NOTES

Resistance to rice blast (*Pyricularia oryzae*) caused by the expression of trichosanthin gene in transgenic rice plants transferred through agrobacterium method

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Abstract The gene of trichosanthin has been transferred into rice plants through agrobacterium method. The single copy insertion and the expression of foreign gene have been proved in

regenerated plants. In antifungal assay the degrees of rice blast (*Pyricularia oryzae*) infection of the transgenic plants expressing trichosanthin and expressing *GUS* gene as control have been evaluated. The differences such as the time of disease symptom observed, the number of infected plants and damaged leaves, the growth of infected plants of the two transgenic plants after being inoculated by rice blast (*Pyricularia oryzae*) are significant. The transgenic plants with trichosanthin gene grew faster than the plants with *GUS* gene, even when humidity environment was removed. The results show that the transgenic plants that expressed trichosanthin are able to delay the infection of rice blast compared with the plants as control. In addition, no damage caused by the expression of trichosanthin gene in transgenic plants has been observed.

Keywords: trichosanthin, antifungi, agrobacterium, rice.

Ribosome inactivating proteins (RIPs) strongly inhibit the synthesis of proteins by removing a specific adenine residue from a conserved stem loop in the 28S rRNA. The depurinated ribosomes are unable to bind the EF-2/GTP complex, so the protein synthesis is blocked at the translocation step[1]. Different RIPs display various activities on intact ribosomes from various organisms. Usually mammalian ribosomes are much more sensitive to RIPs than ribosomes from plants[2]. The expression of RIPs in transgenic plants results in the resistance to infection of some plant viruses and fungi according to recent reports[3]. The RIPs from barley, pokeweed and maize have expressed successfully in tobacco and these transgenic plants showed antifungal activity[1,4]. The enhanced resistance against fungal disease through coexpression of barley RIPs and chitinase (or glucanase) in transgenic plants was also obtained[5] . However, most of these work was carried out in dicot plants instead of monocot such as rice.

There are two classes of RIPs. Type I has only a single chain polypeptide but Type II is a two-chain protein. A chain of Type II is similar with Type I and B chain has lectin properties[6]. Now Type I protein was used for many resistance tests of transgenic plants. Trichosanthin, a kind of Type I RIP isolated from the root tuber of Chinese herb medicine *Tricosanthes kirilowii Maxim*, has 56% of homology compared with the A chain of Ricin[7]. The expression of trichosanthin gene in transgenic tobacco plants has antivirus activity[8, 9]. The previous work in our laboratory showed that trichosanthin had a broad-spectrum antifungal function *in vitro*[10] and was able to resist the infection of *Alternaria longipes in vivo*. We also found that in the transgenic rice plants that expressed trichosathin through bombardment the resistance to rice blast infection has been enhanced.

The advantage of agrobacterium method over bombardment is that many obtained transgenic plants only have a single copy insertion of foreign gene. The transgenic plants with single copy insertion should be useful in genetic analysis of progeny and the breeding in the field later. Here the transgenic rice plants with single copy were obtained through the agrobacterium method and the antifungal activity of them was evaluated.

1 Materials and methods

(i) Plasmid and agrobacterium strain. The 750 bp DNA fragment of trichosanthin gene excluding leading sequence was cloned by our previous work[7]. ATG start coden was added to 5′ terminal of the fragment through PCR. Then the fragment flanked by the cauliflower mosaic virus 35S promotor and nos terminator was inserted into pCambia1300 to obtain pC1301-HY (fig. 1). Plasmid pCambia1301 containing a *GUS* gene was selected as control. Both pCambia plasmids containing a hygro- mycin resistant gene as transgenic rice plants screen and agrobacterium strain EHA105 were kindly provided by Dr. Richard Jefferson of Cambia Centre, Australia.

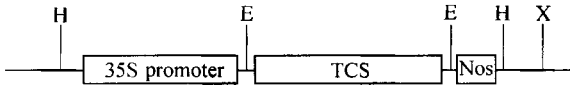

Fig. 1. Expression construct of trichosanthin gene in pC1301-HY. 35S promoter, Cauliflower 35S promoter; TCS, trichosanthin gene; Nos, nos 3′ terminator; E/H/X, *Eco*R I /*Hin*dIII/*Xba* I site. *Xba* I has a single site in pC1301-HY.

(ii) Tissue culture and transformation of rice plants. Japonica rice Zhonghua 8 was provided by Dr. Zhang Wenjun of Institute of Genetics, the Chinese Academy of Sciences. The protocol of tissue

culture was the same as described in ref. [11]. Agrobacterium-mediated transformation method was carried out according to Hiei's report[12].

(iii) Southern and Western blot assay. The isolation of plant total DNA and total proteins were carried out as previous report[11]. Total DNA was digested by *Xba* I and then used for Southern blotting. Southern blot assay (the probe was labeled by α^{32}P-dCTP) was carried out according to the protocol of zeta membrane provided by Bio-Rad Company. The probe was 750 bp trichosanthin gene digested with *Eco*R I in pC1301-HY. Western blot assay was carried out according to the method described in ref. [11]. Anti-TCS serum was prepared by our laboratory.

(iv) Antifungal assay. *Pyricularia oryzae* strain conserved by our laboratory was cultured on potato medium with 10% of glucose for 9 d at 28℃ in the dark. Then the plate was washed by sterile water and continued culturing under light and humidity for 2 d. The spores were washed down by sterile water and counted before being sprayed. The transgenic plants were sprayed with the spores every 3 d. The spray began in the concentration of 1×10^6 spores 20 d after the transgenic plants were transferred into soil. The transgenic plants inoculated with *Pyricularia oryzae* were kept under high humidity environment and the infected symptom was observed 2 weeks later.

2 Result

(i) Transformation of rice. 47/218 (21.6%) of hygromycin resistant rice calli was obtained in 4 weeks after agrobacterium inoculation. The calli were subcultured for 3 weeks on fresh medium and then transferred into pre-regeneration and regeneration medium subsequently. Regenerated plantlets were obtained from 39/47 hygromycin resistant calli. While the plantlets rooted on phytohormone-free medium, they were transfe- rred into soil for Southern and Westhern blot assay later.

(ii) Southern and Western blot assay. Plant total DNA was digested with *Xba* I which had only a single site in pC1301-HY. Fig. 2 shows that the single fragment hybridized with 750 bp trichosanthin gene exists in 5 transgenic rice plants (T1 — T5). Western blot assay showed that trichosathin gene was expressed in these plants (fig. 3). The regenerated plants from the 5 clones were selected for antifungal tests.

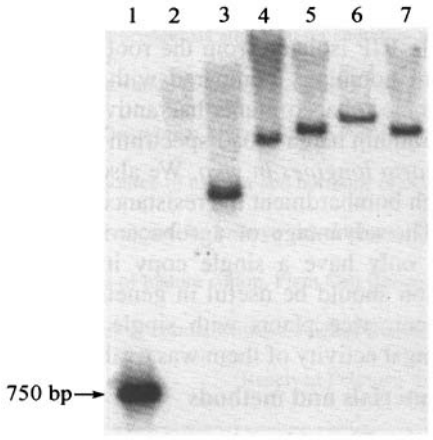

Fig. 2. Southern assay of transgenic rice plants with trichosanthin gene. 1, 750 bp trichosanthin fragment; 2, transgenic rice plants with pCambia 1301; 3—7, transgenic rice plants with pC1300-HY (T1—T5).

(iii) Antifugal tests. The spore suspension of *Pyricularia oryzae* was sprayed out on 13 transgenic plants from the 5 clones (T1—T5) with trichosanthin gene. 10 transgenic plants transferred by pCambia1301 were as control. In 2 weeks the disease symptom was observed on the leaves of control plants. Among them 7 plants showed yellow and brown chlorotic lesion caused by rice blast infection. Meanwhile no disease symptom was observed on the leaves of transgenic plants with trichosanthin gene. One week later disease symptom could be seen on leaves of the 10 control plants. Among them 3 leaves of 1 plant, 2 leaves of 7 plants and 1 leaf of 2 plants were infected. However, no infected symptom was observed on 2 transgenic plants with trichosanthin gene. Among the other 11 infected plants with trichosanthin gene, 3 plants had 2 leaves infected by *Pyricularia oryzae*, others had only a leaf infected (table 1). The degrees of disease symptom had also a great difference between transgenic plants with

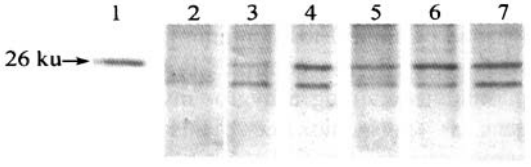

Fig. 3. Western assay of transgenic plants with trichosanthin gene. 1, 26 ku trichosanthin protein expressed in *E. coli*; 2, transgenic rice plants with pCambia 1301; 3—7, transgenic rice plants with pC1300-HY (T1—T5).

trichosanthin gene and with *GUS* gene (fig. 4).

Table 1 Resistance of transgenic plants to infection of *Pyricularia oryzae*

Transgenic rice	No. of plants	Period after inoculation/week	No. of infected plants	Infected degree of a single plant		
				3-leaf	2-leaf	1-leaf
Control (with pCambia1301)	10	2	7	0	0	7
		3	10	1	7	2
		4	10	3	5	2
Plants (with pC1301-HY)	13	2	0	0	0	0
		3	11	0	3	8
		4	13	0	6	7

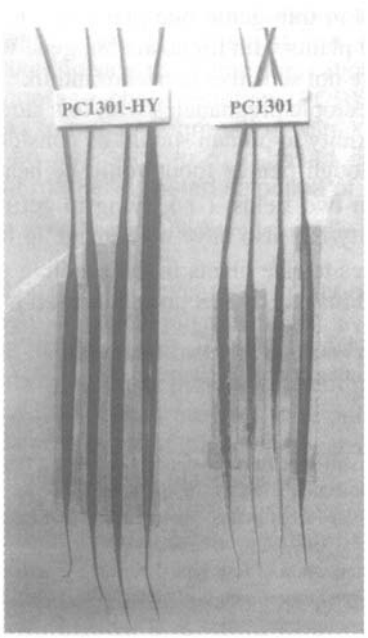

Fig. 4. Comparison of infected symptom between transgenic rice plants with trichosanthin gene and *GUS* gene. Left: Transgenic rice plants with pC1301-HY; right: transgenic rice plants with pCambia1301.

Fig. 5. Growth of transgenic plants with trichosanthin and *GUS* gene 6 weeks after inoculation of *Pyricularia oryzae*. 1 and 2, Transgenic rice plants with pCambia1301; 3 and 4, transgenic rice plants with pC1301-HY.

After removing humidity environment, new shoots from these plants were not able to be infected seriously by *Pyricularia oryzae* again, but the infected leaves began to wither in 4 weeks. Transgenic plants as control were observed to grow more slowly than the transgenic plants with trichosanthin gene. The great difference between the growth of transgenic plants with trichosanthin gene and controls could be observed at this time (fig. 5). However, the harvest period of uninfected transgenic plants with trichosanthin gene was similar to uninfected control plants (data not shown here).

3 Discussion

An alternative method for controlling fungal disease in modern agriculture is to develop plants with a broad-spectrum resistance to fungal pathogens by genetic engineering[5]. RIPs isolated from various plants have such resistance[1,4,13,14]. Trichosanthin has 56% of homology with the A chain of Ricin[7] and has resistance to some plant viruses[8]. Our previous work showed that trichosanthin had a broad range of resistance to various fungi *in vitro*[10] and *in vivo*. Here we firstly reported that transgenic rice plants with single copy insertion of trichosanthin gene obtained through the agrobacterium method

had resistance to rice blast infection.

Southern blot assay confirmed that transgenic rice plants contained a single copy of trichosanthin gene transferred through agrobacterium. The expression of this gene in the transgenic plants was also proved by Western blot assay subsequently. The infection of rice blast in transgenic plants with trichosanthin gene was delayed. The time of disease symptom observed, the number of infected plants and damaged leaves, and the growth of infected plants showed great differences between transgenic plants with trichosanthin gene and with *GUS* gene after they were infected by rice blast (*Pyricularia oryzae*). The results showed that the antifungal activity was not caused by randomly inserted foreign gene and the trichosanthin gene can be used as a candidate gene for plant genetic engineering.

The expression of some RIPs in transgenic plants has toxicity to host plants as previous reports. For example, the high-level expression of pokeweed antivirus protein resulted in chlorotic lesion in transgenic plants, although no serious damage was observed at the low-level expression of this gene in host plants[1]. Lam et al.[8] also reported that the expression of trichosanthin gene somewhat affected plant growth. However the expression of trichosanthin gene in transgenic rice plants has no observed damage to host growth. The harvest period of transgenic rice plants with trichosnathin gene was similar to that of control plants with *GUS* gene. Maybe rice plants are not sensitive to trichosanthin.

Clinically trichosanthin is used to interrupt normal and ectopic pregnancies with few side effects[2], but it has side effects in antivirus test. Therefore possible toxicity to human should be considered while utilizing transgenic rice plants with trichosanthin gene, although rice as food would be heated before entering human digest system. Our next work will focus on two fields: (i) trying to get mutants of trichosanthin gene, which not only keep its antifungal activity but also have no damage to human and animals; (ii) because rice blast has the most serious damages to rice plants in the seedling period, the specific promoter only expressed in rice seedling can be used instead of 35S promotor used in this note.

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