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Insecticidal effect of recombinant endophytic bacterium containing *Pinellia ternata* agglutinin against white backed planthopper, *Sogatella furcifera*

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ABSTRACT

White backed planthopper (WBPH; *Sogota furcifera* Horvath) has become the major threat to rice crops throughout Asia, damaging plants both through its feeding behavior and by acting as a virus vector. Here, we developed a novel method for biologically controlling WBPH by using endophytic bacterium to express anti-pest plant lectins. Strain SJ-10 of an endophytic bacterium, characterized as *Enterobacter cloacae* by morphological, physiological, biochemical and 16s rDNA characteristics, was isolated from rice seedlings. The *Pinellia ternate* agglutinin (PTA) gene was cloned into SJ-10 for expression. The positive transformant, selected by antibiotic resistance, was evaluated using PCR, SDS-PAGE and Western blot assay. After inoculation, rSJ-10 could colonize rice plants so that they expressed PTA, and then the rice was shown to have insecticidal activity against WBPH. The results showed that rSJ-10 could significantly decrease the survival and fecundity of WBPH fed on rice seedlings ($p < 0.01$). At day 19, the fecundity of WBPH inoculated with rSJ-10, or with wild-type SJ-10 was decreased by 86.1%, and 25.6%, respectively. At day 22, numbers of WBPH on rice in the control were 19.4 times greater than on rice inoculated with rSJ-10. At day 26, the rice seedlings all died in the control group, but the seedlings inoculated with rSJ-10 grew well. The results showed that the rice seedlings inoculated with rSJ-10 expressing PTA protein were endowed with the anti-pest activity against WBPH. Further work is needed to investigate whether the rice plants expressing rPTA are toxic to mammals. This research highlights a way to biologically control planthoppers by recombinant endophytic bacteria expressing plant lectins.

1. Introduction

The most important sap-sucking insects of rice (*Oryza sativa* L.) are planthoppers, including white backed planthopper (WBPH; *Sogatella furcifera* Horvath), green leafhopper (GLH) and brown planthopper (BPH; *Nilaparvata lugens* Stål). Sap-sucking homopteran pests, viz., BPH, GLH, and WBPH, all three of which constitute nearly 35% of the insect pests of rice, not only cause severe physiological damage to the rice plant, but also act as vectors for major viral diseases. The WBPH is an important, major pest of the rice in tropics and subtropics of Asia (Heinrichs, 1994). The WBPH feeds by phloem abstraction and causes damage to the rice plant by hopper burn (Khan and Saxena, 1985; Reissig et al., 1986).

Genetic-engineering approaches offer ingenious solutions for introducing alien pest-resistance genes into crops. Artificial-diet bioassays revealed that plant lectins are toxic to homopteran insects (Powell et al., 1993). *Galanthus nivalis* L. agglutinin (GNA) is one such lectin found to be highly toxic to sap-sucking insects (Nagadhara et al., 2003, 2004). It is a mannose-specific, tetrameric protein, consisting of identical subunits of 12 kDa (Van Damme et al., 1987). GNA was found to be most toxic among the lectins tested. Transgenic expression of the gene encoding GNA in rice plants can decrease the survival and fecundity of BPH and WBPH feeding on them (Foissac et al., 2000; Sun et al., 2001).

Pinellia ternata Breit. is a traditional Chinese medicinal plant species, belonging to the family Araceae. Recent studies show that lectins of *P. ternata* have significant insecticidal activities towards cotton aphids (*Aphis gossypii* Glover), peach potato aphids (*Myzus persicae* Sulzer) and WBPH (Yao et al., 2003a; Zhang et al., 2003). The *pta* gene which has significant insecticidal activities is cloned from the inflorescences of *P. ternata* by RACE-PCR (Yao et al., 2001). Transgenic tobacco expressing PTA can significantly inhibit the growth of peach potato aphid (Yao et al., 2003a), and transgenic rice expressing PTA can also significantly resist feeding by BPH (Zhang et al., 2003).

Endophytic microorganisms are those that inhabit the interior of plants, especially leaves, branches and stems, showing no

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apparently harm to the hosts (Azevedo, 1998). The capability of colonizing internal host tissues has made endophytes valuable as a tool to improve crop performance in agriculture. Endophytic microorganisms have received considerable attention in the last 20 years because of their capacity to protect hosts against insects-pests and pathogens. Toxic metabolites produced by endophytic microorganisms in many plants can greatly reduce the populations of associated insects. The extracts of foliar fungal endophytes isolated from *Picea rubens* Sarg. (red spruce) needles were toxic to the forest pest *Choristoneura fumiferana* Clem. (eastern spruce budworm) in dietary bioassays (Miller et al., 2008; Sumarah et al., 2010). Toxic metabolites produced by endophytic fungi (Epichloë and Neotyphodium species) in fescue grasses greatly reduce the populations of associated herbivorous insects. These fungi produce various alkaloids that affect herbivore growth (Clay and Schardl, 2002). Fahey (1988) and Fahey et al. (1991) was the first to introduce a heterologous gene into an endophytic microorganism for insect control. The endophyte *Clavibacter xyli* subsp. *cynodontis*, a Gram-positive xylem-inhabiting bacterium, received a δ-endotoxin gene from *Bacillus thuringiensis*. The genetically modified bacterium is able to secrete toxin inside the plant, protecting it against attacks of target insects. The transformed bacteria were inoculated in corn crops and showed to reduce damages caused by the European corn borer *Ostrinia nubilalis* (Hübner) (Tomasino et al., 1995).

PTA is very effective for controlling Homoptera. Here, we introduced the *pta* gene into the endophytic bacterium of rice, and then used the recombinant endophytic bacterium to inoculate rice plants to control WBPH. We have even isolated the endophytic fungus Chaetomium globosum Kunze from rape seedlings as a living vector for expression of PTA. This recombinant fungus could colonize rape seedlings endowing its host with anti-pest activity against aphids (Qi et al., 2011). However, this fungus could not colonize rice plants. In this paper we report the isolation of endophytic bacteria from rice seedlings as living vectors for expression of PTA against WBPH.

2. Materials and methods

2.1. Isolation and characterization of endophytic bacteria

Rice seedlings were collected from Gongan county, Hubei province, China. Soil adhering to the roots of the seedlings was washed off with water. The seedlings were then cut into 2 cm fragments, surface sterilized by soaking in 75% ethanol for 5 min, then in 0.2% mercury bichloride for 5 min, and finally rinsed five times with sterile water. The surface sterilized materials were adequately ground, centrifuged at 2000 g for 5 min and then the supernatant was collected for spreading on LB (Luria Bertani) agar plates. After incubation at 37 °C for 3 days, bacterial colonies were picked up and saved in 25% glycerol at −70 °C. Among them, a Gram-negative bacterium SJ-10 was selected for further studies.

Physiological and biochemical characters of SJ-10, including phenylalanine deaminase activity, VP (Voges-Proskauer) test, gelatin liquefaction, citric acid, hydrogen sulfide, lysine decarboxylase, malonic acid, indole, methyl red, sucrose and lactose reaction, were detected by methods described by Bergey's manual of systematic bacteriology (Sneath et al., 1986). The 16s *rna* gene of SJ-10 was amplified by PCR (Weisburg et al., 1991), sequenced by the HuaDa Gene Company in Wuhan, China, and then compared with other bacterial 16s *rna* genes in the National Center for Biotechnology Information (NCBI) Blast database.

The morphology of SJ-10 was observed using a transmission electron microscopy (TEM) by the normal established methods (Hoppert and Holzenburg, 1998). Briefly, SJ-10 was incubated on LB agar plates overnight at 37 °C, suspended with 1 ml of deionized water, and then observed with TEM (Hitachi, Japan).

2.2. Construction of recombinant SJ-10

The DNA fragment coding for PTA (GenBank: AY191305.1) was amplified from the plasmid pET-pta (reserved in our lab) by primers pxf (5′-CGCCAAGCTTTTAATTCACCCTCTC-3′) and pxr (5′-TCAGCTGCAGTGGGCACCAATTAC-3′) using an established PCR methodology (Yao et al., 2001). The pxf primer was designed to allow an in-frame fusion of PTA at the N-terminal end with a signal sequence, SP. This amplified fragment was introduced between the sites of *Hind* III and *Pst* I of pP43NMK vector that carried a constitutively expressed P43 promoter (Wang and Doi, 1984), an engineered levansucrase signal sequence (sacB signal peptide; SP) (Wu et al., 1991), and a Kanamycin resistance marker to generate a new plasmid pP43NMK-pta (Fig. 1).

The recombinant plasmid pP43NMK-pta was transformed into SJ-10 according to the methods used for *Escherichia coli* transformation (Sambrook et al., 1989). The positive transformants of SJ-10 were selected by Kanamycin resistance, and then further verified by PCR for amplifying the *pta* gene and DNA analysis for the correct insertion of *pta* gene in the plasmid.

2.3. SDS-PAGE and western blot assay

The recombinant SJ-10 containing pP43NMK-pta was incubated overnight at 37 °C and 200 rpm in a shaker. Then 5 ml of cell culture was centrifuged at 5000 g for 10 min to separate cells and supernatant. Total cellular proteins and proteins in the supernatant of cell culture were analyzed with 15% SDS-PAGE electrophoresis and stained by Coomassie bright blue R-250 according to established methods (Qi et al., 2011).

Anti-PTA mouse serum was used for confirming the PTA expressed by rSJ-10 in a Western blot assay. This serum was made by immunization of mouse with purified PTA from *Pinellia ternate* (Qi et al., 2011). Prepared total cellular proteins and proteins in the supernatant of the cell culture were separated by 15% SDS-PAGE electrophoresis, transferred onto PVDF membrane (Millipore, USA), incubated with the mouse anti-PTA serum (1:1000 dilution) at 4 °C overnight, and then further developed by the established methods (Qi et al., 2011).

2.4. Colonization of rSJ-10 in rice plants

The germinated seeds of rice were sown in pots. After the shoots had grown, seedlings (3-weeks old) were inoculated with 1 ml of bacterium suspension containing 1×10^8 cfu of rSJ-10 by pouring onto the roots. At different time points, the inoculated seedlings were analyzed to determine whether they had been colonized by the bacterium by the previously described methods for isolating endophytic bacteria, and verified by PCR for amplifying the *pta* gene.

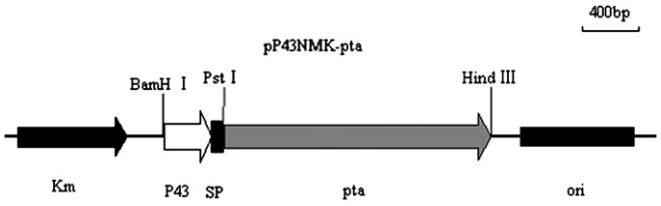

Fig. 1. Schematic map of pP43NMK-pta. The P43 promoter is strong and constitutively expressed to direct the transcription of *pta* during bacterial growth. The SP signal sequence allowed the recombinant PTA to be expressed as a secreted protein.

2.5. Detecting rPTA in rice seedlings

The germinated seeds of rice were sown in pots. After the shoots had grown, the seedlings (3-weeks old) were inoculated with 1 ml of bacterial suspension (1×10^9 cfu/ml) of rSJ-10 by pouring onto the roots. After 2 weeks, a total of 10 g of seedling stems were collected and cut into 1 cm × 1 cm pieces for extracting soluble proteins. These stem pieces were cooled by liquid nitrogen, and then dissolved in 30 ml of phosphate buffer (PBS; 10 mM, pH7.4) at 4 °C for 30 min. After centrifugation, the supernatant was collected and proteins precipitated by addition of trichloroacetic acid to a final concentration of 10% (w/v). The precipitated proteins were then dissolved with 1× SDS-PAGE sample buffer for Western blot assay by the established methods (Qi et al., 2011).

2.6. Anti-planthopper activity assay

Rice seeds were put on three layers of wet sterile gauze in Petri dishes for culture. Ten days after germination, rice seedlings were transferred to pots filled with a mixture of sand, perlite and nutritional substrate (1:2:1). Three seedlings were planted in a pot and maintained under irradiation (16L: 8D), 75% humidity, 25 °C at day and 20 °C at night. After three days, the recombinant SJ-10 were incubated at 37 °C overnight, washed in sterilized water and then resuspended with sterilized water to 1×10^8 cfu/ml. Two ml of cell suspension of rSJ-10 was used for inoculation of rice seedlings by pouring onto the roots. At 3 days, the pre-inoculated seedlings were analyzed to determine whether the bacterium had successfully colonized the stem and leaves. Then, ten 3rd-instar nymphs of WBPH were introduced onto the rice seedlings in each pot. The seedlings were covered with an insect-proof net to avoid escape. Every two days, the number of WBPH was counted for 26 days. The seedlings inoculated with sterile water were used as a control. Additionally, the seedlings inoculated with wild-type SJ-10 were also set up to detect whether the wild-type bacterium had activity against WBPH.

2.7. Statistical analyses

Each experiment in this study was done in triplicate. The statistical differences among groups were analyzed by a one-way analysis of variance (ANOVA) with a Tukey's post hoc test (spss 16.0; SPSS Inc., Chicago, IL). The letter at the $p < 0.05$ and $p < 0.01$ indicates highly significant differences.

3. Results

3.1. Isolation and characterization of endophytic bacteria

A total of sixty-three endophytic bacteria strains were isolated from rice seedlings. Among them, most were Gram-positive *Bacillus*. However, SJ-10 was a Gram-negative bacterium isolated from the stem of a rice seedling, and used in the following studies.

After observed by TEM, we found the SJ-10 cell was in the form of a rod-shaped with many surrounding pili but no flagellum (Fig. 2). After BLAST analysis, it was found the 16s rDNA sequence of SJ-10 had 98% homology with that of *Enterobacter cloacae* Jordan. The physiological and biochemical characters of SJ-10 were also as same as that of *E. cloacae* (Table 1). Therefore, SJ-10 was characterized as a strain of *E. cloacae*.

3.2. Construction of recombinant SJ-10

The amplified *pta* gene was digested with *Pst* I and *Hind* III and then inserted into pP43NMK to construct the recombinant plasmid pP43NMK-pta. The *pta* gene was under the control of promoter p43 and with an SP signal peptide in its N terminal (Fig. 1). The recombinant pP43NMK-pta was transformed into SJ-10. The transformants were selected by Kanamycin resistance, and then the positive transformant was further confirmed by PCR and DNA analysis for verification of the correct insertion of the *pta* gene in the plasmid.

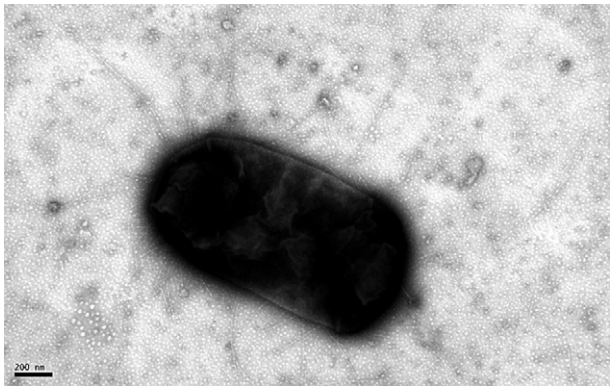

Fig. 2. SJ-10 observed by TEM. The results showed that the SJ-10 cell was in the form of a rod with many surrounding pili, but no flagellum.

The SDS-PAGE analysis showed that the PTA was expressed as a soluble cellular protein in SJ-10 (Fig. 3A). The molecular weight of rPTA was determined to be about 23 kDa, consistent with our previous research (Qi et al., 2008). PTA protein was conferred forming a dipolymer. Because an N-terminal signal peptide was fused with the *pta* gene, the recombinant PTA protein has a larger molecular weight than PTA protein purified from plant. Furthermore, as detected by Western blot assay, the results showed that rPTA could react with the anti-PTA serum (Fig. 3B). This result further verified that PTA was successfully expressed in rSJ-10 as a soluble protein. The results also showed that PTA was only expressed as an intracellular protein in SJ-10, indicating the SP signal peptide didn't work in this bacterium strain.

3.3. Colonization of recombinant endophytic bacterium in plants

After inoculation, the recombinant SJ-10 could be re-isolated from the stems and leaves of rice seedlings, and was verified by amplifying the *pta* gene with PCR (Fig. 4A). The number of rSJ-10 re-isolated from the rice seedlings presented an average of 8.1×10^7 CFU/g^{-1} of fresh tissue after inoculation for one day. As time progressed, fewer bacterial cells were detectable but they did not disappear from the seedlings (Fig. 4B). Twenty six days after inoculation, the bacterium rSJ-10 could still be isolated at an average of 1.96×10^6 CFU/g^{-1} of fresh tissue from the inoculated rice seedlings. Therefore, the rSJ-10 could rapidly infect, colonize and chronically survive in rice seedlings after inoculation. Interestingly, the recombinant SJ-10 could also colonize other crops and

Table 1
Physiological and biochemical characteristics of SJ-10.

Detected item	SJ-10	Detected item	SJ-10
VP	+	Methyl red	−
Sucrose	+	Lactose	+
Gelatin liquefaction	−	Indole	−
Citrate	+	H_2S	+
Malonic acid	+	Phenylalanine deaminase	−
Lysine decarboxylase	−	L-Arabinose	+

Note: "+" positive; "-" negative.

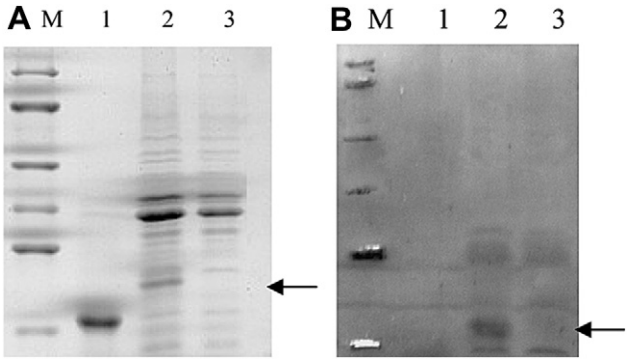

Fig. 3. Analysis of PTA expressed by rSJ-10 with SDS-PAGE and Western blot assay. A. SDS-PAGE. Lane M, molecular weight marker (97kD, 66kD, 45kD, 35kD, 27kD, 20kD, from top to bottom); Lane 1, PTA protein purified from corm of *Pinellia ternate*; Lane 2, total cellular proteins of rSJ-10; lane 3, total cellular proteins of wild-type SJ-10. B. Western blot assay. Lane 1, supernatant protein of rSJ-10; Lane 2, total cellular proteins of rSJ-10; lane 3, total cellular proteins of wild-type SJ-10. The results showed PTA was expressed as an intracellular protein in rSJ-10. The arrows indicate the PTA protein expressed by rSJ-10.

vegetables such as wheat, radish or cabbage for more than 3 months after inoculation (data not shown here).

As shown in Fig. 4C, the proteins from rice seedlings pre-inoculated with rSJ-10 could be recognized by the anti-PTA serum showing a band on the membrane in Western blot assay. However, no band was found on the membrane for the protein samples extracted from rice seedlings inoculated with the wild-type SJ-10. This result indicated that the recombinant endophytic bacterium could express PTA in plants after colonization the stem of rice seedlings.

3.4. Anti-insect activity of rSJ-10

The recombinant SJ-10 was introduced into rice seedlings, and then the anti-insect activity of seedlings was detected in WBPH. As shown in Fig. 5A, there was no significant difference in nymph mortality among seedlings inoculated with rSJ-10, wild-type SJ-10 or sterile water in the early days. After 11 days, WBPH began to produce new nymphs. At day 13, the insects increased rapidly in the control group and the wild-type SJ-10 group but only slowly in the rSJ-10 group. However, the difference was indistinguishable among these three groups. At day 19, the insect number in all three groups arrived at the highest value and the difference between each of the two groups was significant ($p < 0.01$). The insect number in the control group was 1.29 times of that in the wild-type SJ-10 group and 3.38 times of that in the rSJ-10 group. At day 19, the fecundity of WBPH in the groups inoculated with rSJ-10, or wild-type SJ-10 were decreased 86.1%, and 25.6%, respectively. From day 19, WBPH numbers began to decrease in all three groups. At day 22, the insect number in the control group was 19.4 times greater than that in the rSJ-10 group. At day 26, the seedlings all died in the control group and wild-type SJ-10 group, but the seedlings in the rSJ-10 group still grew well (Fig. 5B). The results indicated that the rice seedlings were endowed with an anti-insect activity against WBPH after inoculation with rSJ-10.

4. Discussion

Plant endophytes can survive in many crops. Because endophytes can colonize, propagate and transfer in the interior of plant tissue, they are the perfect vectors to express foreign protein with anti-insect activity. Several heterologous genes have been introduced into endophytic microorganism with the purpose of insect control. The *cry3A* gene from *B. thuringiensis* has been introduced into endophytic nitrogen-fixing bacteria *Gluconacetobacter diazotrophicus* Yamada strain BR11281 and *Herbaspirillum seropedicae* Baldani strain BR11335 of sugarcane. The ability of these bacteria to colonize the plant tissues associated with their poor survival in soil are characteristics that make these recombinants good candidates to be used as vectors for the control of coleopteran and lepidopteran pests in sugarcane (Salles et al., 2000). The *cry1Aa* gene from *B. thuringiensis* was cloned and expressed in a plant-colonizing methylotroph, *Methylobacterium extorquens* Urakami and Komagata. In single-dose assays of the recombinant against the silkworm, *Bombyx mori* Linnaeus, both whole cells and cell lysates caused rapid feeding inhibition followed by mortality (Choi et al., 2008). Endotoxin gene from *B. thuringiensis* was introduced into a nitrogen-fixing bacterium from the genus *Bradyrhizobium*, and the engineered bacterium could improve the nitrogen fixation and protect the host against *Rivelia angulata* larvae on *Cajanus cajan* (L.) Millsp (Nambiar et al., 1990). In China, Li et al. (2007) also isolated an endophytic bacterium *Leifsonia xyli* subsp. *cynodontis* Davis from

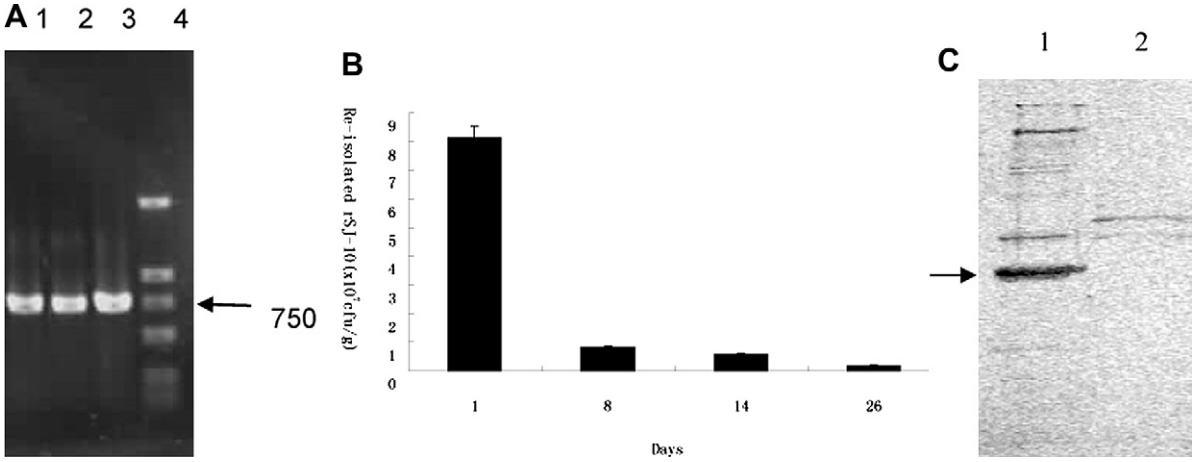

Fig. 4. Colonization of rSJ-10 in rice plants. A. PCR analysis of the recombinant bacteria re-isolated from rice plants. Lane 1, positive control of pNMK43-pta plasmid; 2 and 3, rSJ-10 re-isolated from rice plants. Lane 4, DNA molecular marker (2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp, from top to bottom). B. rSJ-10 re-isolated from rice plants. Twenty six days after inoculation, the bacteria could still be isolated at an average of 1.96×10^6 CFU.g-1 of fresh tissue from the inoculated rice seedlings. C. Analysis of PTA in rice seedlings by Western blot assay. Lane 1, rice seedlings inoculated with the rSJ-10; 2, rice seedlings inoculated with the wild SJ-10. The arrow indicates the rPTA in rice plants.

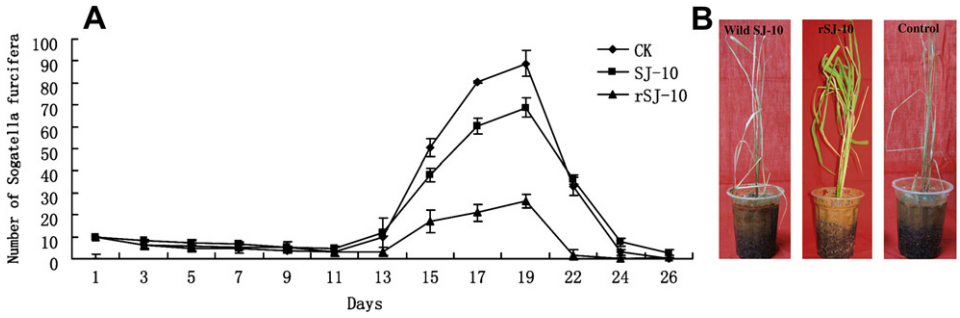

Fig. 5. Insecticidal activities of recombinant endophytic bacteria against WBPH. A. Survival of WBPH on rice seedlings inoculated with rSJ-10. CK, rice seedlings inoculated with sterilized water; SJ-10, rice seedlings inoculated with the wild SJ-10; rSJ-10, rice seedlings inoculated with the rSJ-10; B. Rice seedlings attacked by WBPH. The results showed that rice seedlings inoculated with rSJ-10 could resist attack from WBPH.

rice as a vector for expressing anti-insect proteins against pests on rice plants. Recombinant endophytic fungi *Chaetomium globosum* Kunze expressing *P. ternate* agglutinin has been constructed for control of *M. persicae* Sulzer (Qi et al., 2011). Therefore, endophytic bacteria have the potential to be used as living vectors for expression of different anti-pest proteins. The recombinant endophyte can confer specific insect and disease resistance to plant. Compared to transgenic plant, endophyte has several advantages. a) When inoculating endophyte into plant, the plant genome is not changed, which happens in transgenesis (Shi et al., 2006); b) Recombinant endophyte require a shorter time to be developed because it is easier to modify a microorganism than a plant; c) the modified endophytic microorganism is not transmitted to progeny seed of inoculated plants, and stays restricted to the inner parts of the plant, which is more safe because of no dissemination to next generation or other plants; d) because endophyte can colonize many different plant like SJ-10, which can colonize in rice, wheat, radish or cabbage, it has wide applicability and could be used for many other plant species; e) endophyte can multiply highly inside the plant, often resulting in up to 10^6–10^8 CFU/g of inoculated plant, its anti-insect activity will be as high active as transgenic plant (Fahey, 1988, Fahey et al., 1991; Azevedo et al., 2000). As an alternative to transgenic plants, genetically engineered endophytic microbes might provide a pathway for plants to benefit from foreign genes. Engineered endophyte could improve the resistance of the plant to disease or insects, and could also help the plant to diminish the effect of environmental pollutants (Barac et al., 2004).

Homoptera including aphids, BPH, WBPH, and leafhopper cause serious damage in crops and vegetables around the world (Kerns and Gaylor, 1992; Dupo and Barrion, 2009). Plant lectins including GNA and PTA have obvious lethal action on Homoptera insects such as aphids, BPH and other leafhoppers (Hilder et al., 1995; Powell, 1993; Yao et al., 2003a; Zhang et al., 2003). Transgenic plant expressing PTA showed that PTA agglutinin has obvious inhibitory activity against aphids and planthoppers (Yao et al., 2003a; Zhang et al., 2003). Compared with chemical insecticides, plant lectins cause no contamination of the environment, are stable at high temperatures, and are low toxic to animals. Using plant lectins to control Homoptera pests have hence become of greater interest. But until now, as far as we are aware there is no report about transformation for plant lectin such as with the *pta* gene into endophytic bacteria to improve anti-insect activity of crop such as rice.

In order to express PTA in an endophyte, we isolated endophytic bacterium SJ-10, characterized as *E. cloacae*, from rice seedlings. When re-inoculating SJ-10 into rice seedling, the SJ-10 strain can be re-isolated from rice after 100 days. SJ-10 can also colonize many other crops like corn, wheat and radish. It has been shown that SJ-10 is stable and can colonize many different crop species, and can be used to express foreign protein in crops and vegetables to enhance their stress resistance, so improving quality and yield. In order to construct recombinant SJ-10 strain expressing PTA protein, the *P. ternate* agglutinin (PTA) gene was inserted into an expressing vector pP43NMK-pta and then cloned into endophytic bacterium SJ-10 for expression. The positive transformant, named as rSJ-10, could colonize rice plants and expressed PTA protein at the interior of rice detected with Western blot and SDS-PAGE assay. The results of anti-insect activity analysis showed that rSJ-10 could significantly decrease the survival and fecundity of WBPH fed rice seedlings. At day 19, the fecundity of WBPH inoculated with rSJ-10 was decreased by 86.1%. The anti-insect activity is same as transgenic tobacco expressing PTA (Yao et al., 2003a). From the standpoint of its ability to colonize plants as an endophyte, the modified *E. cloacae* strain SJ-10 would appear to be an ideal host to deliver PTA insecticidal proteins for crop and vegetable protection. Internal density levels of *E. cloacae* in plant tissue can reach as high as 10^6 CFU/g fresh weight. This will favor more PTA protein be expressed and produced in rice.

Here, PTA protein was expressed as an intracellular protein in the bacterial cells. We originally designed to secretory expression of PTA in SJ-10 for strongly improving the host anti-WBPH activity. A SacB signal peptide was added at the N-terminal of PTA protein. However, the signal peptide did not work here, so the PTA was only expressed as an intracellular protein in SJ-10. In the future work, we will try to use other method such as using the hemolysin secretion pathway (Fernandez et al., 2000; Li et al., 2002), or co-expression of bacteriocin release protein (BRP) gene (Fu et al., 2003; Lin et al., 2001). To avoid recombinant plasmid instability under field conditions, we will try to integrate the *pta* gene into the chromosome of *E. cloacae* using a mini-Tn7 transposon system for stable expression of PTA in plants. In addition to stable gene expression, chromosomal integration will obviate the need for selection pressure (Choi et al., 2006).

This is the first report of PTA being transferred into endophytic bacteria and re-inoculated as a recombinant endophyte into rice seedling. Control of WBPH was successfully achieved by expression of the plant lectin gene in SJ-10. It showed that the rice seedlings inoculated with rSJ-10 were endowed with the anti-pest activity against WBPH. Using recombinant endophyte to improve the plant resistance to WBPH is a new method to control WBPH, which is an important pest on rice. Our research highlights that the endophytic bacterium can be used as a living vector for expressing PTA to control WBPH in the future. This research has important implications for controlling sap-sucking pests with a genetically modified endophyte as a microbial biopesticide.

Now, our research is focus on test anti-insect activity of rSJ-10 at greenhouse. But, whether or not rSJ-10 could inhibit WBPH in the complicated field environment needs to be validated in future field

experiments, a subject of our future research. We will test whether the endophytic bacterium will endow the host with the ability against sap-sucking pests in field trials. By then, we can conclude whether this strain could be used to control WBPH as a new microbial biopesticide in the future.

As we mention before, plant lectin can coagulate mammal's red blood cell. We need to consider the possible toxicity of PTA expressed by endophytic bacteria. It is worthwhile to note that *G. nivalis* L. agglutinin (GNA), a mannose-specific lectin, proved to be non-toxic to mammals (Pusztai, 1991). Also, it was found to have negligible effects on the development, survival and fecundity of beneficial insects (Down et al., 2003). Both PTA and GNA belong to the monocot mannose-binding lectin superfamily, with three similar mannose-binding sites (Yao et al., 2003b; Hester et al., 1995, Hester and Wright, 1996). It is speculated that PTA may also be non-toxic to mammals, and the rice expressing PTA was safe to fed mammal. Further work is needed to investigate whether the rice plants expressing PTA are toxic to mammals before this endophytic bacterium was used to control WBPH in field.

We have proved that the recombinant endophytic bacteria expressing PTA can colonize rice stems and leaves. But, we have not yet determined whether this endophytic bacterium can colonize rice seeds. One report by Fahey (1988) and Fahey et al. (1991) showed that the genetically modified endophytic bacterium *C. xyli* subsp. *cynodontis* is not transmitted to progeny seed of inoculated plants. It is speculated that, after inoculated to rice seedling, the endophytic bacteria first enter root, and then transmit to stem and leaf, and may at the end arrive in the rice seed. In our future work, we will analyze whether endophytic bacteria could be isolated from rice seed, whether next generation rice still contains the endophytic bacteria. If it can be proved that the modified *E. cloacae* can not be transmitted to progeny seed, it will be safe to be used in commercial rice production.

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