun et al. 2010; Tsai et al. 2020). Moreover, *P. indica* colonization can also contribute to maintaining the osmotic potential of host plants by modulating proline accumulation under water stress conditions (Saddique et al. 2018).

Infestation of insect herbivores is a severe problem that threatens crop production. Plants develop several defense mechanisms against insect pests or to heal injured tissues. Insect chewing or mechanical wounding usually trigger jasmonic acid (JA) biosynthesis, and sucking insect attack often induces salicylic acid (SA) biosynthesis (Vos et al. 2013; Huot et al. 2014; Singh et al. 2018). The expression of several JA biosynthesis genes such as lipoxygenases and allene oxide synthase (AOS) could be induced by wounding stimuli (Ziegler et al. 2001; Upadhyay and Mattoo 2018). Moreover, the jasmonate resistant gene (JAR) encodes a protein that catalyzes the conversion of JA to JA-Ile, and it is activated in wound-stimulated plants (Lyons et al. 2013). JA and ethylene contents increased significantly in corn attacked by beet armyworm (*Spodoptera exigua*) larvae (Schmelz et al. 2003). In white butterfly (*Pieris rapae*) larvae infested *Arabidopsis*, both JA and JA-Ile contents increased (Vos et al. 2013). Moreover, when plants are subjected to mechanical damage or insect attack, protease inhibitors are often induced to inhibit the activity of insect intestinal proteases and repress digestive systems (Koiwa et al. 1997; Shamsi et al. 2016). Wang et al. (2011b) found that the activity of trypsin inhibitor, a kind of protease inhibitor, could be induced in leaffolder (*Cnaphalocrocis medinalis*) larvae-infested rice plants mediated through ethylene and SA signaling pathways. Increases in JA and trypsin inhibitors were also observed in rice stem borer (*Chilo suppressalis*)-infested rice plants (Liu et al. 2018). In several plant species such as corn, cotton and potatoes, H2O2 induced by mechanical stimuli functions as a secondary messenger to trigger the JA-dependent defense pathway (Orozco-Cardenas and Ryan 1999; Orozco-Cárdenas et al. 2001).

The impact of symbiotic fungus on herbivore resistance has been investigated in arbuscular mycorrhizal fungi (AMF)-colonized plants. Tomato plants preinoculated with *Glomus mosseae* performed stronger expressions of defense genes, such as protease inhibitors, in insect-feeding conditions.
mutants were used as materials. In our study, hemizygous osjar1
collected from AMF-colonized potato plants were not obviously
symbiotic conditions and reduces the
effects of resistance against cabbage loopers
Cnaphalocrocis medinalis (Guenée), commonly called rice
larval feeding treatment. For the experiments analyzing
Aspirin treatment
Aspirin is an inhibitor of allene oxide synthase (AOS), a key
Mann et al. 2008). In our study, hemizygous osjar1
Materials and methods
Plant material and growth conditions
The rice cultivar (Oryza sativa L.) used in this study was Taichung native 1 (TGN). Rice seeds were sterilized using 1% (v/v) NaOCl for 30 min before imbibition at 30°C in the dark for 2 d. Next, seedlings were cultured with Kimura solution (Chu and Lee 1989). The photoperiod for seedling growth was 12 h light/12 h dark, and the temperature cycle was 30°C/25°C (day/night). Leaf counting started from the first leaf that appeared after coleoptile (Chang et al. 2019). A rice osjar1 mutant (no. NG8384) obtained from the National Institute of Agrobiological Sciences in Japan was a Tos17 insertion line and was also applied in this study. The osjar1 mutant presents incomplete husk closure and abnormally developed seeds (Riemann et al. 2008). In our study, hemizygous osjar1 mutants were used as materials.
five larvae was calculated for each treatment. The experiments were repeated three times, and the data were presented as the mean of three experiments.

**Leaf injury score evaluation**

Leaf injury evaluation was performed according to the method of Edilberto (2013). The percentage of the damaged area to the leaf area was counted, and the levels of leaf damage caused by rice leaf folders were classified according to the damage area percentage.

**Chlorophyll fluorescence assay**

Chlorophyll fluorescence of rice leaf tissues was monitored by MAXI-Imaging-PAM (WALZ, Germany). After dark acclimation for 30 min, the minimum fluorescence (F₀) and the maximum fluorescence (Fₘ) of the 4th leaf were measured after the saturated light source was irradiated. The difference between F₀ and Fₘ is variable fluorescence (Fᵥ), and the quantum yield (Fᵥ / Fₘ) of the large photochemistry was calculated. Plants were subjected to light acclimation under photosynthetic active radiation (PAR) with a light intensity of 530 μmol m⁻² s⁻¹ for 3 min, and the steady state fluorescence (Fₛ) and the maximum fluorescence (Fₘ) of the 4th leaf were measured. The actual photochemical quantum yield was calculated according to the formula Φₚₛₛ = (Fₛ/Fₘ - 1) / Fₛ.

**Larvae weight and metamorphosis analysis**

To evaluate the relative growth rate and metamorphosis of rice leaffolder larvae fed on P. indica-colonized and noncolonized rice plants, the 3rd instar larvae of the rice leaffolder were weighed before fed with experimental plant materials, and then larvae were weighed again after feeding for 3 d (grown to 4th instar) and 6 d (grown to 5th instar). The relative growth rate of larvae was estimated according to the changes in weight after feeding. The days of feeding are expressed as D. The larval weights before and after feeding are indicated as B and A, respectively. Relative growth rate = (A – B)/|D|^n*(A + B)/2] (Han et al. 2015). Moreover, the number of pupae and adults were counted after larval metamorphosis, and the percentage of successful metamorphosis of larvae into pupae and adults was calculated. The number of larval samples was 15 for each treatment of an independent experiment, and the average larval weight was calculated. The experiments were repeated three times, and the results are shown as the mean of three experiments.

**Real-time RT–PCR**

The One Step SYBR® PrimeScript™ RT–PCR Kit II (TAKARA, Shiga, Japan) was used for real-time RT–PCR analysis, and the process followed the description of Chang et al. (2019). Primers used for detecting ubiquitin and OsAOS1 mRNA were described by Wang et al. (2011a). Primers used for detecting the expression of P. indica Pitef1 gene were 5'-GAAGAGCGCAGAAAAGGCT-3' and 5'-AAAAC-TACTCTCATCGAGC-3'. Specific primers designed for analysis of OsJAR1 gene expression were 5'-AGGTCTTGT-GAACCATCAACAGC-3' and 5'-AAAATATCTTTTG-CAGCATTGTTAGC-3' (Lu et al. 2015).

**Trypsin inhibitor analysis**

Proteins were extracted from rice leaf tissues according to the method of Chen et al. (2006). Protein samples for each treatment were extracted from leaves collected from four plants. Extracted protein was added to the trypsin inhibitor assay reaction solution which included trypsin and benzoyl-arginine p-nitroanilide (BAPNA, a substrate of trypsin). Trypsin inhibitor amounts in protein samples were determined according to the inhibition level of BAPNA digested by trypsin. Trypsin inhibitor amounts were finally calculated according to the standard curve based on the soybean trypsin inhibitor protein. Experiments were repeated three times, and the data are shown as the mean of three experiments.

**JA content analysis**

Extraction and analysis of plant hormones were performed according to the description by Chen et al. (2014), and LC-MS/MS analysis was performed at the Agricultural Biotechnology Research Center of Academia Sinica, Taiwan. LC-MS/MS analysis was performed using a linear ion trap–orbitrap mass spectrometer (Orbitrap Elite; Thermo Fisher Scientific) and UHPLC system (ACQUITY UPLC; Waters). The detected hormones were separated by a HSS T3 (Waters) column and mass spectrometry was performed by electrospray ionization (ESI). To establish a standard for absolute quantification, stable isotope-labeled hormones such as dihydrojasmonic acid (H2JA, OIChem, cat. no.0145324) were added to samples. The extract buffer included 100 mL 2-propanol, 50 mL D2O and 100 μL 12 N HCl; moreover, H2JA (0.375 μg) were added as standards. Leaf tissues (approximately 60 mg) were ground with liquid nitrogen and then homogenized in 1 mL extract buffer and shaken for 30 min at 4°C. Following, the samples were centrifuged at 13,000×g for 10 min, and then 6 mL dichloromethane was added to the supernatant (700 μL). After shaking for 30 min at 4°C, the samples were centrifuged. Samples were dried by a vacuum concentrator for approximately 1 h before storage at −20°C until LC-MS analysis. The sample was dissolved in 200 μL of methanol and then centrifuged at 4°C before the supernatant was subjected to LC-MS analysis. The data analysis formula was as follows: (JA area /H2JA area) × [1/FW (g)] × [isotope content in 1 mL extraction buffer (ng)]. For JA content analysis, leaf samples were collected from four plants for each treatment in each independent experiment. Three repeat experiments were performed, and the results are shown as the mean of three experiments.

**MDA analysis**

MDA of leaf tissues was extracted by trichloroacetic acid buffer and analyzed according to the method described by Heath and Packer (1968).

**Antioxidant enzyme activity analysis**

The superoxide dismutase (SOD) activity assay was performed according to Paoletti et al. (1986). Rice leaf tissues (50 mg) were homogenized with 2 mL of sodium phosphate buffer (50 mM, pH 7.4) before centrifugation at 12,000×g for 20 min at 4°C. The supernatant (0.2 mL) was mixed sequentially with 1.6 mL Tea-Dea buffer (100 mM triethanolamine-
diethanolamine, pH 7.4), 0.08 mL reduced form nicotinamide adenine dinucleotide (NADH, 7.5 mM), 0.05 mL EDTA (100 mM) and MnCl₂ (50 mM) buffer (pH 7.0). Then, 1 mL β-mercaptoethanol (10 mM) was added to the reaction solution. The reaction was carried out for 10 min before the absorbance was measured at 340 nm. One unit of SOD activity was defined as the amount of enzyme that cause 1 μmol ascorbate degradation. GR activity was measured following the method of Foster and Hess (1980). One unit of GR activity was defined as the amount of enzyme that contributed to the degradation of 1 μmol NADPH.

Statistical analyses

Statistical analyses were performed using R software for Fisher’s least significant difference (LSD) and Excel for Student’s t-test.

**Results**

**Effects of *P. indica* colonization on insect resistance of rice plants**

Changes in the injury levels of leaffolder larvae-induced leaf damage were observed in *P. indica*-colonized rice plants. The 4-leaf-stage noncolonized and *P. indica*-colonized rice plants were given with leaffolder larvae. After 3 d of larval treatment, wilting and growth slowdown occurred significantly in the leaf tissues of noncolonized plants. However, the leaffolder infestation induced damage was alleviated in *P. indica*-colonized plants (Figure 1A). *P. indica* colonization was evaluated according to the expression of *Pitef1* in plant roots (Figure 1B). Furthermore, the fresh weights of noncolonized plant shoots and roots decreased 40% and 17%, respectively, after larval feeding (Figure 1C,D). In *P. indica*-colonized plants, the decrease in fresh weight caused by leaffolder infestation was 28% and 14% in shoot and root tissues, respectively (Figure 1C,D). The influence of *P. indica* on photosynthetic systems was also evaluated under larval chewing stimulation conditions. The maximum photochemical quantum yield (Fv/Fm) value of the plants without the leaffolder treatment was 0.80. The Fv/Fm values of larvae-infested leaves decreased to 0.62. However, the Fv/Fm was still maintained at 0.74 in larvae-attacked leaves of *P. indica*-colonized plants (Figure 1E). In addition, the actual photochemical quantum yield (ΦPSII) was also investigated. The data in Figure 1F show that the ΦPSII of non-*P. indica* inoculated plants significantly decreased from 0.39 to 0.26 after leaffolder chewing. *P. indica*-colonized plants maintained a ΦPSII value of 0.35 even under larvae-infested conditions (Figure 1F).

**Growth retardation of leaffolder larvae fed on *P. indica*-colonized rice plants**

In addition to plant growth, the development of leaffolder larvae fed on *P. indica*-colonized and noncolonized plants was also monitored. In the case of non-*P. indica* inoculated plants, the 3rd-instar larvae grew into 4th instars with a relative growth rate of 0.32, and that of larvae grown on *P. indica*-inoculated plants was 0.28 (Figure 2A). Moreover, the relative growth rate of the 5th-instar larvae grown on *P. indica*-colonized plants was also significantly lower than that of the larvae grown on noncolonized plants (Figure 2A). The number of larvae that successfully pupated or emerged was counted. The data in Figure 2B showed that 82.2% of the leaffolders grown on noncolonized rice seedlings successfully pupated, but only 66.7% of the leaffolders grown on *P. indica*-colonized rice seeds successfully pupated. A total of 68.9% of the larvae grown on *P. indica*-colonized plants successfully emerged into moths; however, only 46.7% of larvae grown on *P. indica*-colonized plants transformed to moths (Figure 2B). Therefore, *P. indica* colonization conducted had an obvious impact on the metamorphosis of leaffolder larvae.

**P. indica modified JA biosynthesis in larvae-infested rice plants**

*P. indica* colonization stimulated both free-form JA and jasmonyl-isoleucine (JA-Ile) increases in leaf tissues (Figure 3).
To reveal the effects of *P. indica* colonization on defensive hormone biosynthesis under herbivory stresses, the amount of JA in leaf tissues of rice plants with or without *P. indica* inoculation was detected after larval infestation for 12 h. The results showed that the contents of both JA and JA-Ile in leaves significantly increased due to larval chewing (Figure 3). On the other hand, in larvae fed leaves, JA level of the *P. indica*-colonized plants was lower than noncolonized plants after infestation, but the JA-Ile content of *P. indica*-colonized plants was significantly higher than that of noncolonized plants (Figure 3).

To further clarify whether JA was involved in *P. indica*-promoted insect herbivore resistance, aspirin, a JA biosynthesis inhibitor, was applied to *P. indica*-colonized rice plants. Rice plants were pretreated with aspirin for 12 h and then given rice leaffolder larvae for 3 d before leaf samples were collected to determine the expression of JA biosynthesis allene oxide synthase 1 gene (*OsAOS1*). The transcript level of *OsAOS1* in *P. indica*-colonized plants was higher than that in noncolonized plants under leaffolder infestation conditions; however, *P. indica*-induced *OsAOS1* expression was significantly repressed in aspirin-treated plants (Figure 4A). Furthermore, the growth rate of larvae fed on *P. indica*- and aspirin-treated rice plants was observed. Under non-aspirin treatment conditions, the growth rate of larvae with non-*P. indica* inoculated plants was 0.31, and it decreased to 0.27 if the larvae were fed *P. indica*-colonized plants. However, *P. indica* colonization induced larval growth retardation was not present in larvae fed on aspirin-treated plants (Figure 4B).

Moreover, *P. indica* effects on leaf damage caused by leaffolder larvae were observed on JASMONATE RESISTANT 1 (JAR1) hemizygous mutant plants (osjar1) of the rice cultivar Nipponbare. The JAR1 gene encodes jasmonate-amido synthetase which catalyzes the biosynthesis of JA-Ile conjugates (Suzo and Staswick 2008; Shimizu et al. 2013). OsJAR1 gene expression and JA-Ile content in the osjar1 hemizygous mutant were lower than those in the wild type in both control and leaffolder larvae-infested plants (Supplemental Figure S1A and S1B). The larvae-induced injury states of wild-type and mutant plants under various *P. indica* and larvae-treated conditions were measured according to leaf damage area. In wild-type plants, 43% of the plant population exhibited the most severe damage level (injury score 9) after leaffolder larvae treatment for 48 h. The population with injury score 9 of the osjar1 hemizygous mutant was higher than that of wild-type plants. The population with injury score 9 of *P. indica*-colonized wild-type plants decreased to 20%. However, the severely damaged population of osjar1 plants increased to 30%, even though the plants were colonized with *P. indica* (Supplemental Figure S2).

**Effects of *P. indica* colonization on enhancing rice leaffolder-induced trypsin inhibitor expression**

To further investigate the mechanism of *P. indica*-enhanced leaffolder resistance, trypsin inhibitor activities of rice leaf tissues were measured. The results showed that trypsin inhibitor levels were obviously induced by leaffolder chewing and were further enhanced in *P. indica*-colonized plants (Figure 5). On the other hand, the *P. indica*-induced trypsin inhibitor expression in larvae-infested plants was repressed.

**Figure 2.** Growth of rice leaffolder fed on *P. indica*-colonized rice plants. (A) Relative growth rate of larvae fed on *P. indica*-colonized and noncolonized rice plants. Larvae (3rd instar) were fed plants for 3 d (the larvae grew to 4th instar) and 6 d (larvae grew to 5th instar), and the relative growth rate of larvae was calculated according to the weight of 3rd, 4th and 5th instar larvae. (B) Percentage of pupation and emergence of larvae. Control, larvae fed on noncolonized plants. The results are shown as the mean ± SE of three independent experiments. Significant differences were analyzed by Student’s t-test. *p < 0.05, ** p < 0.01.*

**Figure 3.** JA and JA-Ile content in leaf tissues of *P. indica*-inoculated plants under rice leaffolder infestation conditions. The 4-leaf-stage *P. indica*-inoculated and noninoculated plants were treated with leaffolder larvae, and leaves were collected after 12 h of larval feeding for detection of JA and JA-Ile content. Data are shown as the mean ± SE of three independent experiments. Control, non-*P. indica* inoculated and nonlarvae-feeding treatment. LF, rice leaffolder larvae. The different letters indicate statistically significant differences among group samples (*p < 0.05).*
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![Graph](image1.png)

**Figure 4.** JA biosynthesis gene expressions and larval growth rate in aspirin-treated plants. (A) Effects of aspirin on OsAOS1 gene expression in leaves of *P. indica*-inoculated and larvae treated plants. (B) Relative growth rate (RGR) of larvae fed on aspirin-treated and *P. indica*-inoculated plants. The 4-leaf-stage plants were pretreated with aspirin for 12 h before larval feeding. Leaf samples were collected to analyze OsAOS1 expressions after 3 d of larval feeding treatment. Larvae were weighed before being moved to the target plants and after 3 d of feeding, and RGR was calculated based on the changes in weight. Data are the mean ± SE of three independent experiments. The different letters indicate statistically significant differences among group samples (P < 0.05). Control, non-inoculated and nonlarvae-feeding treatment. LF, rice leaf folder larvae.

by exogenous aspirin (Figure 5). The results demonstrated that trypsin inhibitor expression enhanced in *P. indica*-colonized plants under insect herbivore stress conditions was mediated through JA signaling.

**P. indica increased antioxidant capacity of rice plants under herbivore stresses**

MDA content is an indicator of oxidative stresses. To monitor the status of oxidative stresses caused by leaffolder larvae chewing, the MDA content of leaf tissues was measured at 2, 24 and 48 h after larval feeding. MDA content was significantly increased in larval infested leaves, and the herbivore-induced MDA was significantly repressed in *P. indica*-colonized plants after larvae-infested treatment for 48 h compared with noncolonized plants (Figure 6). To clarify the regulation of antioxidation in *P. indica*-colonized plants, the activities of the antioxidant enzymes SOD, APX and GR in leaf tissues were measured. In nonlarvae-infested plants, SOD activity was not obviously promoted by *P. indica* colonization; however, SOD activity was enhanced 1.8-fold in *P. indica*-colonized plants compared with noncolonized plants under herbivore-attacking conditions (Figure 7). Compared with the noncolonized control samples, APX and GR activities respectively increased 2.1- and 1.8-fold in *P. indica*-colonized plants under nonherbivore-treated conditions (Figure 7). In leaffolder-infested plants, APX activities in *P. indica*-inoculated plants were still significantly higher than those in non-*P. indica* inoculated plants (Figure 7).

**Discussion**

**P. indica symbiosis reduced pest insect caused plant damage and retarded larval growth**

*P. indica* effects on promoting plant growth and inducing abiotic stress tolerance have been well exemplified in several studies (Lee et al. 2011; Hilbert et al. 2012; Jiang et al. 2020; Tsai et al. 2020). The results presented here showed that both shoot biomass reduction and leaf wilting caused by insect infestation were significantly alleviated in *P. indica*-colonized plants (Figure 1A,C). Moreover, insect herbivore-induced photosynthetic system damage was also relatively decreased in *P. indica*-inoculated plants (Figure 1E,F). These results indicated that rice plants colonized with *P. indica* increased resistance against leaffolder larval infestation. *P. indica* effects on promoting root growth and modifying root architecture have been observed in several plant species (Varma et al. 1999; Bakshi et al. 2015). Furthermore, *P. indica* effects on root growth can also occur under some environmental stresses such as water stresses (Saddique et al. 2018; Tsai et al. 2020). In our study, the root biomass of larvae-infested plants also declined even when the larval attacking site was on leaves (Figure 1D). However, *P. indica*-colonized roots exhibited higher biomass than noncolonized plant roots (Figure 1D). This might have resulted from better growth potential promoted by *P. indica* colonization, and it could have been due to obtaining more carbon sources from shoots if *P. indica*-colonized plants had a higher photosynthetic capacity.

Symbiosis with AMF increased the population of *Tetranychus urticae* on host bean plants (Hoffmann et al. 2009). *Sebacina vermifera* symbiosis repressed defense signals and reduced herbivore resistance (Barazani et al. 2005). In contrast, *P. indica* colonization contributed to increasing insect resistance of rice plants and also impeding growth of leaffolder larvae on *P. indica*-colonized rice plants (Figures 1 and 2). It was suggested that the effects of endophytic fungi on herbivore resistance are species specific. The impact of symbiosis between *P. indica* and rice plants affected not only larval growth but also the metamorphosis of rice leaffolders. Both pupal and moth metamorphosis percentage of leaffolders grown on *P. indica*-colonized rice plants were lower (Figure 2), which may have caused a decrease in the population of rice leaffolders.

![Graph](image2.png)

**Figure 5.** Changes in *P. indica* - and larvae-induced trypsin inhibitor expression in aspirin-treated plants. The 4-leaf-stage plants were pretreated with aspirin (150 μM) for 12 h before larval feeding. Leaf samples were collected for analysis of trypsin inhibitor activity after 48 h of larval-feeding treatment. Data are shown as the mean ± SE of three independent experiments. The different letters indicate statistically significant differences among group samples (P < 0.05). Control, non-*P. indica* inoculated and nonlarvae-feeding treatment. LF, rice leaf folder larvae.
JA is a defense hormone induced by several abiotic and biotic stresses (Yang et al. 2019). The expression of several genes involved in JA biosynthesis and signaling pathways could be induced by insect stimulation (Ye et al. 2012; Wang et al. 2013; Chang et al. 2019). In Spodoptera mauritia saliva-treated rice leaves, OsJAR1 gene expression and JA-Ile accumulation were increased (Wakuta et al. 2011; Fukumoto et al. 2013). In our study, the results showed that both JA and JA-Ile levels were significantly increased in leaves after leafhopper infestation (Figure 3). Larvae-induced JA-Ile levels were promoted in P. indica-colonized rice plants; in contrast, JA levels in P. indica-colonized plants were lower than that in noncolonized plants (Figure 3). The osjar1 hemizygous mutant contain lower JA-Ile levels compared with wild-type plants (Supplemental Figure S1B). The data in Figure S2 show that the proportion of plants with injured leaf area exceeding 50% of the complete leaf area (injury score 9) caused by larval infestation in the osjar1 hemizygous mutant was higher than that in the wild-type group. The effect of P. indica colonization in reducing larvae-induced leaf damage in osjar1 mutants was lower than that in the wild type. It was suggested that P. indica symbiosis could enhance the insect defense ability of rice plants by modulating leafhopper-stimulated OsJAR1 expression to promote JA conversion to JA-Ile. Cosme et al. (2016) indicated that P. indica colonization could reduce the plant growth inhibition caused by water weevil larval infestation in rice roots mediated by the interaction of GA and JA signaling. Our study showed that P. indica colonization in rice roots can contribute to increasing the tolerance against leaffolder and reducing leaf tissue damage through JA signaling. Thus, it was suggested that modulation of the JA signaling pathway in the leaf tissues of P. indica-colonized plants could be a systemic response. Endophyte-induced systemic disease resistance was observed in barley (Deshmukh et al. 2006). Moreover, in nonlarvae-infested rice plants, it was also demonstrated that P. indica-induced JA accumulation in leaves (Figure 3). Therefore, P. indica colonization may stimulate priming effects to induce stress-related hormone biosynthesis. The priming effect of P. indica colonization on inducing JA accumulation also occurred in leaf tissues of P. indica-inoculated sweet potato plants (Li et al. 2021). In P. indica-inoculated cucumber, increased of pest tolerance against nematodes and salicylic acid accumulation in roots were observed (Atia et al. 2020). However, the effects of P. indica on salicylic acid accumulation were not observed in leaf tissues of P. indica-colonized sweet potato (Li et al. 2021).

The effects of protease inhibitors on herbivore larval growth have been demonstrated in several plant species such as sweet potato, taro and peanut (Yeh et al. 1997; Senthilkumar et al. 2010; Lokya et al. 2020). In P. indica-colonized rice plants, trypsin inhibitor accumulation stimulated by leafhopper infestation was further promoted (Figure 5), and leafhopper larvae fed on P. indica-colonized rice performed a lower growth rate (Figures 2A and 4B). It was considered that JA-induced trypsin inhibitor may play a role in the growth-retarding effects of P. indica colonization on larvae. Our study demonstrated that the trypsin inhibitor induced by larval infestation could be repressed by exogenous aspirin (Figure 5). Moreover, in P. indica-colonized conditions, the reduction in larvae induced trypsin inhibitor expression was also found in osjar1 hemizygous mutants that contained lower JA-Ile (Supplemental Figure S1C). The injury caused by larval infestation in P. indica-colonized osjar1 mutant was more severe than that in P. indica-colonized wild-type rice plants (Supplemental Figure S2). JA-dependent trypsin inhibitor regulations have also been observed in several plant species (Wang et al. 2002; Botelho-Júnior et al. 2008; Li et al. 2017a).
P. indica reduce insect herbivore caused oxidative stresses

Under several biotic or abiotic stresses such as drought, flooding, salt, heavy metals, high light intensity, wounding and pest attack, electron transport chains in the chloroplasts and mitochondria in plant tissues are usually blocked and accompanied by a large amount of ROS production (Hodges et al. 2001). MDA is a product of lipid peroxidation and is often used as an indicator of oxidative stresses (Shulaev and Oliver 2006; Han et al. 2016). Accumulation of MDA in leaffolder larvae-infested leaves was significantly lower in P. indica-colonized rice plants (Figure 6). It was indicated that symbiotic interaction with P. indica can reduce oxidative stresses induced by larval chewing. The damaging effects of ROS could be prevented by enzymatic and nonenzymatic antioxidants that function as free radical scavengers (Ahmad et al. 2010). In barley root systems, P. indica colonization conducted antioxidative and glutathione-ascorbate cycle activities to increase resistance against salt and disease stresses (Waller et al. 2005). Li et al. (2021) indicated that P. indica colonization could increase the activities of catalase but not APX, peroxidase and SOD in sweet potato. SOD is considered as the first line of defense to against ROS by removing superoxide ion (O$_2^-$) radicals (Alschler et al. 2002). The effects of SOD overexpression on increasing antioxidative capacity have been investigated in plants (Li et al. 2017b). APX is a cytosolic H$_2$O$_2$-scavenging enzyme that functions as a regulator of ROS levels (Davletova et al. 2005). The Arabidopsis apx1 mutant exhibited more severe wound-induced oxidative damage in both chloroplasts and the nucleus than the wild type, and it was indicated that APX plays a role in regulating ROS levels and protecting organelles in wound-induced oxidative stresses (Maruta et al. 2012). Our study showed that APX and SOD activities in P. indica-inoculated plants were significantly higher under herbivore-stressed conditions (Figure 7). It was indicated that P. indica colonization could promote O$_2^-$ conversion to O$_2$ and H$_2$O$_2$. Furthermore, the efficiency of H$_2$O$_2$ conversion to H$_2$O was also increased. Increasing SOD and APX activities in P. indica-colonized rice plants might contribute to eliminating ROS and further reducing oxidative damage caused by leaffolders. On the other hand, even though the increase in GR activity was not significant in P. indica-colonized plants under herbivore stress conditions, it was obviously enhanced as well as APX by P. indica colonization in nonlarvae-infested plants. It was suggested that P. indica colonization might induce priming effects against environmental stresses.

In conclusion, P. indica colonization functions to maintain photosynthetic efficiency and plant growth under pest threat conditions. P. indica colonization contributes to increased herbivore resistance of rice plants by mediating JA signaling and reducing oxidative stresses. JA-induced trypsin inhibitors might also play an important role in repressing larval growth and metamorphosis in P. indica-colonized plants.

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Notes on contributors
Chih-Yun Chen obtained Master’s degree from Department of Agronomy, National Taiwan University.
Po-Hsun Huang obtained Master’s degree from Department of Agronomy, National Taiwan University.
Kai-Wun Yeh is professor and senior researcher at National Taiwan University. His research interests focus on insect-resistant mechanism of plants and also flowering physiology.
Shu-Jen Wang is professor of crop physiology in Department of Agronomy at National Taiwan University. Her research interests focus on regulatory mechanisms of plant response to biotic and abiotic stresses, and the interaction between beneficial endophytes and plants.

ORCID
Shu-Jen Wang http://orcid.org/0000-0002-1095-7055

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