260—269

30164(13)

第12卷第3期 中 国 病 毒 学 Vol.12 No.3
1997年9月 VIROLOGICA SINICA Sep. 1997

Resistance to Rice Stripe Virus Conferred by Expression of Coat Protein in Transgenic Indica Rice Plants Regenerated from Bombarded Suspension Culture*

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悬浮细胞再生表达外壳蛋白的转基因 Indica 水稻对水稻条纹病毒的抗病性

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提 要 合成、克隆了水稻条纹病毒中国株的外壳蛋白基因并进行了序列分析,由 Indica 水稻成熟胚的愈伤组织形成胚性悬浮细胞。用含有 CP 基因的 pROK2 表达载体的 DNA 包被 1.09 μm 直径钨粉颗粒轰击培养细胞。被轰击的培养物在含有 G418(40 mg/mL)的培养基中进行选择培养,由对 G418 抵抗的愈伤组织中获得 10 株再生株。用 ^{32}P – dCTP 标记的 CP 基因作为探针,以 Southern blot 测定其转化特性。由抗病的和对照的植株抽提基因组 DNA 用 EcoRI 和 BamHI 进行酶切,其中两个植株显示出 0.6 kb 和 0.7 kb 两条杂交带,其大小与 CP 基因相对应。Western blot 和 ELISA 测定进一步证明 CP(32 kDa)在转基因水稻中表达。16 株转基因植株和 100 株对照植株用带毒的叶蝉接种,接种病毒后 24 d 只有 37.5% 的 CP 转基因植株产生病毒症状,而对照植株为 96%。进一步证明转基因水稻植株具有对 RSV 的抗病性。转基因植株 T1 代 CP 的表达分离比例为 3.6:1。

关键词 转基因 Indica 水稻,悬浮细胞转化,外壳蛋白,抗病性,水稻条纹病毒

转基因籼稻;

Abstract The coat protein (CP) gene of a Chinese isolate of rice stripe virus (RSV) was synthesized, cloned and sequenced. Embryogenic suspension cultures were initiated from calli which derived from Indica rice mature embryo. For bombardment of cell suspension culture, tungsten particles of diameter of 1.09 μm were coated with DNA of the expression vector pRoK Ⅱ harbouring CP coding sequence downstream CaMV 35S promoter. Bombarded cultures were selected by growing in the medium containing G418 (40 mg/mL). Ten plantlets regenerated from G418 resistant calli were analysed by Southern blot

Date of receiption: 1996 – 09 – 13, date of correction: 1997 – 02 – 26

* This work was supported by grant RF 95001 # 309 from the Rockfeller Foundation

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using ^{32}P – dCTP labelled CP gene as probe. The genomic DNAs extracted from resistant as well as the control plants were digested with EcoRI and BamHI. Samples from two plants showed two hybridization bands with the sizes of 0.6 kb and 0.7 kb corresponding to the RSV CP gene. Western blot and ELISA further demonstrated that RSV CP (32 kDa) was expressed in transgenic rice plants. Sixteen transgenic plants and 100 control plants were fed by planthoppers carrying RSV. Only 37.5% of the CP gene transformed plants developed viral symptoms compared to 96% of control plants 24 days after inoculation with RSV, indicating that the transgenic rice plants were engineered with resistance to RSV. The CP expression of T1 progenies from the transgenic rice plants segregated into 3.6:1 ratio.

Key words Transgenic Indica rice, Bombarded cell suspension, Coat protein, Resistance, Rice stripe virus

Rice stripe virus (RSV) is a type member of the Tenuvirus, which is transmitted by *Laodelphax striatellus* and three other species of planthopper, and causes epidemic disease in rice-growing areas of China, Japan, Korea and the Commonwealth of Independent States. In China the disease occurred in Jiangsu, Zhejiang, Shanghai, Shangdong, Beijing, Liaoning, Yuannan and Taiwan. During epidemic years the disease could cause 10～20% loss of harvest, and in severely infected areas the losses could reach up to 60～70%. RSV infects many species of the *Gramineae* such as rice, barley, maize, wheat, oat and wild grasses. RSV has four species of single-stranded RNA and four species of double-stranded RNA, which are named RNAs 1～4 in order of decreasing molecular sizes[1,2]. In infected rice plants exist 2 proteins, one is coat protein encoded in RNA3 and the other is major nonstructural protein (NS) which is encoded in RNA 4. Many rice varieties used by farmers are susceptible to RSV disease, therefore, it is necessary to engineer rice plants with viral resistance. The expression vector containing the CP gene of RSV Chinese isolate was used for transforming rice suspension culture via bombardment. The transgenic rice plants thus obtained exhibited certain extent of resistance to RSV infection.

Materials and Methods

1 Virus, planthopper, rice and tobacco variety

A Chinese isolate of rice stripe virus (RSV-C) was used and maintained in barley. RSV-C is a severe strain, which causes leaf chlorotic stripe in rice at early stage and leaf necrotic stripe in the late stage of infection. A colony of viruliferous brown planthopper (*Laodelphax striatellus*) was cultured on barley. Every two weeks the viruliferous planthoppers were transferred to healthy barley seedlings, because seedlings fed by the leafhoppers became severely infected and eventually died. Annong s-1 variety (Indica type) of rice was selected from 18 rice varieties including Japonica and Indica rices, which produce highly embryogenic suspension culture. Tobacco variety used was *Nicotiana tabacum* var. G140.

2 Rice supension culture

The fine embryogenic suspension culture was obtained from rice seeds.

3 Synthesis, cloning and sequencing of RSV-CP gene

The total RNAs were extracted from purified RSV by SDS-phenol and proteinase-K. Poly A were added to the 3′-terminal of RSV-RNAs. The reaction was catalysed with polyA polymerase and incubated at 37℃ for 10

min. The first and second strand of DNA were synthesized by using BRL cDNA synthesis kit.

After being digested with restriction endonuclease, RSV CP gene fragments were subcloned into M13 mp18, 19. CP gene sequence was determined by dideoxynucleotide chain termination method.

4 Construction of expression vector

The recombinant clone (pUC19－146) harbouring RSV－C CP gene was digested by Kpn I and BamHI to recover sequence containing RSV CP gene. Plant expression vector pROKII was digested by KpnI and BamHI and ligated with RSV CP gene by T4 ligase (Fig. 1).

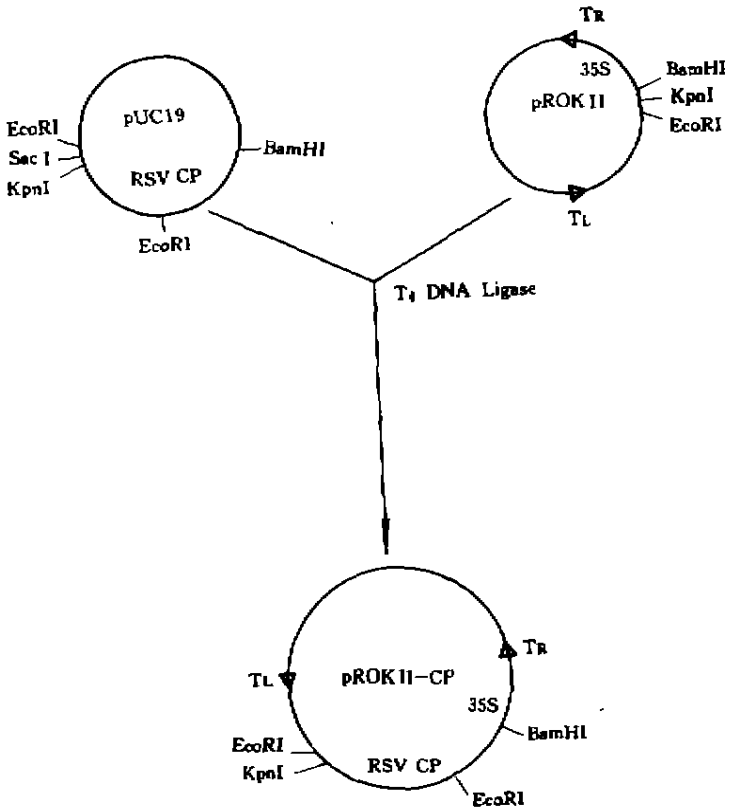

Fig 1 The construction of plant expression vector harbouring RSV CP gene

5 Transformation of rice suspension culture

The plasmid harbouring RSV CP genes was extracted from *E. coli* MC1022 basically as described by Sambrook *et al*[3], and purified by PEG precipitation method. Plasmid DNA was sterilized by precipitating from ethanol and dried in a laminar－flow hood for an hour. The DNA was suspended in 0.1 × TE at a concentration of 1 μg/μL. 25 μL of tungsten particles and pipetted into 1.5 mL Eppendorf tube, to which 2.5 μL DNA solution, 25 μL 2.5 mol/L $CaCl_2$ solution, 10 μL 0.1 mol/L spermidine (free base) were added and finger vortexed. The mixture was left at room temperature for 10 minutes. After spinning, 30 μL supernatant was discarded. The coated microcarriers mentioned above would be enough for 3 bombardments. Immediately prior to loading microcarriers onto macrocarrier, the mixture was finger vortexed.

6 Bombardment and regeneration

On the aperture of the centre of stopping plate 2.5 μL of the microprojectile solution was carefully placed. About 50 mg suspension culture was placed in a filter paper in a petri dish with diameter of 7.5 cm. The culture was dispersed to form a thin layer. Pressure in sample chamber was 0.1 atm and speed of macrocarrier was 450 m/s. Bombarded tissue was washed once with 3 mL fresh medium and then transferred to a flask containing 10 mL of fresh medium with agitaion at 26 ℃.

After 2 days, bombarded culture was transferred to 10 mL of fresh medium with G418 (40 mg/mL). Every week the culture was subcultured once to fresh medium containing G418. Being subject to G418 selection for 2 months, white and fast-growing clones were emerged from brown culture. The resistant clones were transferred to differentiation medium (N6 inorganics, MS vitamins, BAP 2 mg/L, NAA 0.1mg/L, 3% maltose, 0.7% agar) for plant regeneration under continuous light at 26 ℃.

7 Southern blot

Extraction of plant genomic DNA was carried out according to the method described by Junghans *et al*[4]. The DNA was digested with BamHI and EcoRI and seperated in agarose/TAE gel electrophoresis system. DNA denaturation, transferring to zeta-probe blot membrane, immobilization and hybridization reactions were performed according to the procedure of Bio-Rad. The fragment containing the RSV CP gene and with the size of 1.3 kb was recovered from recombinant plasmid pUC19 digested with BamHI and SacI, and used as probe for hybridization after ^{32}P-dCTP random elongation labelling[5].

8 Western blot

Western blot was carried out according to our previous methods[6].

9 ELISA

The accumulation of CP in transformed plants was detected by PAS-ELISA as described by Edwards *et al*[7] using a polyclonal rabbit antiserum against RSV. The absorbance value was detected at 490 nm.

10 Analysis of virus protection in transgenic plants

The transgenic and control nontransgenic regenerated rice plants were inoculated with RSV-C-carrying brown leafhopers in the same cage. ELISA assay showed that most of the adult leafhoppers carried RSV-C. After inoculation for 6 days under natural light, the plants were removed from the cage and sprayed with insecticide to kill the planthopper. After 10 days the typical chlorotic and stripe symptoms were developed in control rice plants. The disease incidence was measured on a scale of 0—III. Disease grade 0 means that the plants grew normally and there were no chlorotic stripes in the leaves. Disease grade I means that there were sparsely chlorotic stripes in the leaves and the growth of plants was basically normal. Disease grade II means that plants were stunted, and there were significant chlorosis in the leaves, especially young leaves. Disease grade III means that the young leaves became chlorotic, curved or even dried, the plants ceased growing and died off finally.

Results

1 Establishment of embryogenic rice suspension culture and its sensitivity to various antibiotics

To obtain embryogenic and finely dispersed rice suspension is an important precondition for transformation experiments. Two months after the "Annong" seeds were transferred to callus induction medium, small amounts of bright brown colored callus, as well as firm callus, were derived from the scutellar region. The fast-growing, compact and white-yellow embryogenic callus would be recovered from the original induced callus being reformed in the medium containing

3% sorbitol. The suspension culture was initiated by inoculating this embryogenic callus into suspension medium on a shaker (120 r/min) at 26 ℃ in the dark. The finely dispersed suspension culture would be obtained in about six months. When the suspension culture was transferred to differentiation medium, a large number of green spots would be emerged in the rapidly amplifying cell aggregates. If the cell aggregates with green spots were further cultured in differentiation medium, green plantlets would be developed in most of the aggregates. To select transformed cells, and to determine the sensitivity of rice suspension cells to antibiotics, the cultures were exposed to various concentrations of kanamycin, G418 and hygromycin. The suspension cultures were treated with different antibiotics for 20 days and then transferred to differentiation medium. Table 1 showed the effect of different antibiotic treatments on cell growth and differentiation. G418 is most effective in inhibiting cell growth and differentiation. The culture treated with G418 (40 mg/mL) for 20 days turned dark－brown. When the culture thus treated was transferred to antibiotic－free medium for 1 month, the culture remained dark－brown and without recovering. Hight concentration of kanamycin (200 mg/mL) did not substantially inhibit cell growth. When the suspension culture treated with kanamycin was transferred to kanamycin－free medium, there were some growth of the treated suspension culture. When the recovering cell aggreagates were transferred to differentiation medium, only limited amounts of albino plants derived. Hygromycin at a concentration of 100 mg/mL was not effective for inhibiting the growth of non－transformed cell. The culture treated with hygromycin not only recovered growth to great extent, but also differentiated green spots or plantlets when being transferred to differentiation medium. This indicated that "Annong" suspension culture was very resistant to hygromycin, therefore, in this study NPT－II gene was employed as a selective marker and G418 was chosen as a selective antibiotic.

Table 1 The sensitivity of Annong rice suspension culture to various antibiotics*

Antibiotics (mg/mL)	No. of green spot	The amount of calli growth (mg fw)
Kanamycin 200	0	50
G418 40	0	10
Hygromycin 100	2	140
Control	30	1060

* Having been selected by G418 for 20 days, 10 mg of the suspension culture was transferred to differentiation medium and cultured at 26 ℃ under continuous light for 1 month. The growth and differentiation state were then determined and recorded. The results were means of three replicates.

2 Gene transferring

To optimize the conditions for gene transferring using bombardment method, plasmid pAct－D was employed to study GUS transient expression[8]. The plasmid pAct－D harbours GUS coding sequence driven by rice pAct－1 promoter. It was reported that the content of GUS protein in transgenic rice plants transformed with pAct－D can be as high as 3.2% of the total soluble protein[9]. "Annong" suspension culture was bombarded with tungsten particles coated with pAct－D DNA and cultured at dark for 2 days. The bombarded culture was then stained with X

– Gluc and a great number of transient GUS expression cell can be detected under microscope. There were no blue cells in the suspension culture unbombarded. Therefore, the bombardment we used can effectively deliver exogenous gene into rice cell. It is expected that tungsten particles coated with pRoKII harbouring RSV CP gene could also be used to transform suspension culture by the similar approach. One month after "Annong" suspension culture being consecutively selected by G418 (40 mg/mL), the suspension turned significantly brown. After further consecutively selecting by G418 for one month, there would be small amounts of white and living cell aggregates in the severely dark – brown cultures. The white cell aggregates were transferred to antibiotic – free medium, they amplified greatly. While the unbombarded culture was selected under the same conditions for one month, the culture would turn significantly brown. Two months later the suspension culture became dark – brown and there would be no white cell aggregates emerging from the brown culture. Even though the brown culture was transferred to G418 – free medium for a month, there was no recovering cell clone visible.

The resistant clones thus selected were transferred to differentiation medium under continuous light at 26 ℃, at the time of cell amplifying, there emerged many green spots which may develop into buds. When the cell aggregates containing buds were transferred to hormone – free medium, roots would developed. When the plantlets reached 5 cm high, the cover of flasks were removed to harden plantlets which would grow well in the greenhouse. We have already obtained more than 10 independent green rice plantlets from G418 resistant cell clones.

3 Coat protein gene of RSV – C

About 63 AMPs were added to the 3′ – terminal of RSV – C RNAs, the reaction was carried out by addition of 1 unit polyA polymerase and incubated at 37 ℃ for 10 min. cDNA was synthesized by using mercury hydrooxymethyl denatured polyA – virual RNAs. The sizes of 1st and 2nd – stranded cDNA were from 0.5 to 3 kb. After transformation about 500 white clones were obatined, among them 300 clones were selected for single colony electrophoresis on agarose gel, 150 clones with larger inserts, and 50 clones were hybridized with RNA probe. ELISA screening of recombinant clones revealed that only clone 146 has positive reaction. The result was confirmed by Western blot. Sequence analysis indicated that RSV – C CP gene consists of 966 bp with 97% sequence homology to the Japanese isolate[10].

4 Expression of coat protein gene in transgenic plants

4.1 Southern blot assay of RSV CP gene in transgenic rice plants

Genomic DNA was extracted from 10 independently regenerated rice plants from G418 resistant cell aggregates and digested with EcoRI and BamHI. Southern tests were performed by transferring digested DNA into Zeta membrane and using ^{32}P dCTP labelled RSV CP gene as a probe. The purified RSV CP gene digested with EcoRI/BamHI in the Southern blot assay demonstrated clearly 2 hybridization bands with the sizes of 0.6 kb and 0.7 kb, respectively (Fig.2). The genomic DNA from regenerated plant No.3 and plant No.10 were digested by restriction enzymes EcoRI/BamHI, in the Southern blot assay there were also hybridizing bands

with the size corresponding to the purified RSV CP gene. While the genomic DNA from the suspension rice culture without being bombarded gave rise no hybridizing signal in Southern blot assay. It was proved that the RSV CP gene has been integrated into genome of regenerated rice plant No. 3 and plant No. 10. The genomic DNA of the other 8 regenerated plants from G418 resistant clones, however, gave no positive signal of hybridizing to RSV CP probe.

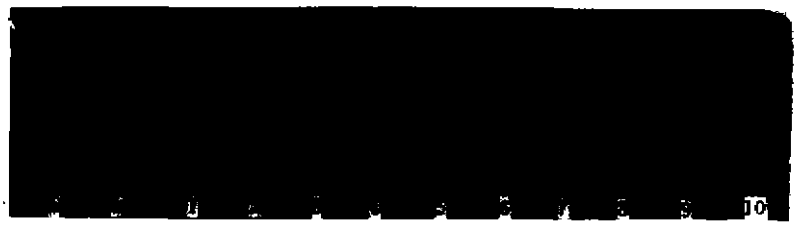

Fig. 2 Southern blot assay of rice plants regenerated from G418 resistant calli. The probe is $\alpha-^{32}$P dCTP labelled RSV CP gene. a. Purified RSV CP gene digested by EcoRI/BamHI b. Untransformed rice plant. 1 – 10. The rice plants regenerated from G418 resistant calli.

4.2 Western blot assay of the expressed RSV CP in transgenic rice plants

The two transgenic rice plantlets with CP gene insertion identified by Southern blot (see above) were transferred to hormone – free medium under continuous light for amplification. About one month later numerous tillers were grown surrounding the plantlets. The newly – derived plantlets were separated from the mother plants and transferred to fresh medium. The plantlets thus obtained were hardened and then transferred to pots in greenhouse. Total soluble proteins were extracted from the transgenic plants. The purified RSV particles showed very strong reaction against CP antibody (32 kD). The protein extracts from untransformed plants showed no reaction to RSV CP antisera in the corresponding position (32 kD). We have assayed 10 plants derived from the Southern blot positive parental plants by Western blot. Among them 8 rice plants showed positive signals in the CP corresponding regions. In figure 3, we demonstrated that 4 plants were Western blot positive. Therefore, the RSV CP gene was precisely expressed in most of the plants with CP gene integration.

Fig 3 Western blot assay of transgenic rice plants. a. RSV CP; b. Untransformed control plant; c – f. Transgenic rice plants.

4.3 ELISA test of the expressed RSV CP in transgenic rice plants

RSV CP gene expression in transgenic rice plants was also studied by ELISA (Table 2). Significant amounts of CP protein can be detected in leaves from transgenic plants estimated by absorbance at OD_{490}. The extract of untransformed plant was set as a control. The percentages of CP protein in total soluble protein in transgenic leaves were 0.22～0.41% estimated according to the standard curve of virus CP concentration as well as the soluble protein content of the leaves.

Table 2 Expression of RSV CP genes in transgenic rice detected by ELISA

Transgeneic gerotype	OD_{490} in ELISA assay of plant extracts	Percentage of CP in soluble protein of plants (%)
Untransformed control rice plant	0.08	0.00
pROK Ⅱ – CP Transgenic rice – 3	0.16	0.22
pROK Ⅱ – CP Transgenic rice – 10	0.17	0.41

5 Virus protection in transgenic rice plants

The assay of rice plant resistance to RSV was studied by feeding viruliferous *Laodelphax striatellus* on young rice plants to be tested. Control plants were the plantlets regenerated from unbombarded suspension culture. One hundred control rice plants and 16 transgenic rice plants derived from plants showing CP expression in fourth – leaf stage had been inoculated in a nylon – mesh cage by viruliferous planthoppers. The planthoppers in the cage were unrested 2 times each day to enable the planthoppers to evenly distributed in plants. In each rice plants there were approximately 2 – 3 vector insects feeding on. The plants were inoculated by planthoppers for six days and sprayed insecticide to kill the insects. After 10～12 days, the control plants developed typical chlorosis symptom (Table 3). Twenty four days post inoculation feeding, most of the control untransformed plants developed stripes in leaves, the disease index (DI) being 53%. Fourty days post inoculation feeding, most of the control plants showed severe symptoms, being stunted, the leaves developing brown necrotic streaks, with the DI being 88%. While the transgenic rice plants subjected to inoculation feeding developed no or mild symptoms. The disease index of the transgenic plants is 15% and 25% respectively 24 and 40 days post inoculation feeding (Table 3 and Fig.4).

Table 3 Resistance to RSV in transgenic rice plants expressing CP

Transgenic genotype	24 days post inoculation feeding					40 days post inoculation feeding				
	No. of plants of each disease grade				Disease index	No. of plants of each disease grade				Disease index*
	0	Ⅰ	Ⅱ	Ⅲ		0	Ⅰ	Ⅱ	Ⅲ	
pROK Ⅱ – CP	10	5	1	0	15	7	7	1	1	25
Untransfored control	4	50	29	17	53	3	7	13	77	88

6 Expression and resistance in progenies of transgenic rice plants

T1 progenies of transgenic rice plants were obtained, and their CP expression and resistance to RSV were tested. Table 4 showed the CP expression in transgenic rice plants detected by Western blot. The CP expression of T1 progenies from the transgenic rice plants segregated into 3.6:1 ratio. Most of the progeny plants expressed CP showed delay of stripe symptom expression.

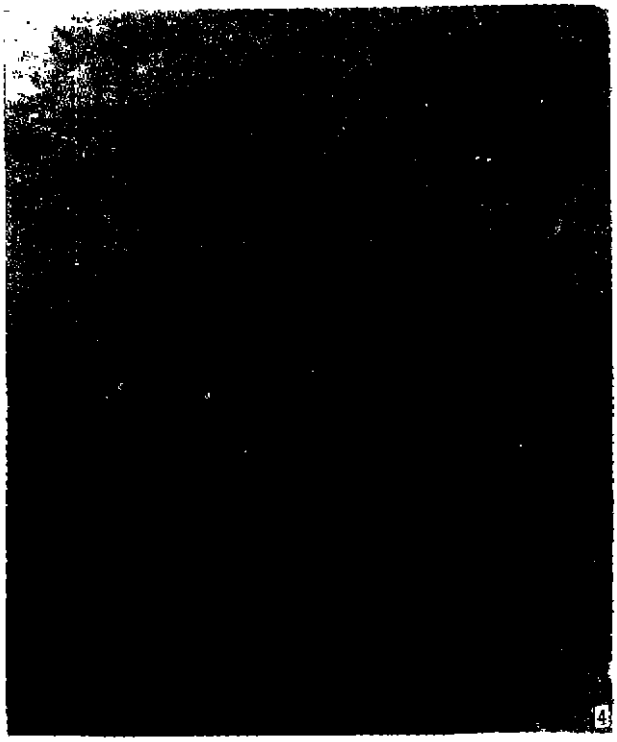

Fig 4 Comparison of resistance to RSV between transgenic rice plants (right) and untrangsenic control (left) after 24 days post inoculation

Table 4 RSV－CP expression in T1 progenies of transgenic rice plants

Catalog number of transgenic plants	Total numbers of test	Number of plants expressing CP	Number of plants not expressing CP	Expressing CP / not expressing CP
t－3	42	33	9	3.60:1
t－10	39	32	7	3.55:1
Untransformed control	50	0	50	0

Discussion

Hayakawa *et al*[11] reported that the CP gene of a Japanese isolate of RSV was introduced into two varieties of Japonica rice by electroporation of protoplasts. We have introduced RSV CP gene into Indica rice genome via biolistic process and the CP gene expressed in transgenic rice

plants. The transgenic rice plants expressing CP gene show significant level of viral resistance in comparison with the nontransgenic plants. Biolistic mediated plant transformation technique has some advantages over the protoplast system, it is simple, efficient, without strict dependence on certain genotype as protoplast mediated transformation method. The gene we introduced into rice plants is not only selectable gene but also gene encoding functional protein which may confer viral resistance. This results lay foundation for further rice genetic engineering for crop improvement. From the ELISA results the CP expression level was not very high, only 0.22～0.41% of the total soluble protein, which was approximately one third of the CP level in transgenic tobacco plants. Therefore, it is necessary to increase CP expression in transgenic rice plants. Enhancers can be employed to enable the CaMV 35S promoter directed high expression[9].

Acknoledgemant We are grateful to Professor R. Wu for kindly providing plasmid pAct－D.

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