Plant Cell Tiss Organ Cult (2013) 113:571–578
DOI 10.1007/s11240-013-0289-z

RESEARCH NOTE

# RNA interference-mediated resistance to maize dwarf mosaic virus

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Received: 28 August 2012 / Accepted: 4 January 2013 / Published online: 11 January 2013


**Abstract** Maize dwarf mosaic virus (MDMV) is a widespread pathogen that causes serious yield loss to maize crops. A hairpin RNA expression vector was constructed herein to overcome the low efficiency of cultural protection against MDMV and to improve the MDMV resistance mediated by a shorter transgenic inverted-repeat sequence. This expression vector contained a 451 bp inverted-repeat sequence, homologous to the protease gene (*P1*) of MDMV. It was used for the *Agrobacterium tumefaciens*-mediated transformation of maize calli induced from a susceptible inbred line. A total of 17 $T_2$ transgenic lines were identified by both specific PCR amplification and Southern blot hybridization. Of these lines, 15 were evaluated for MDMV resistance in inoculation field trials under two environments. The relative replication levels of the *P1* gene were analyzed by quantitative real-time (qRT)-PCR. Results demonstrated that all of the 15 $T_2$ lines showed an enhanced resistance to MDMV in comparison with that of the non-transformed parent line. Six lines were deemed to be 'resistant' with an average disease index below 25 %, which was not significantly different from that of the resistant control. The relative replication levels of the virus gene were significantly reduced in these resistant $T_2$ transgenic lines. The efficiency of virus gene silencing was directly related to the transgene copy numbers presented in these transgenic lines.

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**Keywords** Maize dwarf mosaic virus · Resistance · RNA interference · Transgene

## Introduction

Maize dwarf mosaic disease, a worldwide epidemic, is caused by the B, C, D, E and F strains of sugarcane mosaic virus, which are collectively known as maize dwarf mosaic virus (MDMV) (Achon et al. 2011; Hohmann et al. 1998; Jiang and Zhou 2002; Trzmiel and Jezewska 2008). MDMV is transmitted predominantly by several genera of aphids, but can also be transmitted through infected seed and mechanical injury. The infected plants can be severely implicated in development, resulting in significant yield loss (de Oliveira et al. 2003; Kong and Steinbiss 1998; Meyer and Pataky 2010). To overcome the low efficiency of cultural protection, genetic improvement of maize varieties has become an effective and environmentally sustainable way for controlling this virus (Redinbaugh et al. 2004; Williams and Pataky 2012). Some resistant germplasms, such as 'H9-21' which was used as a resistant control in the field trial of this study, do not meet the requirements of maize production because of their poor agronomic characteristics (Xi et al. 2008). Along with the efforts to identify quantitative trait loci (QTL) and develop promising marker-assisted selection (MAS) protocol for MDMV resistance (Jones et al. 2011; Sun et al. 2010; Uzarowska et al. 2009; Wu et al. 2007), transgenic lines have been developed to introduce cross protection. The latter has been achieved by introducing an antisense sequence homologous to *CP*, *P1*, *NIa*, *Hc-pro*, and other critical genes involved in viral replication, transmission, and particle coating in the single-stranded RNA viral genome (Liu et al. 2009; Murry et al. 1993; Ritzenthaler 2005).

Of these targeted genes, *P1* encodes protease that is crucial for cell-to-cell transmission of the virus (Cronin et al. 1995). RNA interference (RNAi), mediated by self-complementary hairpin RNA (hpRNA) transcribed from the transgenic inverted-repeat sequence, has been proven to be more effective at generating virus resistance because of its straight-forward natural protection mechanism (Fahim et al. 2010; Jiang et al. 2010; Ma et al. 2011; Praveen et al. 2010; Pradeep et al. 2012; Reyes et al. 2011; Shimizu et al. 2011; Zhang et al. 2010, 2011, 2012; Zhou et al. 2012). Moreover, leaf spraying of homologous double-stranded RNA expressed in a bacterium was found to be effective against MDMV infection in maize (Gan et al. 2010). In most of these studies, however, the lengths of the transgenic inverted-repeat sequences were shorter than 100 bp (Jiang et al. 2010; Praveen et al. 2010; Shimizu et al. 2011; Zhang et al. 2010; Zhou et al. 2012). The transgenic lines still showed a wide range of susceptibility, from highly resistant to highly susceptible. Apart from the influence of transgene integration patterns, a longer hpRNA expressed by the transgenic inverted-repeat sequence was found to be more effective in silencing the virus gene than a shorter one (Chen et al. 2004; Clarke et al. 2008; Hily et al. 2007; Praveen et al. 2010; Zhang et al. 2011).

In a previous study, maize virus resistance was enhanced following transfer of a 150 bp inverted-repeat sequence homologous to MDMV protease gene (*P1*). However, the resistance of the $T_2$ lines was deemed to be 'intermediate' or lower than that of the resistant control line (Zhang et al. 2010). The present investigation achieved the following: the construction of an hpRNA expression vector containing a 451 bp inverted-repeat sequences of MDMV protease gene (*P1*); the transformation of maize calli isolated from a MDMV susceptible inbred line; the molecular marker assisted selection of the regenerated lines; and the resistance evaluation of the transgenic lines.

## Materials and methods

### Construction of hpRNA expression vector

A 451 bp DNA fragment of MDMV protease gene (*P1*) (GenBank accession number NC003377.1) was artificially synthesized at Sangon Co. (Shanghai, China). Appropriate restriction sites, necessary for directional insertion into the sense arm (*Xho*I/*Hin*dIII) and the antisense arm (*Bam*HI/*Pst*I) of cloning vector pSK-int (Stratagene, USA), were introduced by PCR amplification (Table 1). The products were inserted into cloning vector pSK-int in the sense and antisense orientations, spaced by an intron of maize actin gene, in order to generate the hpRNA expression construct that contained inverted-repeat sequences homologous to the MDMV protease gene (*P1*). This construct was then sub-cloned into plant expression vector pCAMBIA1390 under the control of *ubiquitin* promoter and *nos* terminator, ultimately generating the hpRNA expression vector pASC451 (Fig. 1).

### Maize transformation and regeneration

Maize calli were induced from immature embryos of inbred line '18-599' on the improved N6 medium (Anami et al. 2010; Fu et al. 2011; Rakshit et al. 2010). This inbred line is an elite parent for many commercial hybrids in southwest China, but it has no MDMV resistance (Fu et al. 2011; Zhang et al. 2010). Embryonic calli were subcultured and transformed by co-cultivation with the *Agrobacterium* strain EHA105 containing the expression vector pASP451. After culturing for 7 days, the calli were screened for 20 days on a selection medium containing hygromycin B at concentrations of 5, 10 and 15 mg $L^{-1}$. The positive calli were then differentiated and regenerated on the N6 regeneration medium at 27 °C under a photon flux density of 50 μmol $m^{-2}$ $s^{-1}$ for a photoperiod of 12 h light/12 h dark.

### Molecular marker screening for transgenic lines

The leaves of the regenerated plants were sampled and used for DNA extraction following the method as described by Saghai-Maroof et al. (1984). The transformed plants were screened by PCR amplification of the 451 bp fragments of the *P1* gene using the forward (5′-GCAGG AACTTGGACCC-3′) and reverse (5′-GCGTGCCACA GTGTAAG-3′) primers. The 20 μL reaction mixture contained 40 ng template DNA, 2 μL 10 × PCR buffer, 200 μmol $L^{-1}$ dNTPs, 1.5 mmol $L^{-1}$ $MgCl_2$, 0.5 μmol $L^{-1}$ each primer and 1 U of Pfu DNA polymerase (Takara, Japan). The temperature cycling was 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s; 72 °C for 10 min. The positive plants were transferred to a greenhouse for the production of $T_1$ seed by bagged self-pollination.

The $T_1$ plant lines were planted in the field. Genomic DNA was extracted from their leaf samples. After quantification using a spectrophotometer (DU-800, Beckman Coulter, Germany), 30 μL of each DNA sample was digested by the restriction enzyme *Bam*HI, separated on a 0.8 % (w/v) agarose gel, and transferred to Gene Screen Transfer membrane (Millipore Co., Bedford, USA). The membrane was hybridized with digoxigenin-labeled (Roche Diagnostics, Germany) probes of the sense fragments of the hpRNA expression construct. The positive plants were used to produce $T_2$ seed by bagged self-pollination.

**Table 1** PCR primers used to construct hpRNA expression vector

| Primer | Restriction site | Sequence |
|---|---|---|
| F1 | *Xho*I | Sense: 5′-AAACTCGAG GCAGGAACTTGGACCC-3′ |
| R1 | *Hin*dIII | Antisense: 5′-GTGAAGCTT GCGTGCCACAGTGTAAG-3′ |
| F2 | *Bam*HI | Sense: 5′-AAAGGATCC GCAGGAACTTGGACCC-3′ |
| R2 | *Pst*I | Antisense: 5′-AAACTGCAG GCGTGCCACAGTGTAAG-3′ |

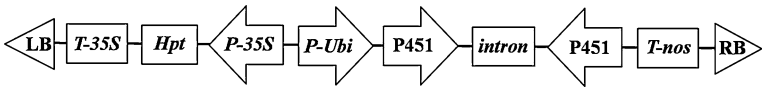


**Fig. 1** T-DNA region of hpRNA expression plasmid pASC451. An inverted-repeat sequence homologous to MDMV protease gene *P1* was spaced by an intron of maize actin gene. *LB* left border, *RB* right border, *Hpt* hygromycin phosphotransferase gene, *P-35S* cauliflower mosaic virus *35S* promoter, *T-35S* cauliflower mosaic virus *35S* terminator, *P-Ubi ubiquitin* promoter, *T-nos* terminator of nopaline synthase, *intron* intron of maize actin gene, *P451* 451 bp fragment of MDMV protease gene *P1*

## Inoculation field evaluation

The $T_2$ lines derived from the positive $T_1$ lines, together with the non-transformed parent line '18-599' (negative control), a susceptible line 'Mo17' (Li et al. 2007) and a resistant line 'H9-21' (Xi et al. 2008), were planted in a randomized block design with three replications, at Xinzhou in northern China in 2009 and Ya'an in southern China in 2010. The 'H9-21' is regarded as MDMV-free under ordinary non-inoculated conditions (Xi et al. 2008). Mechanical inoculation was conducted twice within 1 week at the 4–5-leaf stage, using inoculum sap extracted from the leaves showing mosaic symptoms in 0.1 mol $L^{-1}$ potassium phosphate buffer (pH 7.0). To simulate the aphid pinprick under natural conditions, the leaves of the seedlings of each plot were punctured with a glass fiber brush. This was performed in an effort to avoid the inevitable artificial error introduced by the typical method used in phytopathological research: finger rubbing with quartz sands (Kuntze et al. 1995; Louie 1986). Following this, the diluted (1:10) inoculum was immediately sprayed to the leaves. The disease incidence and symptom scale were investigated at the adult stage according to the standard proposed by Lin (1989). The disease index was calculated as:

$$\text{Disease index} = \frac{\Sigma(\text{Number of infected plants} \times \text{symptom scale}) \times 100}{\text{Number of total plants} \times \text{maximum symptom scale}}$$

Resistance was classified into five levels: highly resistant (HR, disease index 0–10), resistant (R, disease index 10.1–25.0), intermediate (I, disease index 25.1–40.0), susceptible (S, disease index 40.1–60.0), and highly susceptible (HS, disease index > 60.1). One-way analysis of variance test was conducted using SPSS 18.0 software (http://www.spss.com/).

## Relative quantification of virus gene replication

At the pollination stage, the third leaf from the top was sampled from five random inoculated plants in each plot, and immediately frozen in liquid $N_2$. The total RNA was extracted using a Trizol RNA extract kit (TaKaRa, Japan), and reverse transcribed into cDNA using a PrimeScriptTM RT reagent Kit (TaKaRa, Japan). Quantitative real-time PCR was conducted to measure the relative replication levels of the MDMV *P1* gene, using primers complementary to the 451 bp fragment of this gene (forward: 5′-AG CAACAGCCTACCAAACCA-3′; reverse: 5′-GCGTGCC ACAGTGTAAGA-3′). A 250 bp fragment of 18S ribosomal RNA gene (forward: 5′-CTGAGAAACGGCTA CCACA-3′; reverse: 5′-CCCAAGGTCCAACTACGAG-3′) was used as the internal standard gene for normalization. The 25 μL mixture contained 1 μL of the cDNA samples, 12.5 μL of SYBR premix Ex Taq II and 0.4 μmol $L^{-1}$ of the forward and reverse primers. The reaction was conducted in an iQ™5 (Bio-Rad, USA) using the following thermal cycling profile: 95 °C for 1 min; 40 cycles of 95 °C for 10 s, 59 °C for 20 s and 72 °C for 20 s. At the end of the last cycle, the temperature was increased by 0.5 °C $s^{-1}$ to 95 °C, so that a melting curve could be calculated and the amplified product could be detected.

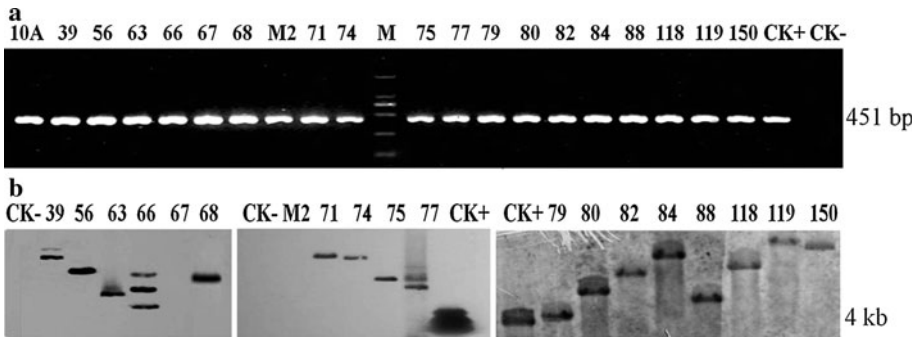


**Fig. 2** Identification of potential positive transgenic lines. **a** PCR detection of $T_0$ plantlets. *M* DNA marker DL2000. CK + , CK − , hpRNA expression vector pASC451 and the non-transformed parent line '18-599', respectively. The other lanes were independent regenerated $T_0$ plantlets. The specific amplified bands were 451 bp long. **b** Southern blotting of $T_1$ plant lines. CK − , the non-transformed parent line '18-599'. Lanes CK + were hpRNA expression vector pASC451. The bands in these two CK + lanes were 4 kb fragments digested from vector pASC451 by restriction enzyme *Bam*HI. The other lanes were the putative transgenic $T_1$ plant lines positive in PCR detection

The sample with the lowest absolute replication level in each amplification was automatically used as a check by the iQ5 software (Bio-Rad) (Vandesompele et al. 2002). The relative replication levels of the virus gene in the other $T_2$ plant lines were calculated.

## Results and discussion

### Transgenic plant lines

After hygromycin B selection, 872 pieces of positive calli were obtained from the transformed embryonic calli. Of these, 268 $T_0$ plantlets were regenerated. After PCR amplification, 20 of the regenerated plantlets were identified as putative transformants and transferred to a greenhouse (Fig. 2a). Nineteen of the 20 produced $T_1$ seeds. After repeating the PCR screening, fourteen, two, and one of the nineteen $T_1$ plant lines were identified to have one (73.7 %), two (10.5 %), or three (5.3 %) bands in the Southern blot, respectively (Fig. 2b). Because the restriction enzyme *Bam*HI had a single recognition site in the hpRNA expression construct sequence, each of the bands represented a different integration and could be used to estimate the number of transgene copies.

### MDMV resistance of $T_2$ transgenic lines

After the pollination stage, obvious differences in MDMV symptoms could be observed among fifteen of the seventeen $T_2$ transgenic lines that produced enough seeds for inoculation field evaluation. Systemic infection of MDMV was observed in the susceptible line 'Mo17' and the non-transformed control '18-599', while the resistant control 'H9-21' and several $T_2$ transgenic lines showed no symptoms of MDMV (Fig. 3). The disease indices of the different $T_2$ lines and the controls matched one another under the two environments (Table 2). Six (68, 71, 75, 79, 84, and 119) of the fifteen $T_2$ lines were deemed to be 'resistant' (R) with an average disease index between 10.1 and 25.0, showing non-significant difference from the resistant control 'H9-21'. Eight lines (74, 63, 88, 77, 56, 150, 80, and 39) showed an 'intermediate resistance' (I) with an average disease index between 25.1 and 40.0. One line (66) was classified as 'susceptible' (S) with an average disease index between 40.1 and 60.0. The enhanced resistance of the six resistant $T_2$ lines was sufficient to meet the requirements of maize production because their resistance was not significantly different from that of the resistant control 'H9-21', which is regarded as MDMV-free under ordinary non-inoculated conditions (Xi et al. 2008). Although all of the fifteen evaluated $T_2$ lines showed an enhanced resistance in comparison with their non-transformed parent line '18-599', they exhibited different degrees of resistance to MDMV. This may be explained by the different numbers and patterns of the transgene integration into the maize genome. A comparison of the results of the resistance evaluation and the Southern blotting analysis revealed that all six resistant $T_2$ lines were derived from the transgenic events with a single-copy integration of the inverted-repeat sequence. All $T_2$ transgenic lines derived from the transgenic events with more than one transgene integration were evaluated to be 'intermediately resistant' or 'susceptible'. The influences of the number and patterns of transgene integration on expression have been shown by numerous studies (Rai et al. 2007; Shou et al. 2004; Travella et al. 2005; Clarke et al. 2008; Vega et al. 2008; Yang et al. 2011; Zeng et al. 2010; Zhu et al.


**Fig. 3** Symptom difference among the transgenic $T_2$ lines and the controls. **a** Resistant control 'H9-21'. **b** Susceptible control 'Mo17'. **c** Non-transformed parent line '18-599'. **d** Transgenic $T_2$ line 75

**Table 2** MDMV resistance of $T_2$ plant lines

| Lines | Transgene copy number | Disease incidence (%) | | | Disease index (%) | | | Level of resistance/ susceptibility |
|---|---|---|---|---|---|---|---|---|
| | | Xinzhou | Ya'an | Average value | Xinzhou | Ya'an | Average value | |
| 75 | 1 | 38.0 | 33.3 | 35.7 | 15.5 | 10.0 | 12.8 k | R |
| 119 | 1 | 43.3 | 40.0 | 41.7 | 16.2 | 13.3 | 14.8 jk | R |
| 84 | 1 | 48.0 | 46.7 | 47.4 | 19.8 | 15.0 | 17.4 ijk | R |
| 79 | 1 | 52.5 | 43.3 | 47.9 | 20.3 | 17.8 | 19.1 hij | R |
| H9-21 | | 42.0 | 35.0 | 38.5 | 20.5 | 18.3 | 19.4 hij | R |
| 68 | 1 | 58.5 | 60.0 | 59.3 | 23.0 | 21.1 | 22.1 ghi | R |
| 71 | 1 | 62.0 | 56.6 | 59.3 | 25.5 | 22.5 | 24.0 fgh | R |
| 74 | 1 | 62.8 | 63.3 | 63.1 | 27.0 | 25.8 | 26.4 efg | I |
| 63 | 1 | 56.7 | 63.3 | 60.0 | 26.5 | 26.7 | 26.6 efg | I |
| 88 | 1 | 66.3 | 60.0 | 63.2 | 30.3 | 27.5 | 28.9 def | I |
| 77 | 2 | 62.4 | 53.3 | 57.9 | 33.7 | 30.0 | 31.9 de | I |
| 56 | 1 | 68.5 | 66.7 | 67.6 | 34.3 | 31.1 | 32.7 d | I |
| 150 | 1 | 66.0 | 60.0 | 63.0 | 35.0 | 31.1 | 33.1 d | I |
| 80 | 1 | 70.1 | 66.7 | 68.4 | 34.1 | 34.2 | 34.2 d | I |
| 39 | 2 | 69.3 | 60.0 | 64.7 | 33.6 | 35.0 | 34.3 d | I |
| 66 | 3 | 75.5 | 70.0 | 72.8 | 45.2 | 41.3 | 43.3 c | S |
| 18-599 | | 88.4 | 80.5 | 84.5 | 55.3 | 51.3 | 53.3 b | S |
| Mo17 | | 100.0 | 100.0 | 100.0 | 80.9 | 73.3 | 77.1 a | HS |

In the column of average value of disease index, the same lowercase letters indicate non-significance, and the different lowercase letters indicate significance at possibility level of 0.05. R, I, S, and HS correspond to resistance, intermediate resistance, susceptible, and highly susceptible to MDMV, respectively

2010). The influence of the transgene integration patterns on improving MDMV resistance has also been reported in a previous study (Zhang et al. 2011).

## Relative replication levels of MDMV *P1* gene

In all fifteen $T_2$ transgenic lines inoculated with MDMV, the relative replication levels of MDMV *P1* gene were several orders of magnitude lower than those in the non-transformed parent line '18-599' and the susceptible line 'Mo17'. The resistant lines 75, 79, 84, and 119 exhibited low relative replication levels similar to those of the resistant control 'H9-21'. For resistant lines 68 and 71, the relative replication levels were much lower than those in the intermediately resistant or susceptible lines (Fig. 4). This explained the improvement in MDMV resistance (Table 2), and confirmed the effectiveness of gene silence triggered by the transgenic inverted-repeat complementary sequence.

## Comparison of resistance between two transformants containing inverted-repeat sequences of different lengths

A previous report demonstrated that the improvement of MDMV resistance in maize could be mediated by a 150 bp

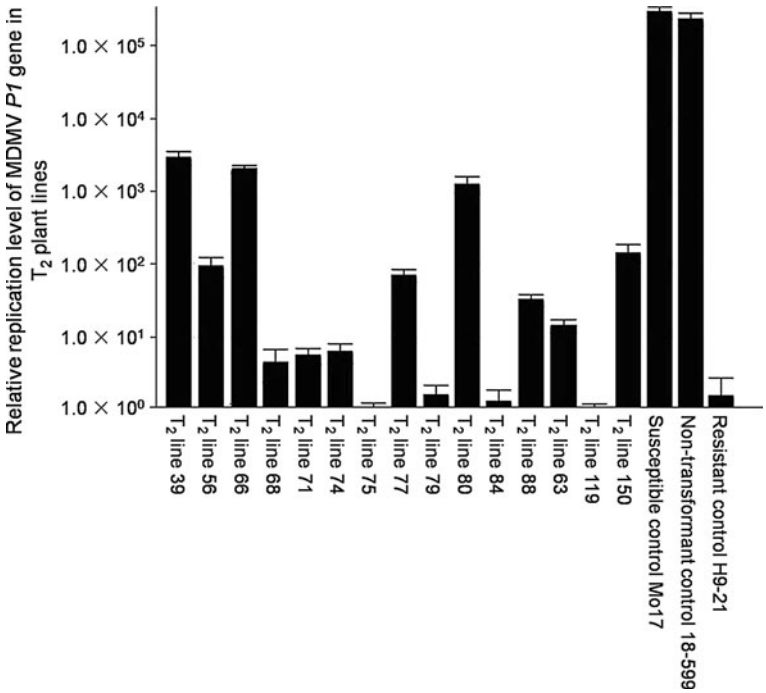


**Fig. 4** Relative replication levels of MDMV *P1* gene in $T_2$ transgenic lines displayed by quantitative real-time PCR. From *left* to *right* are independent $T_2$ plant lines 39, 56, 63, 66, 68, 71, 74, 75, 77, 79, 80, 84, 88, 119 and 150, susceptible control 'Mo17', non-transformed parent line '18-599', and resistant control 'H9-21', respectively

**Table 3** Influence of hpRNA length on virus resistance

| hpRNA length (bp) | Positive transgenic lines | MDMV resistance | | |
|---|---|---|---|---|
| | | Number of resistant $T_2$ lines | Number of intermediate $T_2$ lines | Number of susceptible $T_2$ lines |
| 150 | 9 | 0 | 3 | 6 |
| 451 | 15 | 6 | 8 | 1 |

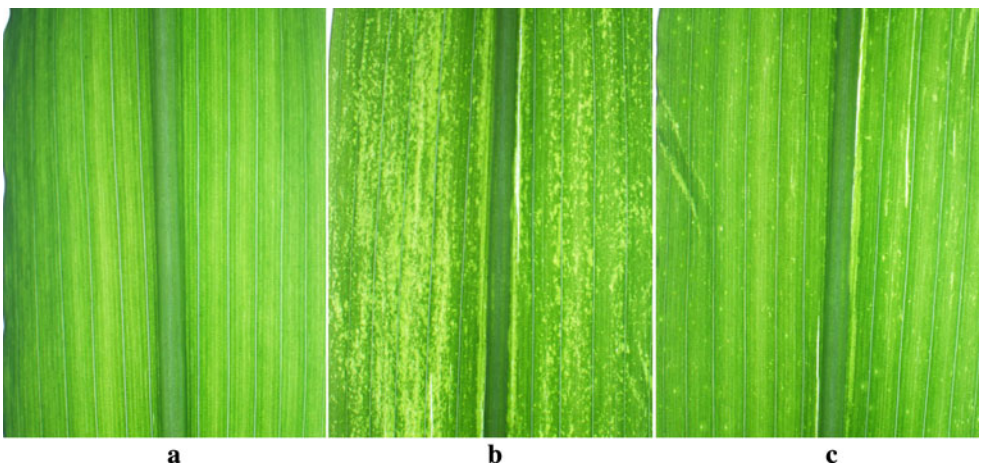


**Fig. 5** Blades of non-transformant parent line 18-599 treated with two methods of mechanical inoculation under dissecting microscope. **a** Untreated control. **b** Excessive mechanical injury caused by using finger rubbing with quartz sands. **c** Puncturation injury by a glass fiber brush

inverted-repeat sequence of the MDMV *P1* gene (Zhang et al. 2010). Of the nine $T_2$ lines, three lines were deemed to be 'intermediately resistant', and the others were deemed to be 'susceptible' or 'highly susceptible', although most of the transgenic lines showed an improvement in resistance when compared with their non-transgenic parent

line '18-599' (Table 3). In the present study, a significant improvement in MDMV resistance was obtained from the transformants of a 451 bp inverted-repeat sequence of MDMV *P1* gene. Six (68, 71, 75, 79, 84, and 119) of the fifteen $T_2$ lines were deemed to be 'resistant', eight were deemed to be 'intermediately resistant', and one line remained 'susceptible' (Tables 2, 3). This comparison indicates that a longer intact hpRNA is more effective than a shorter one at inhibiting virus gene expression, and implies that the gene silencing mediated by RNAi is dose-dependent (Gordon and Waterhouse 2007; Tinoco et al. 2010). The effective length of hpRNA needed to trigger RNAi in transgenic plants is believed to be 300–800 bp (Chen et al. 2004; Clarke et al. 2008; Hily et al. 2007; Praveen et al. 2010; Zhang et al. 2011).

## Improvement of inoculation technique

Uniform inoculation is the key to objective evaluation of virus resistance. In this study, the process of inoculation by finger rubbing with quartz sands was replaced with the method of leaf puncturing with a glass fiber brush. Dissecting microscope examination showed that the punctured leaves resembled the damage caused by aphids (Fig. 5). This improvement in the inoculation method allows for avoidance of the inevitable artificial error and excessive mechanical injury that is often caused by the method most typically used in phytopathological research: finger rubbing with quartz sands (Kuntze et al. 1995; Louie 1986).

**Acknowledgments** The authors sincerely appreciate the financial support from the Projects of Development Plan of the State Key Fundamental Research (973 Project 2009CB118401), the National Key Science and Technology Special Project (2009ZX08003012B and 2013ZX08003-004), and the technical support from Key Laboratory of Biology and Genetic Improvement of Maize in Southwest Region. The authors thank the anonymous reviewers for their critical comments and suggestions, and Ms Dominique Thomas at Cornell University for her help in improving the English writing.

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