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H. H. Zheng · Y. Li · Z. H. Yu · W. Li · M. Y. Chen
X. T. Ming · R. Casper · Z. L. Chen

Recovery of transgenic rice plants expressing the rice dwarf virus outer coat protein gene (S8)

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Abstract The coding region of the eighth largest segment (S8) of the rice dwarf virus (RDV) was obtained from a RDV Fujian isolate. It was then cloned into pTrcHisA for expression in *E. coli* and into vector pE3 for plant transformation. By using callus derived from mature rice embryos as the target tissue, we obtained regenerated rice plants after bombardment of the former with plasmid pE3R8 containing the RDV S8 gene and the marker gene neomycin phosphotransferase (NPT II). Southern blotting confirmed the integration of the RDV S8 gene into the rice genome. The expression of the outer coat protein in both *E. coli* and rice plants was confirmed by western blotting. The recovery of transgenic rice plants expressing S8 gene is an important step towards studying the function of the RDV genes and obtaining RDV-resistant rice plants.

Key words RDV · Segment S8 · Rice · Transgenic plants · Particle bombardment

Introduction

Rice dwarf virus (RDV), the pathogen causing rice dwarf disease, is a member of the phytoreovirus genus, family of Reoviridae (Fauquet and Martelli 1995). It infects graminaceous plants inducing dwarfing and white flecks on plant leaves, and replicates in its natural vectors, *Nephotettix cincticeps* and *Recilia dorsalis*. It is transmitted by these two leafhopper species through their eggs but not through the seed of the host plants. The virus genome is composed of 12 dsRNA segments that are designated S1–S12 in increasing order of mobility in polyacrylamide gel (Boccardo and Milne 1984). The eighth largest segment (S8) encodes a 46-kDa outer coat protein (Suzuki and Sugawara 1991). Since the reoviruses themselves are not mechanically transmissible, the extracted dsRNA preparations without the essential RNA-dependent RNA polymerase would not be infectious. There is no good way to introduce infectious virus transcripts or cDNAs driven by 35S promoter directly into plants by inoculation. The expression of viral genes *in vivo* through transformation could be the most convenient way to study the functions of viral genes and to obtain virus-resistant plants.

Rice (*Oryza sativa* L.) is one of the most important crops in the world. Biolistic-mediated transformation has proven to be an effective transformation method in cereals, such as in rice (Christou et al. 1991), maize (Gordon-Kamm et al. 1990), wheat (Vasil et al. 1992) and oat (Somers et al. 1992). Many transgenic rice plants expressing exogenous genes, such as GUS, HPH (Li et al. 1993; Christou et al. 1991), and BAR (Cao et al. 1992), have been recovered using this method. The transformation of RDV outer coat protein (S8) using electroporation was mentioned by Matsumura and Tabayashi (1995) without further analysis. In this paper, we report the cDNA cloning of the outer coat protein gene (S8) from RDV and the introduction, stable integration, as well as expression of this gene in rice plants.

Materials and methods

Two japonica rice (*Oryza sativa* L.) varieties, 'Zhonghua 8' and 'Zhonghua 10', which are commercially important cultivars in

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H. H. Zheng (✉) · Y. Li · Z. H. Yu · W. Li· M. Y. Chen · X. T. Ming · Z. L. Chen
The National Laboratory of Protein Engineering and Plant Genetic Engineering, Peking University, Beijing 100871, China

Y. Li (✉) · Z. L. Chen
De Montfort University, The Gatoway Leicester, LE1 9BH, UK

R. Casper
Federal Biological Research Centre for Agriculture and Forestry, Institute of Biochemistry and Plant Virology, D-38104 Braunschweig, Germany

Chinese agriculture were used for transformation. The RDV used in this experiment was a Fujian isolate (Gao et al. 1990). Plasmid pGEM3Zf(−) was used as the cloning vector and *E. coli* DH5α as the host strain. Standard molecular manipulations were carried out as described by Sambrook et al. (1989).

cDNA synthesis, cloning, and expression in *E. coli*

RDV and viral RNA was extracted as described by Gao et al. (1990). RDV genomic RNAs were analyzed on 1.25% agarose gel electrophoresis, and S8 RNA was recovered from the gel. The dsRNA denaturation, and cDNA synthesis and amplification were carried out as described by Li et al. (1994). Two primers, which were designed as 5′ end primer 5′-CAA$\underset{\underset{Bgl\text{II}}{}}{\overset{(A)\quad(G)}{AGATCT}}$CCACCTGCCACTATG-3′ (complementary to minus strand 2–26 nt with two nucleotide exchanges) and 3′ end primer 5′-GCG$\underset{\underset{Xho\text{I}}{}}{\overset{(T)(G)}{CTCGAG}}$ATTCAGGACCG-3′ (complementary to plus strand 1377–1396 nt with two nucleotide exchanges), were used in cDNA synthesis and amplification.

The polymerase chain reaction (PCR) products were digested with *Bgl*II and *Xho*I and cloned into linearized pGEM3Zf(−) vector by the same two enzymes, yielding the recombinant plasmid pGR8. For expression of RDV S8 in *E. coli*, the RDV S8 gene fragment was released from pGR8 with *Bgl*II/*Xho*I and inserted into *Bam*HI/*Xho*I-linearized expression vector pTrcHisA. A total of 114 nonviral nucleotides including a 5′ ATG were thus added to the 5′ end of the RDV S8 gene fragment. The resulting recombinant plasmid pTrcAR8 was used to transform *E. coli* DH5α. Extracts both from bacteria transformed with the pTrcAR8 and from bacteria carrying an empty vector were analyzed by western blotting.

Construction of the plant expression vector

The RDV S8 gene fragment was released from pGR8 with *Bgl*II/*Xho*I and inserted into the plant transformation vector pE3 linearized by the same two restriction enzymes. The resultant recombinant plasmid pE3R8 contains the NPT II gene that confers resistance to G418. In this construct, the RDV S8 gene fragment was driven by the 35S promoter of cauliflower mosaic virus (CaMV) in a sense orientation and nopaline synthase (nos) was used as a terminator (Fig. 1).

Explants for transformation

Calli derived from mature rice embryos were used as the explants for particle bombardment. Dehusked mature rice seeds were surface-sterilized with 20% NaOCl for 15–20 min and rinsed three times with sterile water. The seeds were then placed on GM medium for callus induction. Primary calli or short-term subcultured calli were used as target tissue for bombardment.

Plant culture media

N6 medium (Chu et al. 1975) was used for rice cell culture with the following supplements: proline (500 mg/l), enzymatic casein hydrolysate (300 mg/l) and sucrose (30 g/l). The pH of the medium was adjusted to 5.8 before autoclaving. The callus induction and growth medium (GM) consisted of the modified N6 medium supplemented with 2 mg/l 2,4-D; the regeneration medium (RM) contained 3 mg/l BAP and 0.5 mg/l NAA in the modified N6 medium. MS (Murashige and Skoog 1962) medium without hormone was used for plantlet growth. Sorbitol (0.4 *M*) was added to the GM medium for the osmotic treatment of callus during bombardment (4 h before bombardment and 12–16 h after bombardment, respectively).

Biolistic transformation

Forty to fifty pieces of calli were placed in the center (2–3 cm in diameter) of each petri plate containing the osmotic treatment medium. The plasmid DNA was coated onto gold particles with an average size of 1.0 μm following the procedures described in the device instructions. Bombardments were performed with a PDS-1000/He Biolistic Device (BioRad). Bombardment parameters were as following: helium pressure, 1100 psi; gap distance, 1 cm; fly distance, 6 mm; target distance, 6 cm. After bombardment, the calli were transferred to GM medium.

Selection and regeneration

After culture for 1 week on GM medium, the calli were transferred onto the same medium containing 50 mg/l G418 (Sigma). After 4 weeks, visible fresh G418 resistant ($G418^r$) calli were moved to fresh selection medium containing 100 mg/l G418. After approximately 6–8 weeks of strong selection with 100 mg/l G418, the $G418^r$ calli were transferred onto the regeneration medium containing 100 mg/l G418. Regeneration was performed under fluorescent illumination with a 16 h photoperiod. About 2 weeks later, the first somatic embryos appeared, and then shoots and roots developed. The shoots were transferred onto MS medium without hormone. As soon as the shoots were 6–10 cm long, they were transplanted into soil in the greenhouse and grown to maturity.

DNA isolation, PCR amplification and Southern analysis

Genomic DNA was extracted from the leaf and stem tissues of the transgenic and control rice plants according to Dellaporta et al.

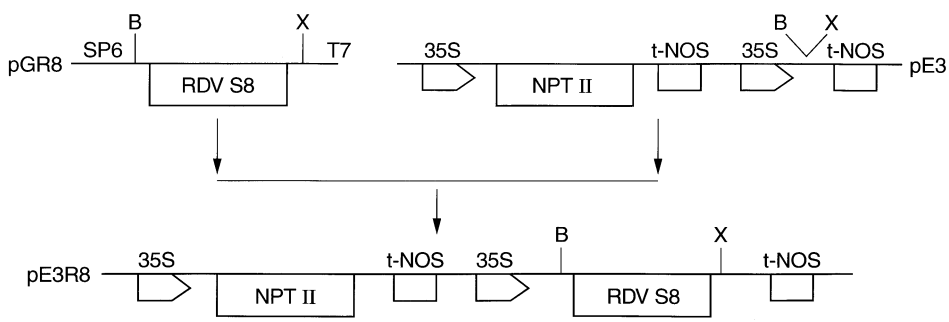

Fig. 1 Construction of the plant expression vector pE3R8. *B Bgl*II, *X Xho*I

(1983) with minor modifications. The presence and integration of the RDV S8 gene in transgenic rice were verified by the polymerase chain reaction (PCR) and Southern analysis.

The PCR assays were carried out as described by Li et al (1994), and the amplified products were analyzed on a 1.2% agarose gel.

For Southern analysis, the genomic DNA, either undigested or digested with *Eco*RI and *Eco*RV, and the plasmid pE3R8 DNA were separated on a 0.8% agarose gel in 1 × TAE. The samples were transferred to Zeta-Probe GT Blotting membrane (BioRad) using the alkaline capillary procedure. Hybridization conditions were performed according to the manufacturer's instructions (BioRad). A 1.4 kb fragment of the RDV S8 coding region was cut from pGR8 with *Bgl*II/*Xho*I and purified via agarose gel electrophoresis. The S8 fragment was labelled with α-[^{32}P]-dCTP using a random primer labelling kit from Promega.

Detection of RDV S8-encoded product by western blotting

For protein extraction, 400 mg of rice leaves (freshweight) was ground in 500 µl extraction buffer (0.625 *M* TRIS-HCl, pH 6.8, 10% SDS, 13% β-mercaptoethanol, 26% glycerol), and the extracts were heated to 100°C for 5 min and centrifuged at 13 000 rpm for 10 min. The supernatant of the extracts was mixed with 1 volume of 2 × SDS loading buffer and boiled for 2 min. The samples were applied onto a 5%/12% SDS-polyacrylamide disk gel. Electrophoresis, transfer of proteins to one nitrocellulose membrane (Amersham), and immunological detection of RDV S8 product were carried out as described by Li et al. (1995). A rabbit anti-RDV S8 antibody and alkaline phosphatase conjugated anti-rabbit IgG (Promega) were used as primary and secondary antibodies, respectively.

Results

Cloning and expression of the S8 gene in *E. coli*

To study the expression of RDV gene in *E. coli*, we extracted total proteins from 3 ml of an overnight culture of recombinant bacteria carrying pTrcAR8. The immunological reactivity of the expression products was detected by western blotting with antiserum against intact RDV. Due to the 114-nonviral nucleotide insertion in the frame of the RDV S8 ORF 5′ end, a slower comigrating peptide was detected in the expression products when these were compared with the outer capsid protein from partially purified RDV virions and RDV-infected rice total proteins (Fig. 2). In addition to the major peptide band at 46 kDa encoded by S8, a fast moving band was also detected in the *E. coli* expression products. This band was also detected in partially purified RDV virions and RDV-infected rice total proteins.

Recovery of G418 resistant rice plants

A preliminary study for determining G418 dose for effective selection showed that when G418 was added to the GM medium at a concentration of 50 mg/l, callus growth was strongly reduced; at 100 mg/l G418 significantly inhibited callus growth during the 3 following weeks of culture. After bombardment with plasmid pE3R8, the calli were first moved onto G418-free GM medium for 1 week and then transferred to the GM medium with 50 mg/l G418. Three or 4 weeks later, actively growing calli were initiated. No G418-resistant callus developed in the control calli, which had been bombarded without pE3R8. After subculture for 3–4 weeks, the new calli were propagated on the GM medium with 100 mg/l G418, then transferred to regeneration medium. Two weeks later, somatic embryos appeared, and then shoots and roots developed. As soon as the shoots were 6–10 cm long, they were transplanted into soil in the greenhouse and grown to maturity. G418 was also added to the regeneration and plantlet growth media at a concentration of 100 mg/l. The results of these three separate experiments are summarized in Table 1.

DNA integration analysis

Plants regenerated from G418-resistant cell clusters were designated as T_0 plants. Seeds of self-pollinated T_0 plants gave rise to T_1 plants.

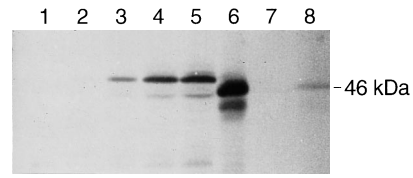

Fig. 2 Western blot detection of the RDV S8-encoded product in extracts from different sources with antiserum against intact RDV particles. *Lane 1* Extract from bacteria transformed with the empty vector pTrcHisA and induction for 2 h, *lane 2* extract from bacteria transformed with pTrcAR8 without IPTG induction, *lanes 3–5* bacteria transformed with pTrcAR8 with IPTG induction for 1, 2 and 3 h, respectively, *lane 6* extract from partial purified RDV viron, *lanes 7 and 8* total protein extract from healthy (*7*) and RDV-infected rice (*8*)

Table 1 Frequencies of selection and regeneration of G418 resistant rice plants

Experiment	Number of calli bombarded	Number of G418 resistance cell clusters	Number of plant lines with G418 resistance	Number of plant lines confirmed as transformants
1	160	47	5	5
2	80	23	3	3
3	240	40	14	11

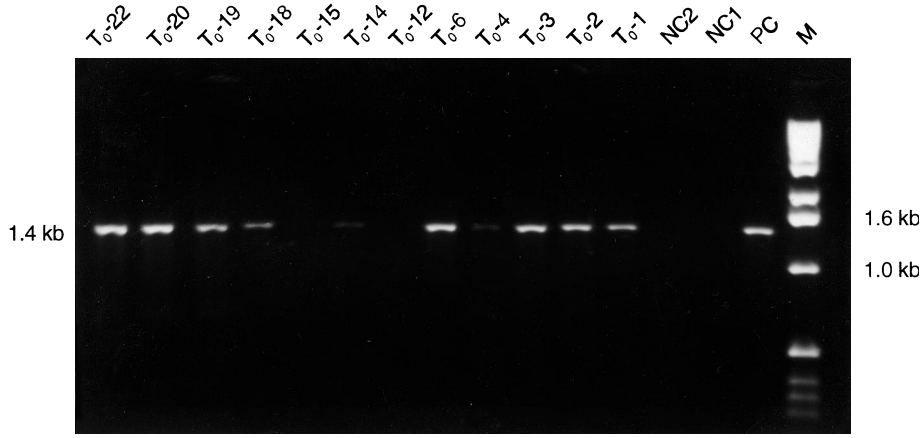

Fig. 3 PCR analysis of putatively transformed rice lines. Ethidium bromide-stained agarose gel of PCR-amplified DNA for the RDV S8 gene (1.4 kb). *M* 1 kb DNA ladder, *PC* plasmid pE3R8, *NC1* and *NC2* nontransformed rice plants, *T_0-1 to T_0-22* different putatively transformed rice lines

Total DNA from the putatively transformed plants were analyzed by PCR using primers specific to the coding region of the RDV S8 gene. Of the 22 T_0 plants that survived selection 19 showed the expected size (1.4 kb) of the amplified DNA fragment. Fig. 3 shows the result of PCR assays of 12 putative transgenic plants.

Southern blot analysis was carried out to confirm the presence and integration of the RDV S8 gene in the genome of transgenic rice lines that showed positive reactions in PCR assays. Genomic DNA from 2 plants (T_0-2 and T_0-4) were either undigested or digested with *Eco*RI or *Eco*RV. A high-molecular-weight hybridizing band in undigested genomic DNA from T_0-2 and T_0-4 demonstrated the presence of the RDV S8 gene. When the genomic DNAs were digested with *Eco*RI (which cuts internally in the S8 gene), an expected fragment of 800 bp together with other fragments hybridized with the S8 probe. When the genomic DNAs were digested with *Eco*RV, T_0-2, and T_0-4, they showed different hybridization patterns with the RDV S8 probe. These results suggested that the RDV S8 gene had intergrated into the genomes of the transgenic rice plants derived from two separate transformation events. As expected, neither undigested nor digested genomic DNA from nontransformed plants hybridized with the α-[^{32}P]-labelled RDV S8 probe (Fig. 4).

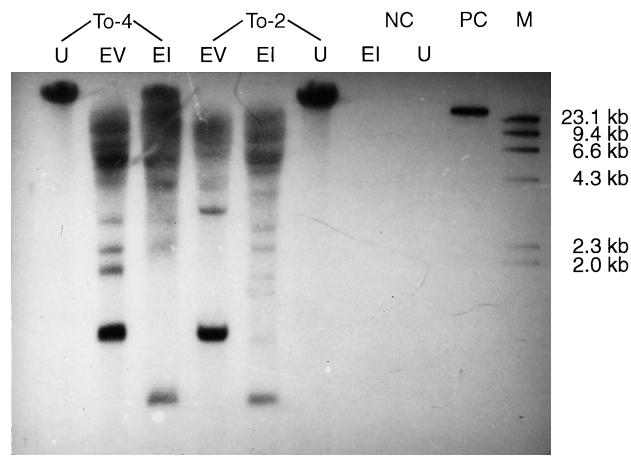

Fig. 4 Southern blot analysis of transgenic rice lines. The RDV S8 gene fragment was used as the hybridization probe. *M* λ/*Hin*dIII marker, *NC* genomic DNA from nontransformed rice line Zhonghua 8, *PC*, plasmid pE3R8, *T_0-2* and *T_0-4* are the primary transformed plants (T_0) of lines No. 2 and No. 4, respectively. *Sublanes U*, *EI*, *EV* correspond to undigested, and *Eco*RI- and *Eco*RV-digested genomic DNA, respectively

RDV S8 gene expression analysis

Total protein from a nontransformed healthy rice plant (negative control, labelled as NC), from a RDV-infected rice plant (positive control, labelled as PC) and from 6 transformants were further analyzed by western blotting. T_0-1, T_0-2, T_0-4, T_0-5, and T_0-6 showed various degrees of positive immuno reactions compared with RDV-infected rice (PC) and nontransformed, non-infected rice (NC) developed with RDV antiserum and visualized by alkaline phosphatase labelled anti-rabbit IgG (Fig. 5).

In addition to the major product (46 kDa) encoded by RDV S8, a fast-moving protein band was also detected in the transgenic rice plants. This band appeared in the western assays of the products encoded by the S8 gene in *E. coli*.

Inheritance of the introduced RDV S8 gene

To date, 2 T_0 plants have set seed. Total DNA from T_1 seedlings of transgenic plants were analyzed by PCR using primers specific to the coding region of the RDV S8 gene. In the case of plant line T_0-3, 29 out of 40 T_1 seedlings showed the expected size (1.4 kb) of the amplified DNA. χ^2 tests indicated (a) good agreement with segregation ratios of 3:1. This is consistent with

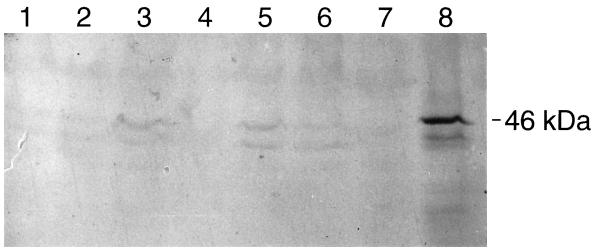

Fig. 5 Western blot detection of RDV S8-encoded product in protein extracts from different transgenic rice lines. *Lanes 1–6* Total protein extracted from transgenic T_0 rice lines T_0-6, T_0-5, T_0-4, T_0-3, T_0-2 and T_0-1, *lane 7* total protein extracted from healthy nontransformed rice, *lane 8* total protein extracted from RDV infected rice

Mendelian inheritance of a single dominant locus. In 1 case (T_0-2), 16 plants were positive in PCR assays out of 32 T_1 seedlings, showing a 1:1 segregation ratio. This segregation could be explained by the passage of the transgene exclusively through one gamete (Cooley et al. 1995). An insertion mutation of an essential gene required for pollen or ovum viability may account for this aberrant inheritance (Cooley et al. 1995; Christou et al. 1989).

Discussion

Our work demonstrates that the biolistic transformation of rice using primary calli derived from mature seeds is feasible. We have shown that $G418^r$ cell clusters can be obtained from embryogenic calli at the ratio of 1:4.4 after bombardment. In our experiment, G418 at a concentration of 100 mg/l was used as the selectable marker for the NPT II gene because rice is relatively tolerant to kanamycin. From a total number of 110 G418-resistant cell clusters, 22 plant lines were regenerated, and 19 were confirmed to be transgenic. Data from Southern analysis and western blotting proved that the RDV S8 gene was stably integrated into the host genome and expressed. The segregation of this gene in the T_1 generation further confirmed gene integration into the plant genome.

In the western assays of the products encoded by the S8 gene in the *E. coli* expression system, transgenic rice plants, RDV-infected rice plants and partially purified RDV virons we detected a fast-moving protein in addition to the major product (46 kDa) encoded by S8. This fast moving protein, reported as 45 kDa (Suzuki and Sugawara 1991), has been noticed in purified RDV virions also by Suzuki and Sugawara (1991).

Protection from virus infection mediated by the expression of viral coat proteins (CP) in transgenic plants has been demonstrated for a number of viruses; most of them were ssRNA (Abell et al. 1986; Fitchen and Beachy 1993), while RDV is dsRNA. Therefore, the study of RDV S8-transformed rice may provide valuable information about CP-mediated viral resistance and the mechanisms involved in virus infection and spread in phytoreoviruses. On the other hand, the system we described here for *in vivo* expression of viral genes is now becoming routine. Therefore, it should be possible to use this technique and classical breeding methods for the expression of different combinations of plant reovirus gene segments in host plants and to identify the function of each viral segment and to study virus replication.

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References

Abell PP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738–743

Boccardo G, Milne RG (1984) Plant reovirus group. CMI/AAB Descriptions of Plant Viruses No. 294

Cao J, Duan XL, McElroy D, Wu R (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep 11:586–591

Cooley J, Ford T, Christou P (1995) Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration. Theor Appl Genet 90: 97–104

Christou P, Swain WF, Yang NS, McCabe D (1989) Inheritance and expression of foreign genes in transgenic soybean plants. Proc Natl Acad Sci USA 86:1991–1995

Christou P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9:957–962

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on nitrogen sources. Sci Sinica 18:659–668

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version I I. Plant Mol Biol 1:19–21

Fauquet CM, Martelli GP (1995) Updated ICTV list of names and abbreviations of viruses, viroids, and satellites infecting plants. Arch Virol 140:393–413

Fitchen JH, Beachy RN (1993) Genetically engineered protection against viruses in transgenic plants. Annu Rev Microbiol 47:739–763

Gao Q, Qu YX, Liu W, Chen ZL (1990) Partial cDNA cloning and nucleotide sequence of rice dwarf virus genome. Acta Bot Sinica 32:13–18

Gordon-Kamm WJ, Spencer TM, Mangana ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WRJ, Willetts NG, Rice TB, Mackey CJ, Kruegger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618

Li LC, Qu RD, Kochko A, Fauquet C, Beachy RN (1993) An improved rice transformation system using biolistic method. Plant Cell Rep 12: 250–255

Li Y, Xue ZH, Liu YF, Chen ZL (1994) cDNA synthesis, molecular cloning and sequence analysis of rice dwarf virus segment S12. Chin Virol 10: 339–345

Li Y, Wang Y, Liu YF, Liang XW, Chen ZL (1995) cDNA cloning, sequencing and expression in *E. coli* of RNAs of beet necrotic yellow vein virus. Acta Microbiol Sinica 35: 421–426

Matsumura T, Tabayashi N (1995) Transformation of rice plants by plant reovirus genes. Seminars Virol 6: 133–139

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473–497

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Somers DA, Rines HW, Gu W, Kaeppler HF, Buchmell WR (1992) Fertile, transgenic oat plants. Bio/Technology 10: 1589–1594

Suzuki N, Sugawara M (1991) Outer capsid protein heterogeneity of rice dwarf phytoreovirus. J Gen Virol 72: 2239–2242

Vasil V, Castillo AM, Fromm ME, Vasil IK (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embrogenic callus. Bio/Technology 10: 667–674