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Changes in Rice Allelopathy and Rhizosphere Microflora by Inhibiting Rice Phenylalanine Ammonia-lyase Gene Expression

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Abstract Gene expression of phenylalanine ammonia-lyase (PAL) in allelopathic rice PI312777 was inhibited by RNA interference (RNAi). Transgenic rice showed lower levels of *PAL* gene expression and PAL activity than wild type rice (WT). The concentrations of phenolic compounds were lower in the root tissues and root exudates of transgenic rice than in those of wild type plants. When barndyardgrass (BYG) was used as the receiver plant, the allelopathic potential of transgenic rice was reduced. The sizes of the bacterial and fungal populations in rice rhizospheric soil at the 3-, 5-, and 7-leaf stages were estimated by using quantitative PCR (qPCR), which showed a decrease in both populations at all stages of leaf development analyzed. However, PI312777 had a larger microbial population than transgenic rice. In addition, in T-RFLP studies, 14 different groups of bacteria were detected in WT and only 6 were detected in transgenic rice. This indicates that there was less rhizospheric bacterial diversity associated with transgenic rice than with WT. These findings collectively suggest that *PAL* functions as a positive regulator of rice allelopathic potential.

Keywords Allelopathy · Phenylalanine ammonia-lyase · Rhizospheric microbes · Rice

Introduction

Rice is one of the most important cereal crops in the world, and 90 % of worldwide rice cultivation takes place in Asia (IRRI, 1993). The annual loss in rice yield attributable to weeds is estimated to be 10 %, amounting to about US $30 billion (Bastiaans and Kropff, 2003). This explains why rice operations are willing to spend so much on weed control. During the past few years, herbicides have been the main strategy for weed control, but this has raised concerns over the evolution of herbicide-resistant weeds. Better weed control can be achieved by increasing doses of herbicides, but this also can increase costs and result in crop phytotoxicity (Johnson et al., 2004; Chauhan and Johnson, 2011).

Allelopathy is an environmentally friendly method of weed control for crops that can potentially be subjected to integrated crop production. Many laboratory and field experiments have addressed allelopathy, including important recent studies (Lehoczky et al., 2011;Wang et al., 2012; see also in this issue, Gealy et al., 2013; Kato-Noguchi and Peters, 2013; Worthington and Reberg-Horton, 2013)

Although there is disagreement among researchers regarding which compounds should be classified as rice allelochemicals, a number of putative candidates, such as long-chain fatty acid esters, benzaldehydes, terpenoids, momilactone, steroids, and phenolic acids, have been reported (Mattice et al., 1998; Seal et al., 2004; Macias et al., 2006; Kato-Noguchi, 2011; He et al., 2012a). Although allelochemical toxicity can inhibit plant growth, little is known about the cellular, biochemical, and transcriptional changes involved in this response (Chung et al., 2006). Many of these compounds are synthesized via

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the phenylpropanoid pathway, which gives rise to a wide range of secondary metabolites, such as flavonoids, catecholamines, phenolic and phenylpropanoic acids, phenols, lignins, and tannins (Ferrer et al., 2008). All phenylopropanoids are derivatives of phenylalanine and cinnamic acid, which are synthesized by transamination and oxidative deamination.

The first step in the branched phenylpropanoid metabolism is the conversion of L-phenylalanine (Phe) into *trans*-cinnamate. This is catalyzed by phenylalanine ammonia lyase (PAL, EC 4.3.1.5) (MacDonald and D'Cunha, 2007). A number of studies have established a correlation between PAL and the phenolic pathway. This connection is especially visible when rice is exposed to abiotic factors such as exogenous salicylic acid (SA), low nutrients, or UV light (Bi et al., 2007; Song et al., 2008; Fang et al., 2009). In rice subjected to boron deficiency, a positive correlation has been observed between the concentrations of phenolics and PAL activity after 5–7 day of boron starvation (Camacho-Cristal et al., 2002). In addition, some defense-related proteins and the PAL enzyme, which is associated with phenylpropanoid metabolism and plant defense, have been found to be up-regulated in allelopathic rice (Song et al., 2008; Fang et al., 2009). The results of differential proteomics analyses also have confirmed a positive role of PAL in phenolic acid metabolism and allelopathic potential in rice. He et al. (2005) used deferential proteomics and bioinformatics to investigate the molecular mechanism of rice allelopathy under biotic stresses induced by barnyardgrass. They found that the enhanced inhibitory effect of allelopathic rice on target weeds was correlated to the up-regulated gene expression of the key enzyme, PAL, in the phenylpropanoid metabolism.

Functional analysis of PAL in allelopathic rice has yet to be performed. This is largely because PAL from allelopathic rice has not yet been purified and characterized. In this study, we used RNAi to silence *PAL* in the allelopathic rice PI312777, and we measured phenolic compounds in root tissues and root exudates. To analyze rice allelopathic potential, the inhibitory effect of phenolics released by the transgenic plants on barnyardgrass also was determined. The size and diversity of the microbial populations in the rhizosphere soil of two rice lines were compared to identify the interactions between allelochemicals and rhizospheric microbes.

Methods and Materials

Plant Materials Rice (*Oryza sativa* L.) accession PI312777 (high allelopathic potential) introduced from the USDA-ARS was selected as the donor plant and barnyardgrass (*Echinochloa crus-galli* L.) collected from a paddy field was used as the receiver plant (Dilday et al., 1994).

Construction of RNAi Vector Total RNA from the root of PI312777 was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Genomic DNA was digested using DNase I (Takara). Complementary DNA (cDNA) was synthesized using the ExScript RT reagent Kit (Takara). A 500 base pair (bp) partial PAL coding region 2,106 bp downstream of ATG (GenBank accession No. AK102817) was amplified with a forward primer 5′-CGGGATCCAATCTTCGGCACTGGCTCC-3′ and a reverse primer 5′-GGGGTACCGGGTCAGGTGGTCGGTGTA-3′ containing BamH I and Kpn I sites (underlined), respectively. The same fragment was amplified again with a forward primer 5′-GGACTAGTGGGTCAGGTGGTCGGTGTA-3′ and reverse primer 5′-CGAGCTCAATCTTCGGCACTGGCTCC-3′ containing recognition sites Spe I and Sac I, respectively. Before construction of the stability vectors, all gene fragments were cloned into pMD18-T vector (Takara), transformed into *Escherchia coli* (strain DH5α), and sequenced at Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. China. The obtained DNA sequences then were analyzed on the NCBI website (www.ncbi.nlm.nih.gov) to confirm its sequence similarity with *PAL*. Both fragments were inserted into pTCK303 to create a *PAL*-RNAi stability vector (pTCK303-*PAL*).

Mobilization of Constructs in Agrobacterium tumefaciens The recombinant vectors pTCK303-*PAL* were mobilized into *Agrobacterium tumefaciens* disarmed strain EHA105 by using the freeze and thaw method (Höfgen and Willmitzer, 1988). Transformants were selected on YEB-agar containing kanamycin (50 $\mu g \cdot ml^{-1}$) and rifampicin (20 $\mu g \cdot ml^{-1}$).

Transformation of Rice Plants The protocol for *A. tumefaciens*-mediated transformation of rice plants was as described by Supartana et al. (2005) with minor modifications. Briefly, grains of allelopathic rice PI312777 were sterilized with ethanol and sodium hypochlorite at 20 °C for 36 hr. The water was replaced once during the sterilization process. After 36 hr of soaking, the embryo region of the grain turned white. At this stage, neither shoots nor roots had appeared. To inoculate *A. tumefaciens* containing the recombinant vector pTCK303-*PAL* into the embryonic apical meristem in the soaked grain, the area on the husk where the shoot would later emerge was pierced to a depth of 1–1.5 mm with a needle (Φ0.70 mm; Kangyou Medical Instrument Co., Ltd. Jiangsu, China) that had been dipped in the *A. tumefaciens* inoculum. The inoculated grains then were placed on filter paper on wet vermiculite in flasks covered with aluminum foil and incubated at 23 °C in the dark for 5 day (Fang et al., 2011). Germinated seedlings were transplanted to a Styrofoam plate (with holes spaced at 5×6 cm). This was floated in a container (45×35×15 cm^3) filled with 10 L culture. To select positive transgenic rice lines, histochemical analysis of *GUS* gene expression was

carried out according to the method described by Jefferson (1989). The stability of the transgenic plants was checked by germinating in hygromycin B water solution.

Detection of PAL Gene Expression and PAL Enzyme Activity in Transgenic Rice Lines

All identified transgenic lines and wild type (WT) were transplanted into containers ($45\times35\times15$ cm^3) containing 10 L Yoshida nutrient solution (Yoshida et al., 1976), which was changed every week. After 7 day of recovery in the nutrient solution, total RNA was isolated from the transgenic lines and WT, and cDNA was synthesized as described. Expression levels of *PAL* were detected in both transgenic rice lines and WT by quantitative PCR (qPCR) and semi quantitative RT-PCR using the forward primer 5′-tcgccgacttcttccctc-3′ and reverse primer 5′-atcgctccggtgaactgc-3′. Further, PAL activity in the roots of transgenic lines and WT were determined following the protocols described by Dickerson et al. (1984). Protein content was measured using the Folin-phenol method (Lowry et al., 1951).

Determination of Total Phenolics in Root Tissue and Root Exudates Root tissue and exudates were sampled from the recovered transgenic lines and WT plants as described. For root extracts, 1 g of root tissue was immersed in 25 ml methanol containing 1 % HCl for 24 hr at 24 °C, and the procedure was repeated twice. These methanolic extracts were centrifuged at 4,000 g for 15 min, and the supernatants were pooled and stored at 4 °C (Shen et al., 2009). For root exudates, hydroponic solution was collected and then filtered using a 0.2 μm filter. The resulting solution was stored at 4 °C. The total content of phenolics was determined using a minimally modified Folin-Ciocalteu method (Singleton et al., 1999). Root exudates or root tissue extracts (3.25 ml) were added to test tubes with 0.5 ml of Folin-Ciocalteu's reagent and 1.25 ml of sodium carbonate (2 M). After mixing, the tubes were allowed to stand for 40 min in dark. Then OD was measured at 760 nm. The total phenolic compounds was expressed in tannic acid equivalents in micromole per gram fresh root or per liter of root exudate.

Bioassays on Barnyardgrass Using Rice Root Exudates This experiment was conducted in a greenhouse in which the temperature ranged from 22 °C (night minimum) to 30 °C (day maximum), and humidity ranged from 40 % (day minimum) to 60 % (night maximum). Every 20 germinated barnyardgrass seeds were transplanted on filter paper to the bottom of a Petri dish, and 15 ml root exudates were added as treatment. The dishes were placed in glass boxes to prevent water loss. Germinated seeds grown in sterile ddH_2O were used as controls. Each treatment was repeated four times. The dry weight of barnyardgrass was measured after 10 day of treatment, and inhibition rates were calculated as follows: $IR(\%) = [(CK - T)/CK] \times 100\%$, with IR>0 showing inhibitory effect and IR<0 showing a promotive effect (Lin et al., 2007).

Nucleic Acid Extraction from Rice Rhizospheric Soil This experiment was performed using a pot test. Soil was collected from the teaching field belonging to the Fujian Agriculture and Forestry University in Fuzhou, Fujian in southeastern China (26.05N, 119.18E). The soil was classified as acidic (pH=5.5) with a 6.65 % organic matter content and then air-dried and powdered. Plastic pots with a capacity of 2.5 L were used to hold the soil, and each pot was fertilized with fertilizer (N, 9; P_2O_5, 4.5; K, 7.2 $g{\bullet}m^{-2}$) followed by flooding the soil for 2 day. After this, 6 seedlings with uniform germination were selected from each line and transplanted individually into pots. The soil was irrigated with 250 ml dd H_2O every day throughout the plant growth period. When plants reached the 5-leaf stage, rhizospheric soil was sampled. Roots were shaken vigorously to remove soil adhering to them. Nucleic acids were extracted from the soil samples by using a moderately modified version of the method described by Peršoh et al. (2008). Briefly, 0.5 g of each rhizospheric soil sample was placed in a 2 ml tube, and the humic substances in the samples were removed by adding 0.2 M $Al_2(SO_4)_3$ and 4 M NaOH for the flocculation of humic substances with Al $(OH)_3$. To determine the optimal volumes of $Al_2(SO_4)_3$ for each soil sample, we set up a series with different gradient volumes of $Al_2(SO_4)_3$ within a range of 30 to 300 μl. After precipitation of humic substances, 325 μl extraction buffer (0.4 M LiCl, 100 mM Tris–HCl, 120 mM EDTA, pH=8) and 10 % SDS (pH=8) each were added, followed by vortexing with beads and purification with phenol:chloroform:isoamylalcohol (25:24:1) and chloroform:isoamylalcohol (24:1). Then, nucleic acids were precipitated using cold ethanol in the presence of 3 M sodium acetate (pH=5.2) at −20 °C for 10 min, and the precipitate was collected by centrifugation and washed with 75 % ethanol. The pellet was dried and resuspended in sterile ddH_2O.

Assessment of the Size of the Rice Rihzospheric Microbe Population Using qPCR For the qPCR standards, gene fragments of bacteria and fungi were amplified from the extracted DNA by using the following primers, F: 5′-agagtttgatcctggctcag-3′ and R: 5′-ggttaccttgttacgactt-3′ for bacteria, and ITS1: 5′-cttggtcattttagagaagtaa-3′ and ITS4: 5′-tcctccgcttattgatatgc-3′ for fungi. Both purified fragments were ligated into pMD-18 T (TAKARA) and then transformed into competent *E. coli* and plated on 50 $\mu g ml^{-1}$ ampicillin-containing LB agar plates that had been overlaid with 10 mM X-Gal and 50 mgml-1 IPTG. After incubation overnight at 37 °C, white colonies (putative positive clones)

were picked and cultured to isolate plasmid DNA. These were used as a template to prepare the standard curve. qPCR was performed using a *realplex*^{4} Real-Time PCR System (Eppendorf) using the SuperReal PreMix (SYBR Green) (Tiangen Biotech (Beijing) Co., Ltd). The PCR mixture consisted of 15 ng template DNA, 7.5 μl SuperReal PreMix, 4.5 μM each of the forward and reverse primers, and RNase-free ddH_2O in a total volume of 15 μl. The mixture was denatured initially at 95 °C for 15 min, followed by 41 cycles of 95 °C for 10 sec, 55 °C for 20 sec, and 68 °C for 40 sec for bacteria, and 95 °C for 10 sec, 50 °C for 20 sec, and 68 °C for 40 sec for fungi. Melting curve analysis was performed after amplification to verify the specificity of the amplified product under the following conditions: 95 °C for 15 sec, 60 °C for 15 sec, followed by an increase to 95 °C in 10 min and holding at 95 °C for 15 sec. Threshold cycle values (Ct) were recorded for each different microbe in both standard and test samples. A standard curve was established using the Ct values and copy numbers of plasmid DNA. The size of the microbe population was estimated by using the standard curve.

Analysis of Terminal Restriction Fragment Length Polymorphism (T-RFLP) Gene sequences of 16S rRNA were amplified using DNA from the rhizosphere soil of 6-carboxyflurescein-labeled 27 F (5′-agagtttgatcctggctcaG-3′) and 907R (5′-ccgtcaattcctttgagttt-3′). The PCR reaction volume was 30 μl. This contained 10×Taq buffer, 0.25 mM dNTP, 50 pM of each primer, and 1.5 U Taq DNA polymerase (Takara). Amplified PCR products were purified using the Gel Extraction Kit and digested for 5 hr at 37 °C with *Msp I* and *Hae III* in a 20 μl reaction volume containing 2 μl 10×buffer, 2 μl 10×acetylated bovine serum albumin (Takara), and 1 μl restriction endonuclease (5 U). Two microliters of the digested product were then mixed with 12 μl formamide and 0.5 μl DNA size markers (GeneScan-1000ROX, Applied Biosystems), denatured at 96 °C for 4 min and stored on ice. The length of the restricted fragments was then determined by using an automated ABI DNA sequencer (model 3730, Applied Biosystems). According to the SoftGenetics Application, fluorescently labeled 5′ terminal restriction fragments were detected and analyzed using the GeneMarker Version 1.2 (Applied Biosystems). Terminal restriction fragments ranging in size between 50 and 600 bp were determined for each given T-RFLP pattern. The microbe species were identified by comparing the results obtained here to the Ribosomal Database Project II (http://wdcm.nig.ac.jp/RDP/trflp/#program).

Statistical Analysis All data were subjected to analysis of variance (ANOVA) using SPSS. Means were assessed using the least significant difference at $P<0.01$ (LSD0.01).

Results

PAL Gene Expression in Transgenic Rice lLnes GUS dye and amplification of the GUS gene were used to identify transgenic rice strains (Fig. 1). To detect the expression level of *PAL* in transgenic lines, qPCR and semi quantitative RT-PCR were performed on the roots of transgenic and WT rice. As expected, *PAL* expression was down-regulated 5.1 fold in transgenic lines relative to WT (Fig. 2I, II). Similarly, PAL activity in these *PAL*-RNAi rice roots (53.8 U · mg · protein · h^{-1}) was significantly lower than in WT (118.8 U · mg · protein · h^{-1}) (Fig. 2II) suggesting that *PAL*-RNAi successfully interfered with transgenic plants, leading to a comparative reduction in *PAL* gene expression and PAL enzyme activity.

Determination of the Concentrations of Phenolic Compounds The total concentration of phenolic compounds as measured using the Folin-Ciocalteu Colorimetry method was significantly lower in root tissues (13.89 μmol · g^{-1}) and root exudates (4.62 μmol · L^{-1}) in *PAL*-RNAi rice than in WT plants, which contained 17.06 μmol · g^{-1} in root tissues and 6.88 μmol · L^{-1} in root exudates (Fig. 3). The results suggested that silencing *PAL* gene in allelopathic rice PI312777 leads to a reduction in PAL activity, which in turn decreases the release of phenolic compounds.

Bioassays of Barnyardgrass Performance of the allelopathic potential of rice was determined by the suppression of weed growth. The inhibitory effect of rice root exudates on dry weight of BYG was investigated. The allelopathic potential of *PAL*-RNAi rice to BYG was reduced to 15.7 % compared to 33.3 % in WT (Fig. 4). These results are consistent with the concentrations of phenolics in the two rice strains. The down-regulation of *PAL* exerts a negative influence on the synthesis and release of phenolics, thus leading to a reduction in the allelopathic effect of transgenic plants on barnyardgrass (Fig. 4).

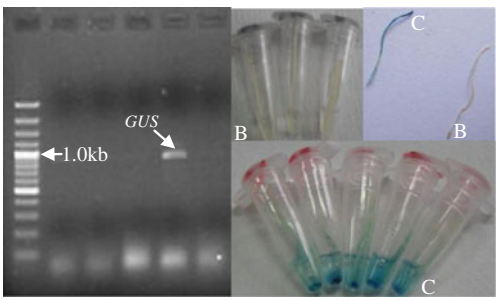

Fig. 1 Identification of *PAL*-RNAi transgenic rice through β-glucuronidase activity and PCR amplification. *A* Amplification of GUS gene in transgenic rice *B* β-glucuronidase activity in transgenic rice *C* Wild type

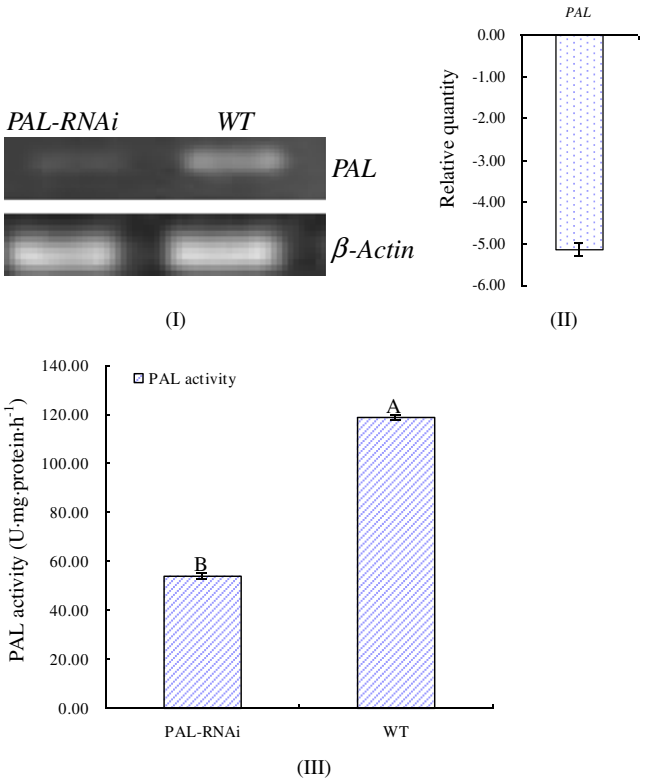

Fig. 2 Expression of *PAL* (I) by semi-quantitative RT-PCR and qPCR (II) and determination of PAL activity (III) in transgenic and wild type (WT) rice. WT: Wild type plant of allelopathic rice PI312777, *PAL*-RNAi: transgenic rice expressing *PAL*-RNAi

Microbial Population in the Two Rice Rhizospheres To determine the sizes of the bacterial and fungal populations in rice rhizospheric soil, standard curves related C_T values to the amounts of bacteria and fungi. These were first established by qPCR. Each standard represented from 10^5 to 10^8 copies of *E. coli*. The calibration curve for the bacterial copies was linear (R^2=0.9952) and spread over 4 orders of magnitude (CT1=−1.4553×log10 [16S rRNA]+30.312; data not shown) and CT 2=−5.16×log10 [ITS] +43.87 (R^2=0.9943) for fungi. The levels of bacteria and fungi in the rhizosphere of the two rice lines and control soil without rice plants were then calculated based on the standard curve. The levels of all three microbes decreased in the various seeding stages. Levels were the highest in PI312777, but the transgenic strain with reduced *PAL* expression showed fewer soil microbes (Fig. 5). In the 3, 5, and 7-leaf stages of PI312777, the bacterial density was $1.06\pm0.26\times10^8$ cells · g^{-1}, $2.89\pm0.16\times10^6$ cells · g^{-1}, and 128 cells · g^{-1} soil, respectively, and it was $2.81\pm0.34\times10^7$ cells · g^{-1}, $2.89\pm0.16\times10^6$ cells · g^{-1}, and 125 cells · g^{-1} soil, respectively, in the *PAL*-RNAi rice strain. Plants of the same age were used for this experiment,. A similar trend was observed with fungal cells (Fig. 5). This suggested that inhibition of *PAL* gene expression in PI312777 led to a reduction in phenolic exudates, which may have decreased microbial diversity.

Bacterial Diversity of Two Types of Rice Rhizospheric Soil A technique widely used in the study of microbial communities was used to confirm the results of qPCR and assess the diversity of bacteria in the rhizosphere of transgenic and WT rice T-RFLP. The results showed that the bacterial microflora from WT rhizospheric soil could be divided into 14 groups based on their taxonomic properties: Proteobacteria (19.3 %, 40), Actinobacteria (7.2 %, 15), Firmicutes (49.3 %, 102), Bacteroidetes (4.8 %, 10), Cyanophyta (1.4 %, 3), Spirochaetes (2.4 %, 5), Nitrospirae (0.5 %, 1), Fibrobacteres (0.5 %, 1), Deinococcus-Thermus (0.5 %, 1), Tenericutes (5.6 %, 12), Fusobacterium (1.0 %, 2), Clostridium(5.3 %,11), Deferribacteraceae (1.0 %, 2), and unknown bacteria (1.0 %, 2). In transgenic rice rhizosperic soil, only 6 groups were detected. These included Proteobacteria (45.9 %, 17), Firmicutes (35.1 %, 13), Spirochaetes (5.4 %, 2), Tenericutes (2.7 %, 1), Clostridium (8.1 %, 3), and unknown bacteria (2.7 %, 1) (Fig. 6). The total number of bacterial species in WT rice rhizospheric soil was 207, compared to a total of 37 in transgenic rice soil. These results confirmed that inhibition of *PAL* in PI312777 decreased the size and diversity of rhizospheric microbial community.

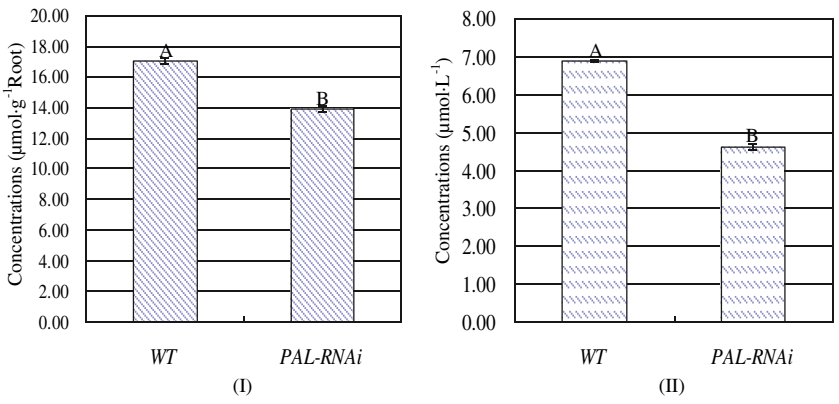

Fig. 3 Determination of the content of phenolic compounds in rice root tissues (I) and root exudates (II)

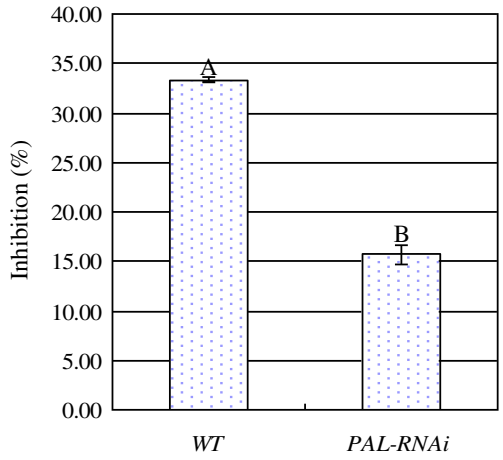

Fig. 4 Inhibition ratio of root exudates from transgenic and WT rice to the dry weight of barnyardgrass

Discussion

PAL is one of the key enzymes in phenol acid metabolism. It catalyzes L-phenylalanine to ammonia and trans-cinnamic acid, from which phenolic compounds are produced. In this study, we determined a role of the gene *PAL* in the regulation of rice allelopathy by using RNAi to inhibit *PAL* gene expression in allelopathic rice accession PI312777. The transgenic plants were validated and *PAL* gene expression and PAL enzyme activity were analyzed. *PAL* transcription levels were down-regulated and PAL activity was decreased in *PAL*-RNAi transgenic plants. The concentrations of phenolic compounds in root tissues and root exudates also were lower in transgenic plants than in controls. An increase in the dry weight of barnyardgrass was observed with root exudate bioassays of the RNAi transgenic rice.

Taken together, the results suggest that the down-regulation of *PAL* gene expression decreases the gene expression of phenolic metabolism-related enzymes and lowers the level of phenolics that in turn reduces the allelophatic potential of rice on baryarngrass. Thus, the *PAL* gene may play a role in the regulation of gene expression in phenolic metabolism-related enzymes, and synthesis and release of phenolics.

Rice allelopathy takes place through the release of allelochemicals into the environment (Xu et al., 2003). The accumulation of root allelochemical exudates contributes to enhanced rice allelopathy. This takes place when allelopathic rice responds to external biotic and abiotic factors, such as pest attack, weak solar radiation, high-temperature shock, and nutrient starvation (Belz, 2007; Song et al., 2008; He et al., 2012b). Rice has been reported to contain putative allelochemicals, such as long-chain fatty acid esters, benzaldehydes, terpenoids, momilactone, steroids, blumenol A, grasshopper ketone, and phenolic acids (Mattice et al., 1998; Seal et al., 2004; Macias et al., 2006; Kato-Noguchi, 2011; Kato-Notuchi et al., 2012; He et al., 2012a; Xu et al., 2012).

The biosynthesis of rice allelochemicals is regulated by several genes, which can also be up-regulated by abiotic or biotic stresses. PAL activity in allelopathic rice irradiated with UV light was increased two-fold relative to non-irradiated (Shin et al., 2000). Song et al. (2008) used subtractive hybridization suppression (SSH) to determine which genes are up-regulated in allelopathic rice PI312777. About 24 up-regulated genes were identified. These were classified into five groups: genes related to primary metabolism, genes related to phenolic allelochemical synthesis, genes related to plant growth or the regulation of the cell cycle regulation, genes related to stress response, signal transduction, and protein synthesis or degradation. The *PAL* gene was one of these. The transcript abundance was higher in PI312777 than in the non-allelopathic rice Lemont under low-N conditions. Exogenous SA was shown to induce the accumulation of *PAL* mRNA and thereby the synthesis of PAL protein, increasing PAL enzyme activity and so protecting crop plants from pest and weed infection (Bi et al., 2007; Fang et al., 2009).

Root exudates also are known to play roles in plant–microbial interactions. Microbes can enhance allelopathy (Bertin et al., 2003; Cipollini et al., 2012). Root-microbe communication characterizes the underground zone (Walker et al., 2003). In our study, the populations of rice rhizospheric microbes, including bacteria and

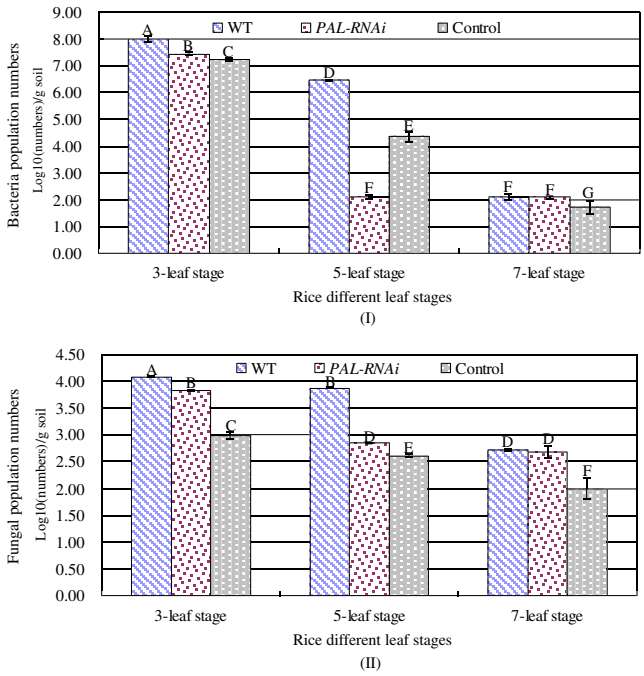

Fig. 5 Bacterial and fungal population numbers in the rhizospheric soil of transgenic and WT rice. (I) Bacterial population; (II) Fungal population

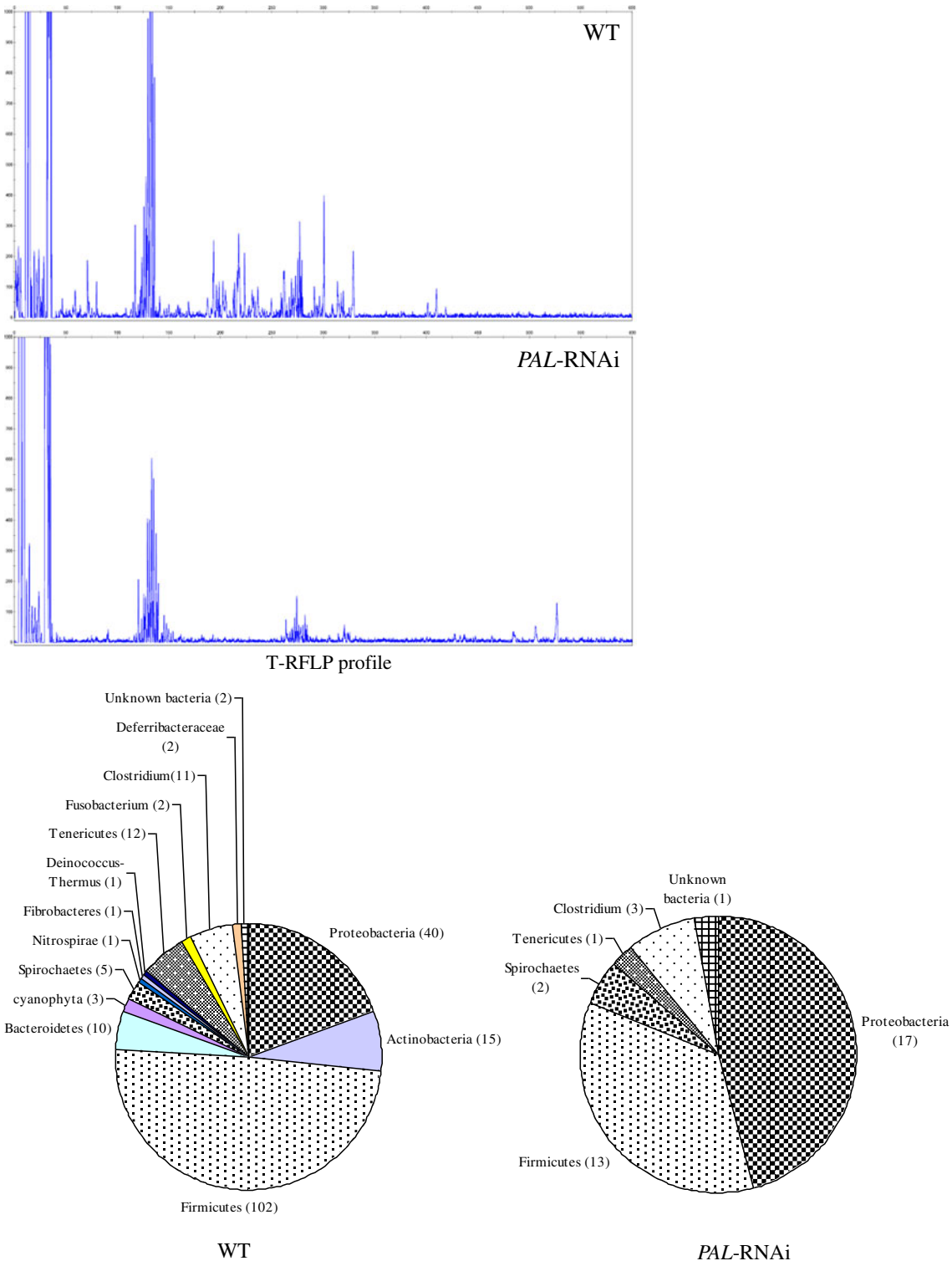

Fig. 6 Bacterial community in rice rhizospheric soil as indicated by T-RFLP

fungi, were smaller in transgenic rice soil than in WT rice plants at various stages of leaf development. This suggests that inhibition of *PAL* in allelopathic rice may have a negative effect on soil microorganisms by reducing rice phenolic exudates. Lin et al. (2011) found that phenolic acids are useful carbon resources that help establish the soil microbial community. The application of phenolics may stimulate the activities of soil microorganisms and improve microbial population in paddy soil.

The present study showed that the concentrations of phenolic acids in the rhizosphere soil of PI312777 were highest at the 4-leaf stage (data not shown here). The microbial population was found to decrease in size at the 5- and 7-leaf stages, correlating with the reduction of the concentration of phenolics in the rhizosphere soil after the 4-leaf stage. At the 5-leaf stage, there were only 6 bacterial groups in transgenic rice soil, while there were 14 groups in rhizosperic soil of the WT rice plant. Again, this suggests that rice phenolic exudates normally have a positive effect on soil microbes which may be in turn increase rice allelopathic potential against weeds. It is, therefore, necessary to better explore the functional relationship between allelochemicals and microbes.

In summary, biosynthesis of rice allelochemicals is closely correlated to the relevant genes, including *PAL*. This vital gene regulates the biosynthesis of phenolics. Inhibition of *PAL* gene expression in rice was here found to reduce the concentrations of phenolics in rice plants and root exudates and their allelopathic potential. Reduction of phenolic exudates in transgenic rice also influenced the quantity of rhizospheric microbes with 8 phyla lesser in transgenic plants compared to WT. Further studies on the microorganisms and their interactions with allelochemicals may reveal the process and underlying mechanism of rice allelopathy.

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