Controlling sap-sucking insect pests with recombinant endophytes expressing plant lectin

We developed a novel pest management strategy, which uses endophytes to express anti-pest plant lectins. Fungal endophyte of *Chaetomium globosum* YY-11 with anti-fungi activities was isolated from rape seedlings, and bacterial endophytes of SJ-10 (*Enterobacter* sp.) and WB (*Bacillus subtilis*) were isolated from rice seedlings. *Pinellia ternate* agglutinin gene was cloned into SJ-10 and WB for expression by a shuttle vector, and YY-11 was mediated by *Agrobacterium tumefaciens*. The positive transformants were evaluated using PCR and Western bolt assay. We found that the recombinant endophytes colonized most of crops, and resistance of rice seedlings, which were inoculated with the recombinant endophytic bacteria, to white backed planthoppers was dramatically enhanced by decreasing the survival and fecundity of white backed planthoppers. Rape inoculated with recombinant endophytic fungi significantly inhibited the growth and reproduction of aphids. Results showed that the recombinant endophytes expressing PTA may endow hosts with resistance against sap-sucking pests.

Key words: endophyte; *Pinellia ternate* agglutinin; sap-sucking pest; *Myzus persicae*;

As a common way to control pests, chemical pesticides have been extensively used in agricultural industries for the past several decades, which, however, may cause serious environmental problems[1]. To reduce the use of chemical pesticides, enhancing the crop resistance to insects provides a more environmental friendly way, which is usually achieved by making transgenic crops with resistance to pests. Resistance genes like δ-endotoxin from *Bacillus thuringiensis* (Bt toxin) have been used to make pest-resistant transgenic crops in recent years[1]. However, it is well known that Bt toxin is only effective to Lepidopteran, Dipteran, and Coleopteran, but not to Homopteran (e.g., aphid and planthopper) that has caused severe losses of crop yields around the world[2].

Several transgenic crops such as transgenic cotton and rice expressing Bt toxin have already been planted in several countries to control Lepidopterous pests. However, studies showed that the pest population may change if transgenic crops are solely planted[3,4]. Particularly, Bt toxin expressed by transgenic plants only kills and/or inhibits Lepidopterous pests, but fails to control sap-sucking pests[2]. As a result, sap-sucking insect pests (e.g., red spiders, cotton plant bugs, and planthopper) are becoming the main threats to agricultural industries with the increasing plantation of transgenic crops expressing Bt toxin[3,4]. Despite that sap-sucking pests may cause severe losses of transgenic and traditional crops, there is still no effectual biopestcide currently available for controlling these pests.

Recent studies showed that some plant lectins were toxic to sap-sucking insect pests. For example, the lectin from snowdrop (*Galanthus nivalis* agglutinin; GNA) is toxic to planthoppers by binding itself to gut epithelium and then passing into haemolymph of pest[5]. The mannose-binding *Pinellia ternate* agglutinin (*pta*) gene was also found with significant insecticidal activities against Homopterans[6,7]. Insect bioassays showed that transgenic plants expressing PTA significantly inhibited aphid growth and exhibited considerable insecticidal activities against rice brown planthopper. However, it still remains controversial to use plant

lectins for making transgenic crops as they can agglutinate mammals' red blood cells. On the other hand, to our best knowledge, there are still no other insecticidal proteins, which are as adequately effective as plant lectins to control sap-sucking insects.

Endophytic microorganisms are those that inhabit in the interiors of plants, especially in leaves, branches, and stems, without apparent harms to the hosts[8]. The capability of colonizing internal host tissues makes endophytes a valuable tool for agricultural industries to improve crop performance. Some endophytic microorganisms have received considerable attentions in the last two decades due to their capabilities to protect hosts from pests and pathogens. The idea of using endophytes to deliver insecticide proteins was first proposed by Fahey et al. in 1991[9]. Lampel et al. used a bacterial endophyte of *Clavibacter xyli* subsp. *cynodontis* for the expression of insecticide protein of crylA(c) from *Bacillus thuringiensis* subsp. *Kurstaki* to control the European corn borer. The authors found that this genetically modified endophyte was toxic to the insect larvae in bioassays, and also discovered significant insecticidal activities in planta[10]. Downing et al. transferred *cry1Ac7* gene of *Bacillus thuringiensis* strain 234 into the endophytic bacterium *Herbaspirillum seropedicae* which was found in sugarcane for controlling the sugarcane borer *Eldana saccharina*, and showed this genetically modified endophyte could inhibit the growth of the sugarcane borer larvae to some degree[11]. In addition to endophytic bacteria, endophytic fungi are also found in the majority of currently known plants and can be isolated from plant tissues after strict surface sterilization. Endophytic fungi can be potentially used for improving plant growth, fixing nitrogen, and particularly biologically controlling pests and plant diseases due to their capabilities to habitat in plants after inoculation[12].

In a previous study, we have isolated two endophytic bacterial strains (WB and SJ-10) from rice.

WB was characterized as *B. subtilis* and SJ-10 was characterized as a strain of *Enterobacter* with 16s rDNA, morphological, physiological, and biochemical characteristics (unpublished data). The endophytic fungal strain of YY-11 was isolated from rape seedlings and characterized as *Chaetomium globosum* (unpublished data). The experimental results clearly showed that WB and SJ-10 improved plant growth, and YY-11 exhibited activities against several phytopathogenic fungi, including *Rhizoctonia solani*, *Botrytis cinerea*, *Fusarium oxysporum f. sp.Vasinfectum*, *F. graminearum, Sclerotinia sclerotiorum*, and *Botryosphaeria berengriana f.sp piricola* (unpublished data). All of these endophytic microorganisms could colonize different types of crops such as rice, wheat, maize, cotton, and radish (unpublished data).

In this study, we used endophytes to deliver and express PTA lectin for developing a novel strategy against sap-sucking pests. Different from transgenic plants, recombinant endophytes can enhance the resistance of the hosts to sap-sucking pests without changing other performances of the hosts. Endophytes usually exist in plants during their growing stages, but are absent in seeds[8]. Therefore, grain crops (e.g., rice, maize, and wheat) inoculated with recombinant endophytes expressing PTA lectin, are relatively safe to consume. We hypothesized that hosts inoculated with recombinant endophytes expressing PTA lectin would be endowed with the resistance to sap-sucking insect pests.

Results

Construction of recombinant endophytes. The plasmid pCAMBIA1301 is a vector usually used for transforming a foreign gene into plants and for making transgenic fungi[13]. The constructed recombinant pCAM1301-PTA was transferred into *A. tumefaciens* and then used for transferring YY-11. The positive transformants selected by hygromycin resistance were further

verified by PCR (Fig 2A). The total soluble proteins in the transformants were analyzed by SDS-PAGE, which showed that PTA was successfully expressed by rYY-11 with the same molecular weight as native PTA protein extracted from *P. ternate* corm (Fig 2B). This protein was then analyzed by Western blot, which showed that the protein was recognized by mouse anti-PTA serum (Fig 2C). These results indicated that PTA was successfully expressed by rYY-11 as a soluble protein.

To construct the recombinant endophytic bacteria, the *pta* gene was inserted into the pP43NMK vector and then used for transforming SJ-10 and WB. The positive transformants were selected by PCR (Fig 3A) and DNA sequencing. Then, the total soluble cell proteins and secreted proteins were analyzed by SDS-PAGE. The results showed that PTA was successfully expressed by SJ-10 as a soluble cellular protein and by WB as a secreted protein in rWB culture (Fig 3B). Western blot assay showed that PTA expressed by rSJ-10 and rWB also reacted with mouse anti-PTA serum (Fig 3C). The p43 promoter and SP signal peptide worked well for expressing PTA in rWB as a secreted protein. However, PTA protein was only expressed as an intracellular protein in rSJ-10, indicating that the SP signal peptide didn't work in this cell.

Recombinant endophytes colonizing crops. Like wild types, the recombinant endophytes could also colonize many crops. The results showed that rYY-11 colonized in rape, cotton, rice, wheat, cabbage, and radish for more than 1 month (Fig 4). Similarly, both rSJ-10 and rWB were respectively re-isolated from rice seedlings at an average of 5.5×10^4 and 2×10^4 cfu.g-1 of fresh tissue after one month of the inoculations (Fig 5). Moreover, rSJ-10 and rWB could also colonize other crops and vegetables such as wheat, radish, and cabbage for more than one month. These results showed that the recombinant endopytic fungi and bacteria could colonize various types of

crops, indicating a potential for controlling pests with piercing-sucking mouthparts on many crops in the future.

Inoculation of rYY-11 protecting hosts from aphids. Insecticidal activities against aphids were detected for the recombinant YY-11 expressing PTA. rYY-11 was used for the inoculation of rape seedlings. Aphids were then introduced on the seedlings and covered by a net for avoiding insect escape. As shown in Figure 6A, the number of aphids on the rape seedlings inoculated with rYY-11 was not significantly different from the one of the controls in the first two days. However, the number of aphids fed on the rape seedlings inoculated with rYY-11 was significantly different from that inoculated with wild YY-11 and PBS from Day 5. The rape seedlings showed an anti-aphid ability after the inoculation with rYY-11. The average number of aphids on the rape seedlings inoculated with rYY-11 was always less than that inoculated with wild YY-11 or PBS before day 25 ($p<0.001$). On Day 25, the average number of aphids on the seedlings inoculated with rYY-11 was only 27, which was significantly less than that on the seedlings inoculated with wild YY-11 (125 aphids per seedling) ($p<0.0001$) or PBS (167 aphids per seedling) ($p<0.0001$). This result indicated that the plant was endowed with the anti-aphid ability after inoculated with rYY-11. The number of aphids in the YY-11 group seemed lower than that in the PBS control group, but there was no statistically significant difference between these two groups, indicating that wild YY-11 had no anti-insect activity by itself.

In addition to the “no-choice” tests, in which aphids were not allowed to freely move among plants, we also detected the anti-aphid ability for seedlings in a greenhouse, where aphids could freely move among plants. The result showed that the number of aphids was significantly less on the rape seedlings inoculated with rYY-11 than that inoculated with wild rYY-11 or PBS (Fig 6B).

At Day 7, aphids were found to move onto plants inoculating rYY-11 and wild YY-11 from the rape seedlings with insects. During this period, the number of aphids was similar among the groups of rYY-11, wild YY-11, and PBS. However, from Day 14 until the end of the experiments, aphids on the seedlings inoculated with rYY-11 was significantly less than that inoculated with wild YY-11 or PBS. At Day 45, the average number of aphids was 9.2±5.3 (mean ± SD) on the seedlings inoculated with rYY-11, which was significantly less than that inoculated with wild YY-11 (116±13) ($p<0.0001$) or that inoculated with PBS (120.7±28.5) ($p<0.0001$), indicating that the rape seedlings inoculated with rYY-11 were resistant to aphids (Fig 6B&C).

Inoculation of rSJ-10 and rWB protecting rice from planthoppers. Rice seedlings were inoculated with rSJ-10 and insecticidal activities against planthoppers were detected. As shown in Figure 7A, there was no significant difference in the mortality rates among the nymphs fed on the seedlings inoculated with rSJ-10, wild SJ-10, and PBS in the early stage. After Day 11, *S. furcifera* began to produce offspring. On Day 13, the number of insects in the control group and wild SJ-10 group rapidly increased, but the number of insects in the rSJ-10 group only experienced a slight increase. On Day 19, the number of insects in all the three groups reached the highest amounts and the differences among those groups were very significant ($p<0.001$). The number of insects in the control group was 1.29 times of that in the wild SJ-10 group and 3.38 times of that in the rSJ-10 group. From Day 19, the number of insects in all the three groups began to decrease. On Day 22, the number of insects in the control group was 19.4 times of that in the rSJ-10 group. From Day 13 to Day 22, the number of insects in the rSJ-10 group was significantly less than that in the wild SJ-10 group ($p<0.001$) or PBS control ($p<0.001$). On Day 26, the seedlings of the control group and the wild SJ-10 group all died, but the seedlings of the rSJ-10

group still grew well (Fig 7C). Thus, after the inoculation with rSJ-10, rice seedlings were endowed with insecticidal activities against *S. furcifera*. Meanwhile, there was no significant difference in insect numbers between the wild SJ-10 group and the PBS control group, indicating that wild SJ-10 had no insecticidal activities against *S. furcifera*.

In another experiment, rice seedlings were inoculated with rWB and insecticidal activities were detected against planthoppers. After the inoculation with wild or recombinant WB, the number of *S. furcifera* fed on the rice seedlings started to gradually decrease from Day 10 (Fig 7B). After Day 13, the number of insects rapidly increased in the control group, while slowly decreased in the wild WB or rWB group. On Day 19, the number of insects in the control group reached the highest amount, which was 104.7 times of that in the rWB group and 58.2 times of that in the wild WB group. Difference among three groups was very significant ($P<0.001$). After Day 19, the number of insects in the wild WB group began to increase, but the number of insects in the rWB group still slowly decreased. On Day 22, the number of insects in the control group was 3.55 times of that in the wild WB group, and 98.7 times of that in the rWB group. On Day 26, all seedlings in the control group died, but the seedlings in the wild WB and rWB groups grew well (Fig 7C). This result showed that rWB could colonize rice to improve insecticidal activities of its seedlings against *S. furcifera*. The wild WB also showed some anti-insect activity, but future studies will be needed to elucidate its anti-inset mechanism.

Recombinant endophytic fungi antagonizing phytopathogenic fungi. On the basis of microscope, it was found that the hyphae of rYY-11 could attach to and wind around the hyphae of *S. sclerotiorum* (Fig. 8A), indicating that rYY-11 had an apparent mycoparasitism during the confrontation with *S. sclerotiorum*, which is consistent with previous studies[14]. Rape

seedlings pre-inoculated with rYY-11 resisted the infection of *S. sclerotiorum*, while seedlings in the control group were seriously infected and killed by this fungus (Fig 8B). This result indicated that YY-11 didn't lose its anti-fungal activity after being transferred with exogenous genes such as plant lectins.

Discussion

For the past several decades, the techniques, such as plant breeding and integrating foreign DNA into plant genomes to make transgenic plants, have been routinely used to enhance plant resistance to pests. However, these methods are generally costly, which may take years to be commercialized depending on plant species. Meanwhile, cloning insecticidal protein genes in transgenic plants provides an alternative way; however, its applications are generally limited in some monocotyledonous plants such as sugarcane, rice, sorghum, and maize. In comparison, beneficial endophytes can be also used to express and secrete useful products, but do not require the integration of foreign DNA into plant genomes. Therefore, this technique is of great use to introduce insecticidal toxin genes into the endophytes with the capability to colonized plants. Here, for the first time, we report the effects of an endophyte recombinant strain on Homopteran. We found that *Enterobacter* sp. SJ-10 and *B. subtilis* WB, and *C. globosum* YY-11 carrying the *P. ternate* agglutinin gene were toxic to white backed planthopper and aphids. The number of aphids and white backed planthoppers on seedlings was greatly suppressed by the endophyte recombinant strain infection.Therefore, plants inoculated with the recombinant endophytic bacteria or fungi contain plant lectin proteins and gain the resistance to insects.

Many endophytes have mutualistic relationships with their host plants, from which they obtain nutrients and in turn provide protection for the host plants from biotic and abiotic stresses.

Alkaloid produced by endophytes can significantly reduce herbivores[15-17]. For instance, some endophytes can produce toxic alkaloids such as *Neotyphodium coenophialum*, which exist inside Tall fescue (*Schedonorus phoenix* (Scop.) Holub) and greatly limits animal feeding and reproductive performance. In banana, naturally occurred endophytic *Fusarium oxysporum* antagonized nematode *Radopholus similis* in vitro through the production of nematode-antagonistic metabolites[18,19]. Inoculation of those endophytes into tissue culture plants resulted in improved plant growth and reduced nematode densities[18,19]. Other benefits of using endophytes may include increased nutrient uptake[20,21] or enhanced photosynthetic rates[22,23]. In our study, the endophytic bacteria *Enterobacter* sp. SJ-10 and *C. globosum* YY-11 exhibited no anti-insect activities. Only after transferring the *pta* gene into endophytes, the recombinant endophytes gained anti-insect activities. However, the anti-insect activities of wild *B. subtilis* WB were less obvious, which needs to be verified by future studies. Some endophytes with anti-insect activities against aphids, *Nilaparvata lugens*, and *Sogatella furcifera* will be isolated from plants in the future work.

Endophytes colonize an ecological niche similarly to that of the phytopathogenes. This could favor endophytes as agents for the control of pathogenic microbes[24]. Some endophytic bacteria or fungi have resistance to phytopathogenic fungi or can promote plant growth, such as *C. globosum* in this study, which showed an excellent anti-fungi activity. Here, the recombinant *C. globosum* YY-11 could reduce the infection of phytopathogenic fungi *S. sclerotiorum* to rape. rYY-11 was able to completely inhibit the development of pathogenic symptoms in the studied seedlings. So, the recombinant *C. globosum* YY-11 could be used to resist both *S. sclerotiorum* and aphids.

The results of the anti-insect detection study demonstrate the feasibility of using endophytic

bacteria or fungi, altered by the plasmid vector introduction of an insecticide-coding gene, for controlling an insect pest on a major agricultural crop. Here, anti-insect activities of the recombinant *C. globosum* YY-11 were similar to that of transgenic tobacco expressing *Pinellia ternata* agglutinin[6], implying that recombinant endophytes could substitute transgenic plants for controlling insects. It is important to note here that before being commercialized, the effectiveness of this technique for controlling aphids and white backed planthoppers still needs to be tested in the field, in which the effect of recombinant endophyte infestation on the yields of inoculated and uninoculated crops can be assessed. The future work involves the construction of strains expressing increased levels of plant lectins that are comparable to current commercial bio-control agents.

There is only a paucity of research that has been done on the suitability of endophytic bacteria as biocontrol agents. A cryL4(c) gene cloned from a *Bacillus thuringiensis* strain showing activity against the sugarcane borer, *Eldana saccharina*, was introduced into an isolate of *Pseudomonas fluorescens*, capable of colonizing sugarcane. Glasshouse trials indicated that sugarcanes treated with *P. fluorescens* were more resistant to eldana damage than those untreated sugarcanes[25]. *P. ternate* agglutinin has been expressed as inclusion bodies in *Escherichia coli* M15[26] but not in endophyte. The recombinant strain, when present at plant, was vulnerable to UV degradation and rain washoff. In contrast to the exposed state of microorganism following spray operations, PTA expressed by endophytic *C. globosum* and *B. subtilis* would remain packaged as an intracellular or intercellular protein within the cell, providing protection against UV effects and washoff. Field applications of insecticidal lectin proteins in recombinant *C. globosum* and *B. subtilis*, therefore, could have the benefit of a longer residual effect. From the standpoint of its

ability to colonize plants as endophytes, the modified *C. globosum* and *B. subtilis* strain would appear to be an ideal host to deliver *P. ternate* agglutinin insecticidal proteins for crop protection. In conclusion, we recorded experimental evidence that endophytes do play an important role in controlling pest insects. Insecticidal activities of bacterial and fungal endophytes recombinant strain on Homopteran were recorded in our study.

Endophytes enter plant tissues primarily through root; however, above-ground portions of plants, such as flowers, stems, and cotyledons, may also be used for entry[27]. Specifically, the bacteria enter tissues via germinating radicles, secondary roots, stomates, or as a result of foliar damage. Here, we found that YY-11 can enter plant tissues in the root (unpublished data). Hyphae of *C. globosum* can enter plant through interspace between root cells.

Endophytic bacteria and fungi are potential candidates for systematic delivery of biopesticides to a host plant without direct manipulation of the plant genome. Endophytes can reproduce in plant tissues and expressed plant lectin protein in plant tissue including stem, leaf, and roots, which can improve anti-insect activity of plants. Control of Homopteran was achieved by the expression of the plant lectin gene in the endophyte. This method may be used to express more useful proteins in the future. In a conclusion, our research confirmed that endophytic fungi and bacteria could be used as a delivery and expressing lectin against pests with piercing-sucking mouthparts. This research has important implications for controlling sap-sucking pests using a genetically modified endophyte as a biocontrol agent.

Acknowledgement

This work was supported by National High Technology Research and Development Program of China (2006AA 10A 210).

Methods

Endophytes. WB (*B. subtilis*) and SJ-10 (*Enterobacter* sp.) are endophytic bacteria isolated from rice, and YY-11 (*C. globosum*) is an endophytic fungus isolated from rape seedlings. All of these endophytes were reserved in our lab.

Construction of vectors and transformation of endophytes. Prokaryotic and eukaryotic vectors were constructed to respectively express PTA in endophytic bacteria and fungi. For the endophytic fungi, the *pta* gene was amplified by PCR from the plasmid of pT-pta containing *pta* gene (GU593718) [6] and then inserted into pCAMBIA1301 plasmid [13] to form a recombinant plasmid of pCAMBIA1301-PTA (Fig 1A). This recombinant plasmid was transformed into *Agrobacterium tumefaciens* and positive transformants were used for transforming YY-11 using the methods by[13]. The resistance of the positive transformants from fungi to hygromycin was further verified by PCR through amplifying *pta* gene.

For the endophytic bacteria, the *pta* gene was inserted into a *Bacillus subtilis* and *Escherichia coli* shuttle vector of pP43NMK to form a new plasmid of pP43NMK-PTA, in which the *pta* gene was fused at the N-terminal end with a signal sequence of SP, and *pta* was directed by a constitutively expressed P43 promoter[28]. The recombinant plasmid pP43NMK-PTA (Fig 1B) was transferred into SJ-10 according to the methods used for *E. coli* transformation, and into WB using the methods by[28]. The transformants of SJ-10 or WB were selected by Kanamycin resistance, PCR, and DNA sequencing.

SDS-PAGE analysis and Western blot assay. To detect whether PTA was expressed in the recombinant endophytic fungi, the selected positive transformants were further analyzed by the Western blot assay. Briefly, YY-11 transformant was frozen using liquid nitrogen and then

dissolved with phosphate buffer (pH 7.2). The supernatant was collected for analysis by 15% SDS-PAGE and then transferred onto nitrocellulose membrane. The membrane was blocked with 2% BSA at room temperature and then incubated overnight with mouse anti-PTA serum (reserved in our lab) at 4 ^{o}C. After being extensively washed by PBST (PBS containing 0.1% Tween 20), the membrane was incubated with goat anti-mouse IgG antibody conjugated with HRP (Sigma, USA) and then developed with TMB (3, 3′,5 ,5′-tetramethylbenzidine) for 15 min.

To detect whether PTA was expressed in the recombinant endophytic bacteria, the positive transformants of WB and SJ-10 were incubated at 37 ^{o}C overnight, respectively. Total soluble cell proteins and secreted proteins were then analyzed by 15% SDS-PAGE and the Western blot assay as described above.

Host specialization of recombinant WB, SJ-10 and YY-11. The used wild-type endophytic microorganisms can colonize different types of crops such as rice, wheat, maize, cotton, and radish. To detect whether the recombinant endophyte could still colonize in these crops, seedlings of rice, cotton, maize, and wheat were cultivated in pots. After two true leaves appeared, each seedling was inoculated with 1 ml ascospore suspension (10^6/ml) of the recombinant YY-11 (designated as rYY-11) or 1ml bacteria suspension ($8x10^6$ cfu/ml) of the recombinant SJ-10 (designated as rSJ-10) or WB (designated as rWB) by pouring seedling roots. After 100 days of the inoculations, the recombinant endophytic fungi and bacteria were respectively isolated from seedlings after strict surface sterilization, and then verified by PCR through amplifying the *pta* gene.

Anti-aphid activity of rYY-11 in rape seedlings. To detect whether the recombinant endophytic fungi YY-11 improve crop resistance against aphids, a total of 24 pots of rape

seedlings (3 seedlings in each pot) were randomly divided into 6 groups with 4 pots for each group, and then inoculated with 5 ml ascospore suspension (10^5/ml) of rYY-11, wild YY-11, or PBS for each pot. After another 15-day growth, three groups of rape seedlings inoculated with rYY-11, wild YY-11, or PBS were inoculated with 20 third-instar nymphs of aphids (*Myzus persicae*) for each pot, and then covered by an insect-proof net to avoid escape. After that, seedlings were maintained in a green house. The number of survived aphids in each group was counted every day for a total of 25 consecutive days.

To detect whether the recombinant endophytic fungi YY-11 protect rape from aphids, an experiment was carried out to mimic a natural environmental condition. Three groups of rape seedlings inoculated with rYY-11, wild YY-11, or PBS were grown in a green house for 10 days. These seedlings were not covered by an insect-proof net and randomly surrounded by other rape seedlings with aphids. These aphids would naturally move onto the inoculated seedlings. Within a total of 45-day growth under a normal condition, the number of aphids on each rape seedling was counted at Day 7, 14, 21, 28, 35 and 45.

Anti-planthopper activity of recombinant endophytic bacteria. Rice (*Oryza sativa*) seedlings were grown in pots filled with a mixture of sand, perlite, and nutritional substrate (1:2:1). Three seedlings were planted in each pot, and then inoculated with 2 ml cell suspension (1×10^8 cfu/ml) of rSJ-10 or rWB on the root of each seedling. Three days later, ten females and ten males of white backed planthopper adults, *S. furcifera*, were inoculated on seedlings in each pot. The seedlings were covered by an insect-proof net to avoid escape, and the number of insects on rice plants was accounted and analyzed for a total of 26 consecutive days. Seedlings inoculated with wild SJ-10, wild WB, and water were used as control experiments.

Anti-fungal activity of rYY-11. YY-11 exhibited activities against several phytopathogenic fungi. To detect whether the recombinant YY-11 also exhibit activities against phytopathogenic fungi such as *S. sclerotioru*, which is an important pathogen of rape. *S. sclerotiorum* was co-cultured with rYY-11 on a glass slide covered by a thin layer of PDA agar at 28 ^{o}C for 4 days. The interactions between those two fungi were determined by a phase contrast microscope. Rape seedlings (3 weeks old) were grown in pots with 3 seedlings in each pot, and then inoculated with 5 ml ascospore suspension (10^5/ml) of rYY-11. After 10 days of the inoculation, 20 mycelial discs (5 mm) of *S. sclerotiorum* were inoculated on the root and leaf of rape seedlings in each pot. Seedlings were maintained under controlled conditions (25 °C and photoperiod of 12 hours per day, and 90% relative humidity) for the development of rape sclerotinia rot. Control plants were inoculated only with PBS buffer ((g/l) NaCl, 8; KCl, 0.2; Na_2HPO_4, 1.4; KH_2PO_4, 0.24) and *S. sclerotiorum*. The symptoms were evaluated after 15 days of disease development.

Statistical analysis. Each experiment in this study had three repetitions. The statistical differences among groups were analyzed by a one-way analysis of variance (ANOVA) with a Tukey post-hoc test (SPSS 16.0 software).

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Figure legends

Figure 1. Schematic map of recombinant plasmid. (A) pCAM1301-PTA. Hygromycin resistance gene (*hygromycin* R) was used as a marker for screening positive transformant on PDA plate with

hygromycin. CaMV35S promoter was used for control expressing of lectin gene and hygromycin resistance gene. (B) pP43NMK-PTA. The exogenous gene was controlled by the p43 promoter, and the signal peptide SP was helpful to secrete the expressed exogenous protein into medium.

Figure 2. Analysis positive transformants of *C. globosum* YY-11. (A) PCR verification of rYY-11. Lane W, wild YY-11; lane M, DNA ladder of DL 2000 (Takara, Japan); lane 1-5, transformants of YY-11. Arrow directed the amplified DNA fragment about 750 bp. (B) SDS-PAGE analysis of recombinant *C. globosum* YY-11. Lane M, standard protein marker (97, 66, 45, 35, 27, 20, 14.4 kDa from up to down); lane P indicated PTA lectin extracted from *P. ternate*; lane 1-2, total cellular proteins of rYY-11; lane 3-4, total cellular proteins of wild YY-11. (C) Western blot analysis of positive transformant of *C. globosum* YY-11. Arrows directed the recombinant PTA protein expressed by rYY-11.

Figure 3. Analysis positive transformants of *Enterobacter* sp.SJ-10 and *B. subtilis* WB. (A) PCR verification of recombinant SJ-10 and WB. Lane M, DNA ladder of DL 2000; Lane 1, transformant of SJ-10; lane 2, transformant of WB; lane 3, positive control. Arrow directed the amplified DNA fragment about 750 bp. (B) Analysis of total cellular proteins expressed by SJ-10 with SDS-PAGE. Lane M, standard protein marker; lane 1, total cellular proteins of rSJ-10; lane 2, total cellular proteins of wild SJ-10. (C) Analysis of secreted proteins expressed by WB by SDS-PAGE. Lane M, standard protein marker; lane 1, secreted proteins expressed by rWB; lane 2, secreted proteins expressed by wild WB. D and E. Western blot analysis of PTA expressed by rSJ-10 and rWB, respectively. Arrows directed the recombinant PTA expressed by bacteria.

Figure 4. rYY-11 could colonize crops. (A) rYY-11 was re-isolated from inoculated crops after 1 month post inoculation. (B) PCR verified the rYY-11 re-isolated from crops. Lane M, DNA ladder

of DL 2000; lane 1-4, PCR amplified the *pta* gene from the YY-11 re-isolated from rape, cotton, rice and wheat, respectively; lane 5-6, positive controls. Arrow directed the *pta* gene about 750 bp.

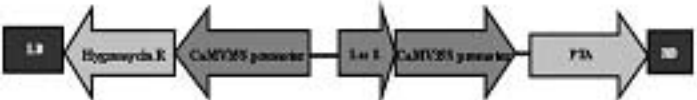
Figure 5. Recombinant bacteria in rice. (A) rSJ-10 and rWB was isolated from rice pre-inoculated these two bacteria post inoculation after 1 month. (B) PCR verified the bacteria re-isolated from rice. Lane M, DNA ladder of DL 2000; lane 1, positive control; lane 2, wild SJ-10 negative control; lane 3, rSJ-10 re-isolated from rice; lane 4, wild WB negative control; lane 5, rWB re-isolated from rice. Arrow directed the *pta* gene about 750 bp.

Figure 6. Anti-aphid activity of rYY-11. (A) Survival of aphid on rape seedlings inoculated with rYY-11. (B) Average number of aphid on each rape seedling in a mimicry natural environment. (C) aphids on rape leaf in a mimicry natural environment.

Figure 7. Anti-planthopper activity of rSJ-10 and rWB. (A) Insecticidal assay of rSJ-10 against *Sogatella furcifera*. (B) Insecticidal assay of rWB against *Sogatella furcifera*. (C) Rice seedlings attacked by *Sogatella furcifera*.

Figure 8. Anti-fungal activity of rYY-11. rYY-11 could infected into *S. sclerotiorum*, and induced the infected hyphae to be calabash-like (A) or enlargement (B) deformation. Rape seedlings pre-inoculated with the rYY-11 could also resist the infection of *S. sclerotiorum* (C).

A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CaMV35S promoter
CaMV35S promoter
PTA

B


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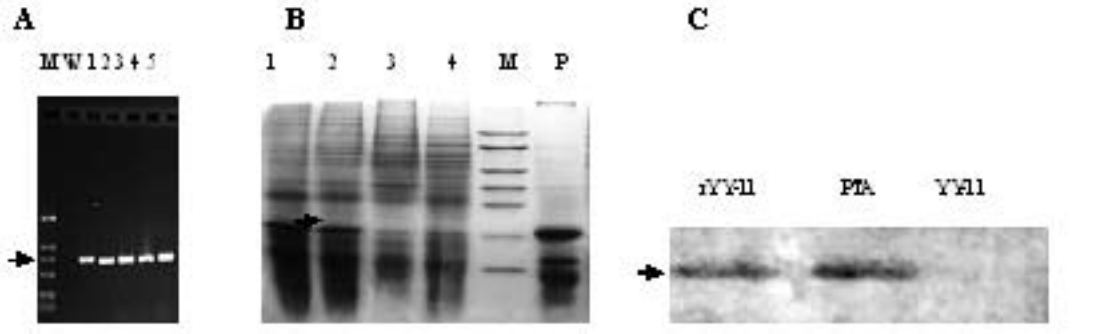
Hind III
Km
Pa2
SP
pta
ori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2
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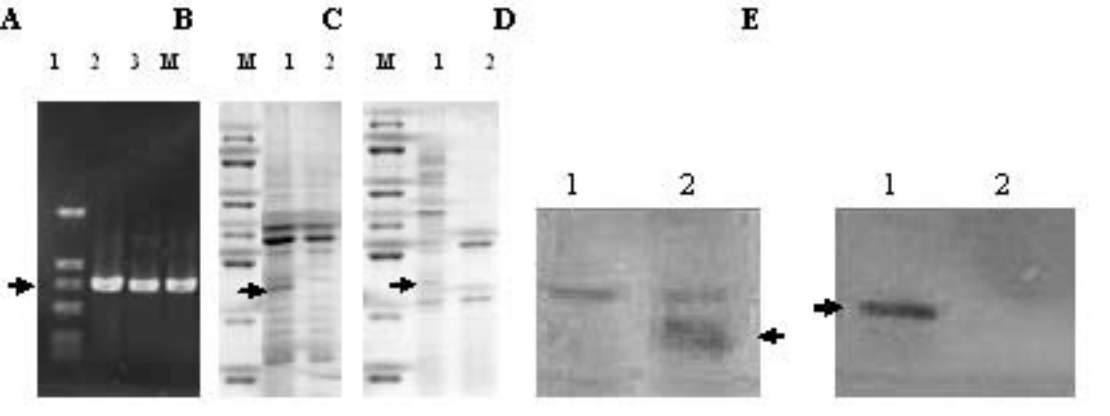
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C
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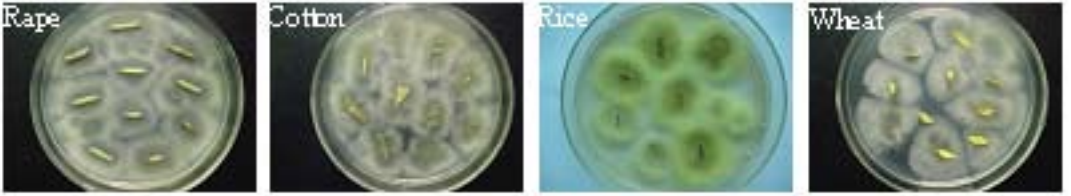
1 2

A



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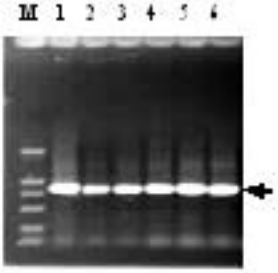

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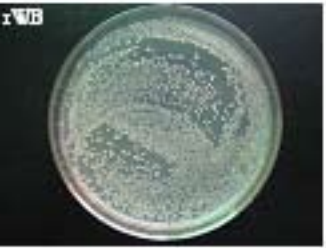

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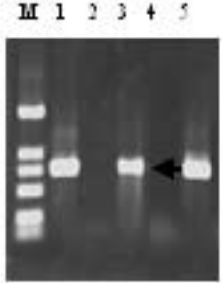

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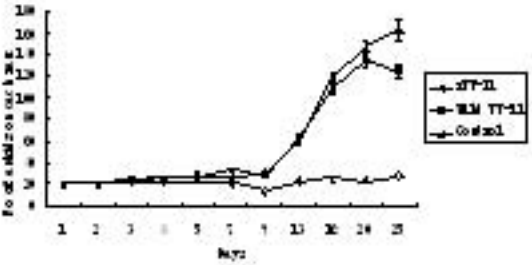
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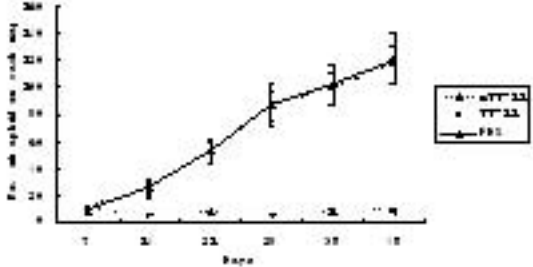
WM TY-11
Control
Days

B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TY-11
PBS
Days

C


Control
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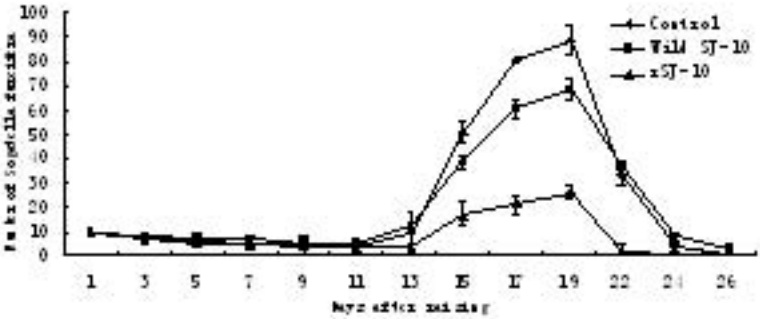
rTY-11

A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rSJ-10
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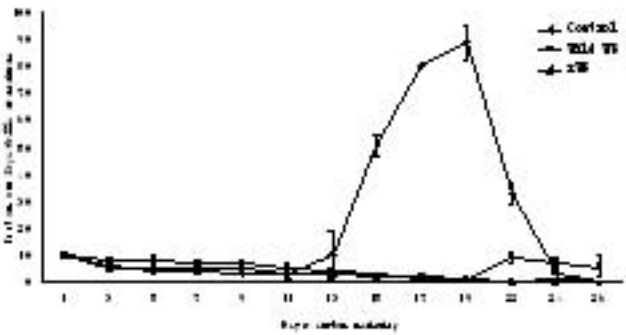
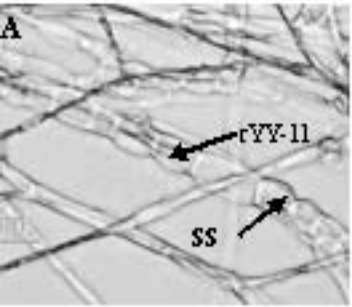
9
11
13
15
17
19
22
26

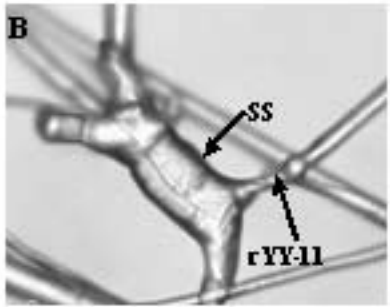
B


Control

C


WM SJ-10
rSJ-10
Control
WM WB
rWB





C