RESEARCH ARTICLE

Yunfeng BAI, Hongchun YANG, Lin QU, Jun ZHENG, Jinpeng ZHANG, Maoyan WANG, Wan XIE, Xiaomei ZHOU, Guoying WANG

Inverted-repeat transgenic maize plants resistant to sugarcane mosaic virus

Abstract RNA silencing is a post-transcriptional gene-silencing phenomenon induced by double-stranded RNA (dsRNA). In an attempt to generate dsRNA-mediated transgenic maize plants resistant to sugarcane mosaic virus (SCMV), we cloned SCMV *NIb* gene-specific sequences and inserted it into the binary vector p3301 in the sense and antisense orientations (named SCMVir-NIb), which could produce RNAs capable of duplex formation in plant cells. Maize immature embryos were co-cultured with *Agrobacterium* carrying two vectors, one marker-free vector harboring the SCMVirNIb and one vector harboring bar gene as the selective marker. Resistant calli were recovered by selection on medium containing Biolaphos. Among the regenerated plantlets from resistant calli, 14 plants have been certified to contain SCMVirNIb by PCR amplification and DNA dot blot. T_1 plants derived from the 14 plants were challenged in a greenhouse with SCMV inoculums and the percentages of resistant plants in 11 T_1 lines were higher than 60%. One plant in the T_1 line was found to carry SCMVirNIb without bar gene by PCR assay.

T_2 plants derived from T_1 SCMV resistant transgenic plants were challenged with SCMV inoculums in field. The percentages of resistant plants from 3 lines, including the line derived from the marker-free transgenic plant, were higher than 85%. The non-transgenic control plants were all susceptible. Further molecular analysis confirmed that the resistant plants from the marker-free transgenic line contained SCMVirNIb but not the bar gene.

Keywords inverted-repeat transgene, marker-free, maize (*Zea mays*), SCMV

Yunfeng BAI
Institute of Crop Genetics, Shanxi Academy of Agricultural Sciences, Taiyuan 030031, China
State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing 100094, China

Hongchun YANG, Lin QU, Jun ZHENG, Jinpeng ZHANG, Maoyan WANG, Wan XIE, Xiaomei ZHOU
State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing 100094, China

Guoying WANG (✉)
Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China
State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing 100094, China
E-mail: gywang@mail.caas.net.cn

1 Introduction

Double-stranded RNA (dsRNA) is not a normal constituent of the cytoplasm of eukaryotic organisms and does not usually occur naturally. When dsRNA occurs in eukaryotic cells, Dicer, a RNase III-like ribonuclease, specifically cleaves dsRNA into small interfering (si)RNAs of 21–25 nucleotides. siRNAs act as a guide to recognize complementary RNAs for their degradation to suppress gene expression, which was a manifestation of posttranscriptional gene silencing induced by dsRNA, named as RNA interference in *Norhabditis elegans* (Fire et al., 1998).

RNA silencing is an intrinsic plant self-defense mechanism and can play an important role in protecting the organism's genome against foreign nucleic acids. In addition to its antiviral function, RNA silencing is also thought to suppress the expression of potentially harmful segments of the genome, such as transposons (Plasterk, 2002; Tabara et al., 1999; Ketting et al., 1999), which might otherwise destabilize the genome by acting as insertional mutagens.

RNA silencing plays an important role as a natural antiviral response in plants. Successful virus infection requires evasion or suppression of RNA silencing. Indeed, many plant viral proteins have been identified as suppressors of RNA silencing (Anandalakshmi et al., 2000; Guo et al., 2002; Andrew et al., 2000, Silhavy et al., 2002).

RNA silencing can be triggered by dsRNA produced during the intermediate steps of positive-strand RNA virus replication, which induced the viral RNAs degradation. DsRNA can be delivered by stably transforming plants with transgenes that express a self-complementary RNA. The resulting transcript hybridizes with itself to form a hairpin structure that contains a single-stranded loop region and a base-paired stem, which mimics the dsRNA structure that induces viral gene silencing. It has been shown previously that extremely efficient and specific suppression of transgenes can be obtained in tobacco (Waterhouse et al., 1998; Kalantidis et al., 2002) and barley (Wang et al., 2000) by the expression of hairpin-like RNA containing transgene sequences.

For the current study, we designed cDNA constructs using inverted repeat of the polymerase gene (*NIb*) segment. The constructs were cloned into a marker-free expressional vector and introduced into the calli derived from immature embryos of maize via *Agrobacterium tumefaciens*-mediated co-transformation system. Many co-transgenic maize lines, containing both the inverted repeat constructs and the marker gene, were regenerated. Several selectable-marker free transgenic maize plant lines were subsequently obtained from the progeny of the co-transformants. Our results indicated that this transgene conferred high resistance to SCMV on the plants.

2 Methods

2.1 Construction of the inverted-repeat gene

A full-length SCMV-polymerase sequence was cloned by RT-PCR in the pGEM-T Eazy (Promega) vector using the DNA oligonucleotides 5′-GAGCGTTGAAGAAC AATGTG-3′ and 5′-CGTTGTTCCAGATCCACTTC-3′. The sequences were verified by direct sequencing. A marker-free expression vector harboring an inverted-repeat sequence from 898 bp of the 5′ half of SCMV-NIb was constructed.

2.2 Transformation and selection

Competent *A. tumefaciens* LBA4404 was transformed with one binary vector harboring the SCMVirNIb and one binary vector harboring *bar* gene as the selective marker, respectively, by using the freeze-thaw method. Immature embryos of maize line Zong 3 were co-transformed. The embryos were then transferred into selective medium containing herbicide phosphinothricin 5 mg·L^{-1} and cultured two weeks and subcultured three times at 2-week intervals, on selective medium containing phosphinothricin 10 mg·L^{-1} to produce PPT-resistant calli which were subsequently transferred to a regeneration medium

without phosphinothricin, and plantlets were then regenerated (Zhang et al., 2001).

2.3 Molecular detection of transgenic plants

Oligonucleotide primers (5′-gct cac gga gtg tat tca gg-3′ and 5′-ggt gct gct gta aaa gtc cg-3′) were used to identify transgenic progeny containing *NIb* gene. These primers amplify a 560 bp fragment. PCR analysis was performed using approximately 100 ng of genomic DNA. PCR reaction was performed as pre-cycling for 5 min at 94°C, followed by 30 cycles of 45 sec at 94°C, 45 sec at 56°C, 1 min at 72°C, and finally, 5 min at 72°C. PCR products were fractionated by electrophoresis in 1% agarose gel, stained with ethidium bromide dye and visualized with a UV illuminator.

About 10 μg DNA was dot blot on to Hybond™-N^{+} positively charged nylon membrane. The membrane was hybridized with a radio-labeled probe from a 560-bp *NIb* gene fragment (Promega). The hybridized membranes were analyzed using a Phosphorlmager (Molecular dynamics).

2.4 Testing transgenic plants for SCMV resistance

SCMV inoculum was produced in the maize line Ye107 (donated kindly by Dr. LI Huifang from China Agricultural University) and mechanically inoculated twice within a week interval to transgenic maize plants at the three-leaf stage. The plants were evaluated for viral symptoms at 10-day intervals, beginning at 10 days after the initial inoculation. The infection degree was scaled by leaf degree of susceptibility.

Leaf degree of susceptibility = Σ (leaf scoring scale of chlorosis × leaf number of the scale)/total leaf number.

The leaf scoring scale of chlorosis: 0 = asymptomatic; 1 = scattered chlorotic dots; 2 = chlorotic stripe; 3 = chlorosis at a larger area; 4 = chlorosis at the whole leaf.

Disease index at adult stage: 0 = symptomless; 1 = chlorosis at upper 3 leaves; 2 = chlorosis at middle and upper leaves and dwarf; 3 = serious dwarf and small ear or earless.

3 Results

3.1 Gene clone and construction of inverted-repeat sequence

A full-length SCMV-*NIb* sequence cloned by RT-PCR was ligated with a pGEM-T Eazy vector and verified by the direct sequencing. The sequencing result indicated that the *NIb* gene contained 1563 bp and coded 521 amino acids with a GDD motif. The sequence contained two *Mun*I sites at 898 bp and 1040 bp site at the 5′ end,

respectively. The vector was digested with *Sac*I and *Mun*I (partially digested). Both 898 bp and 1040 bp fragments from the 5′ half of *NIb* were excised and inserted simultaneously into the *Sac*I site of marker gene-free vector p3301 in the sense and antisense orientations (named SCMVir *NIb*), giving the hairpin gene that is transcribed under the control of maize ubiquitin promoter and could produce RNAs capable of duplex formation in plant cells (Fig. 1)

3.2 Selection of resistant calli and molecular analysis of T_0 plants

A total of 2053 maize immature embryos were co-transformed using the *Agrobacterium* inoculation procedure and 78 resistant calli were obtained after being cultured in a selective medium containing biolophos for eight weeks and the resistant calli rate was 3.8%. Ninety-eight plantlets were regenerated from 68 resistant calli and 88 plantlets were transferred into a greenhouse. Genomic DNA of transgenic plants at 10-leaf period was isolated for PCR and dot blot. Among *bar* gene PCR-positive plants, 14 plants were certified to contain SCMVirNIb by PCR amplification (Fig. 2) and DNA dot blotting (Fig. 3), which were all morphologically normal and show full fertility. Self-pollinated seeds were harvested from them.

3.3 Virus resistance and PCR analysis of T_1 plants

T_1 lines derived from the above 14 plants were challenged in a greenhouse with SCMV inoculums at the three-leaf period. After 10 days, susceptible phenotype and resistant phenotype were clearly observed in all lines and the average percentage of resistant plants were 53.9%. The resistant percentage of 11 T_1 lines was higher than 60%, whereas an average resistant percentage of non-transgenic control lines was 15%. Second and third evaluations were consistent with the initial evaluation (Table 1).

One resistant plant, numbering L9-2 in line 9, was found to carry SCMVirNIb without the bar gene by PCR assay which shows the independent segregation of bar and SCMVirNIb transgenes allowing generation of selectable marker gene-free plants. Of all other resistant plants, the interest gene and marker gene were co-segregating and PCR-positive rates were 87.5%–100% in the transgenic lines.

3.4 Virus resistance and PCR analysis of T_2 plants

The self-pollinated seeds from 68 resistant *NIb* PCR-positive plants in the T_1 populations were harvested and sown in soil. The resulting T_2 plants were challenged with SCMV inoculums in the field and monitored for viral symptoms and virion accumulation. An average percent-
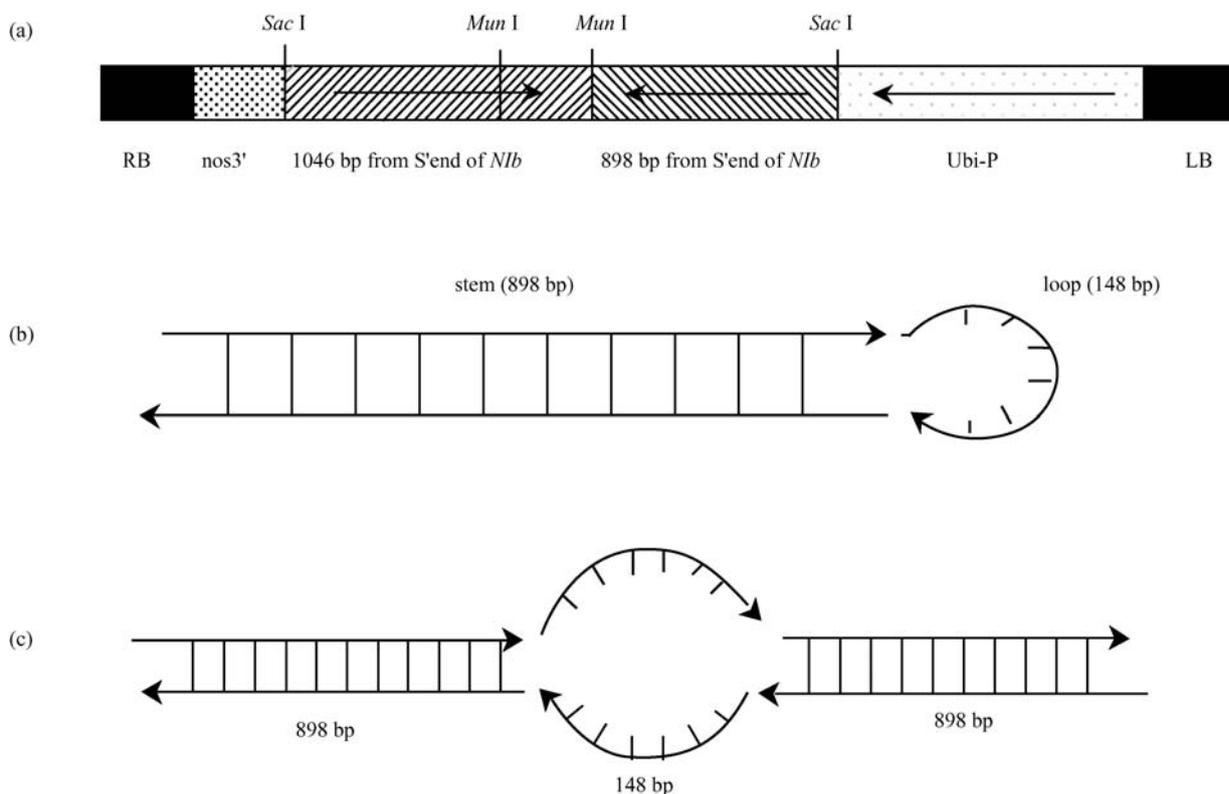

Fig. 1 Design of maker-free pSCMVirNIb and diagram of dsRNA produced by inverted-repeat transgene
Note: (a) Design of inverted-repeat transgene; (b) Diagram of self-complementary (hairpin) RNA produced by inverted-repeat transgene; (c) Diagram of intermolecular-complementary dsRNA produced by inverted-repeat transgene.
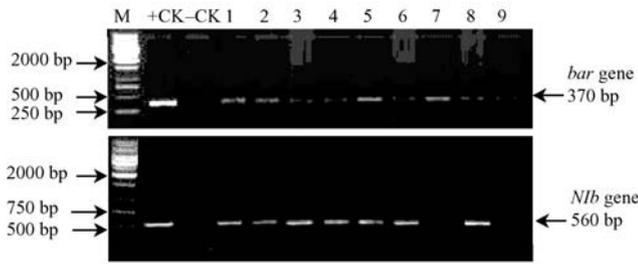
Fig. 2 PCR assay of T$_0$ plants for both *bar* gene and *NIb* gene
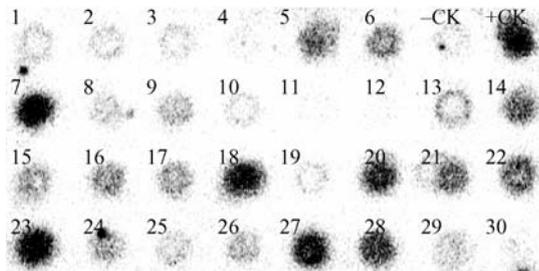Note: M: 1 kb ladder; +CK: plasmid pSCMVirNIb; −CK: non-transgenic control plant; 1–9: transgenic plants.

Fig. 3 The DNA dot-blot detection of T$_0$ plants with *NIb* gene
Note: +CK: plasmid pSCMVirNIb; −CK: non-transgenic control plant; 1–30: T$_0$ plants.

age of resistant plants were 49.8% 10 days later and 56.8% at the adult stage, and an average leaf degree of susceptibility was 3.2 degree from all lines. But resistant percentages were higher than 70% from 12 lines and higher than 85% in 3 lines at the adult stage (Table 2). The resistant

percentage of line 9-2 (L9-2) derived from a marker-free transgenic plant was 90% (Fig. 4).

Furthermore, molecular analysis confirmed that the resistant plants from the marker-free transgenic line L9-2 contained SCMVir *NIb* not *bar* gene (Fig. 5)

Virion accumulation was measured 30 days after inoculation, by enzyme-linked immunosorbent assay (ELISA), using monoclonal antibody diagnosis. The ELISA level (Table 3) of L9-2 suggested that the plants had undetectable levels of SCMV, corresponding to the resistant symptom.

Mixed saps of resistant plants of L9-2 were mechanically inoculated to virus-free plants of maize line Zong 3 and inoculated plants were virus symptom-free, which confirmed that the resistant plants of L9-2 were virus-free or slightly infected by virus.

4   Discussion

RNA interference can be efficiently triggered by dsRNA (Fire et al., 1998). In virus-resistant gene engineering, dsRNA can be produced by self-complementary RNA derived from inverted-repeat transgene. In our study, the SCMV-resistant percentage of maize lines containing inverted-repeat transgenes encoding a hpRNA derived from SCMV *NIb* sequences was higher than those of maize lines containing sense (Murry et al., 1993; Liu et al., 2005; Zhou et al., 2001) or antisense transgenes (Bai et al., 2006). The molecular details of hpRNA-mediated viral resistance have not yet been determined up to now. However, it is

Table 1 Virus incidences of T$_1$ lines

line	No. of inoculated plants	10 DPI		20 DPI		30 DPI		adult stage	
		No. of infected plants	percentage of infected plants/%	No. of infected plants	percentage of infected plants/%	No. of infected plants	percentage of infected plants/%	No. of infected plants	percentage of infected plants/%
1	14	5	35.7	5	37.5	5	37.5	5	37.5
2	10	4	40.0	3	30.0	3	30.0	3	30.0
3	10	9	90.0	8	80.0	8	80.0	8	80.0
4	24	9	37.5	10	41.2	10	41.2	11	41.2
5	25	10	40.0	10	40.0	11	44.4	11	44.4
6	23	9	39.1	10	43.5	10	43.5	10	43.5
7	13	12	92.3	12	92.3	12	92.3	11	84.6
8	15	6	40.0	6	40.0	6	40.0	7	46.7
9	15	6	40.0	6	40.0	6	40.0	6	40.0
10	16	4	25.0	5	31.2	6	37.5	6	37.5
11	13	5	38.5	6	46.1	6	46.1	6	46.1
12	14	12	85.7	12	85.7	12	85.7	12	85.7
13	12	4	40.0	5	50	5	50.0	5	50.0
14	15	6	38.5	7	46.7	7	46.7	7	46.7
total	219	101	–	105	–	107	–	108	–
mean	–	–	46.1	–	47.9	–	48.9	–	49.3
CK	20	17	85	17	85	17	85	18	90

Note: DPI = days post inoculation. The same below.

Table 2 Resistance of T_2 transgenic lines to SCMV

line	No. of inoculated plants	10 DPI		20 DPI		30 DPI		adult stage	
		No. of resistant plants	percentage of resistant plants/%	No. of resistant plants	percentage of resistant plants/%	No. of resistant plants	Percentage of resistant plants/%	No. of resistant plants	percentage of resistant plants/%
L8-10	13	12	92	11	85	11	85	11	85
L8-11	13	11	85	11	85	11	85	11	85
L9-2	10	9	90	8	80	9	90	9	90
CK	13	0	0	0	0	0	0	0	0

Fig. 4 Reaction of transgenic line L9-2 and non-transgenic control plants to SCMV
Note: CK: non-transgenic plants; L9-2: marker-free transgenic line.

shown that the inverted-repeat transgene can be used for SCMV-resistant engineering successfully.

Tobacco was simultaneous challenged with virus and dsRNA (Tenllade et al., 2001), and the results show that the resistance induced by dsRNA depended on the dsRNA dosage. In inverted-repeat transgenic plants, it has not been determined whether the gene silencing

efficiency depends on the transgenic copy number or not. In this study, the resistant rate was higher than 90% in a transgenic line consistent with the result in barley (Wang et al., 2000). It needs to be determined whether the high resistance was correlative with a higher transcript
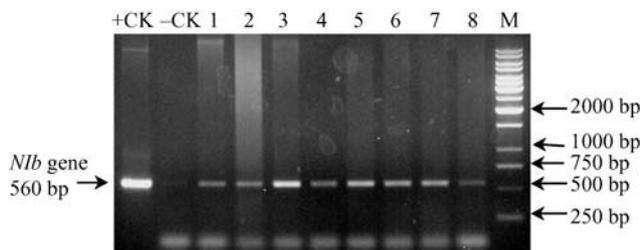

Fig. 5 PCR assay of L9-2 plants with *NIb* gene
Note: +CK: plasmid pSCMVirNIb; −CK: non-transgenic plant; 1–8: 8 plants of L9-2; M: 1 kb ladder.

Table 3 ELISA detection and virus symptoms of T_2 transgenic plants

line	plant	A_{492}	symptom	line	plant	A_{492}	symptom
8–10	1	0.169	−	8–11	4	0.232	−
	2	0.342	−		5	0.255	−
	3	0.409	+		6	0.168	−
	4	0.559	+	9–2	1	0.252	−
	5	0.462	+		2	0.172	−
	6	0.398	−		3	0.257	−
8–11	1	0.354	−		4	0.144	−
	2	0.193	−		5	0.226	−
	3	0.138	−		6	0.206	−
+CK		1.223	+	−CK		0.202	−

Note: +CK: non-transgenic plants; −CK: non-inoculated healthy plants.

level in graminaceous cereal crops because of the ubiquitin promotor.

dsRNA derived from virus gene fragments delivered by stable transformation with inverted-repeat transgene can trigger the intrinsic RNA silencing mechanism of plants and be degraded into 21–25 nt small interference RNA (Missiou et al., 2004; Goldbach et al., 2003). Engineering virus-resistant crops through inverted-repeat transgene takes the advantage of sense or antisense transgene. One important aspect of this strategy to engineer-virus resistant plants is the fact that the transgenic viral sequence is not translated. Moreover, the actual RNA transcript is almost undetectable, most likely, because it gets cleaved quickly into small fragments. These two features limit the environmental risks of this strategy possibly, such as transencapsidation or recombination of the transgene with an incoming virus. Removal of selectable marker-genes is a reasonable strategy for addressing public concerns about the use of marker genes from an ecological and food safety perspective and would certainly contribute to the public acceptance of transgenic crops. In our study, the marker-free inverted-repeat transgenic lines with high SCMV-resistance were regenerated via co-transformation which may be an efficient safe virus-resistant transgenic method.

5   Conclusion

The inverted repeat sequence derived from SCMV *NIb* gene was introduced into maize line Zong 3 and the SCMV-resistant lines were regenerated which shows that introducing virus-derived inverted repeat transgene into maize is an effective virus-resistant technique.

References

Anandalakshmi R, Marathe R, Ge X, Herr J M, Mau C, Mallory A, Pruss G, Bowman L, Vance V B (2000). A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. Science, 290: 142–144

Andrew P (2000). Suppression of posttranscriptional gene silencing by a plant viral protein localized in the nucleus. EMBO J, 19: 1672–1680

Bai Y F, Zhao J F, Zheng J, Zhang J P, Wang M Y, Gou M Y, Dong Z G, Yang H C, Wang G Y (2006). SCMV-resistant transgenic maize mediated by antisense *cp* gene. Acta Agronica Sinica, 32(5): 661–665 (in Chinese)

Fire A, Xu S, Montgomery M K, Kostas S A, Driver S E, Mellow C C (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature, 391: 806–811

Goldbach R, Bucher E, Prins R (2003). Resistance mechanisms to plant viruses: an overview. Virus Res, 92: 207–212

Guo H S, Ding S W (2002). A viral protein inhibits the long range signaling activity of the gene silencing signal. EMBO J, 21: 398–407

Ketting R F, Haverkamp T H, van Luenen H G, Plasterk, R H (1999). Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RnaseD. Cell, 99: 133–141

Liu X H, Zhang H W, Liu X, Liu X J, Tan Z-B, Rong T Z (2005). Isolation of the capsid protein gene of Maize Dwarf Mosaic Virus and its transformation in maize. Chin J Biotechnol, 21(1): 144–148 (in Chinese)

Missiou A, Kalantidis K, Boutla1 A, Tzortzakaki S, Martin Tabler M, Tsagris M (2004). Generation of transgenic potato plants highly resistant to potato virus Y (PVY) through RNA silencing. Mol Breed, 14: 185–197

Murry E, Launie G (1993). Transgenic corn plants expressing MDMV strain B coat protein are resistant to mixed infection of Maize Dwarf Mosaic Virus and Maize Chlorotic Mottle Virus. Bio/technology, 11: 1559–1564

Plasterk R H A (2002). RNA silencing: the genome's immune system. Science, 296: 1263–1265

Silhavy D, Molnar A, Lucioli A, Szittya G, Hornyik C, Tavazza M, Burgyan J (2002). A viral protein suppresses RNA silencing and binds silencing-generated 21- to 25-nucleotide double-stranded RNAs. EMBO J, 21: 3070–3080

Smith N A, Singh S P, Wang M B, Stoutjesdijk P A, Green A G, Waterhou P A (2000). Total silencing by intron-spliced hairpin RNAs. Nature, 407: 319–320

Tabara H, Sarkissian M, Kelly W G, Fleenor J, Grishok A, Timmons L, Fire A, Mello C C (1999). The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. Cell, 99: 123–132

Tenllado F, Diaz-Ruiz J R (2001). Double-strand RNA-mediated interference with plant virus infection. J Virol, 75: 12288–12297

Wang M B, Abbott D C, Waterhouse P M (2000). A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. Mol Plant Pathol, 1: 347–356

Waterhouse P M, Graham M W, Wang M B (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci USA, 95: 13959–13964

Zhang R, Wang G Y, Zhang X H, Zhao H J (2001). *Agrobacterium tumefaciens* mediated maize transformation. J Agric Biotechno, 9(1): 45–48 (in Chinese)

Zhou X M (2001).Transfer of maize dwarf mosaic virus coat protein gene into maize inbred line and the resistance of transgenic plant to MDMV. Dissertation for the Doctoral Degree. Beijing: China Agricultural University (in Chinese)

Zhu J H, Zhu C X, Wen F J, Song Y Z (2004). Comparison of resistance to potato virus Y mediated by direct and inverted repeats of the coat protein gene segments in transgenic tobacco plants. Acta Phytopathol Sin, 34(2): 133–140 (in Chinese)