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Full Length Research Paper

Cloning and transformation of *SCMV CP* gene and regeneration of transgenic maize plants showing resistance to SCMV strain MDB

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The coated protein gene of sugarcane mosaic virus (*SCMV CP* gene) was cloned from maize (*Zea mays* L.) leaves showing dwarf mosaic symptoms by reverse-transcription polymerase chain reaction (RT-PCR) with degraded primers. The results of sequencing and homologous comparison indicated that the cloned gene was from SCMV strain MDB, with 920 bp in length, including an open reading frame (ORF) encoding 219 amino acids and a 3' untranslated region (3'UTR). This gene was constructed into the expression vector p35SCPrBar in the antisense orientation, under the control of CaMV35S promoter, the plasmid p35SCPrBar was introduced into embryogenic calli induced from the immature embryos of maize inbred line 18-599(red) that is highly susceptible to SCMV-MDB via particle bombardment, the results of polymerase chain reaction (PCR) and Southern blotting hybridization demonstrated that the *SCMV CP* gene had been integrated into maize genome. The plants regenerated from T_1 transgenic seeds were challenged with SCMV-MDB by artificial inoculation, as a result, transgenic progenies showed resistance to the inoculated virus to different extent. This work is beneficial for extending the planting regions for the elite inbred line 18-599(red).

Key words: Maize (*Zea mays* L.), sugarcane mosaic virus (SCMV), coat protein gene, cloning and transformation.

INTRODUCTION

Maize (*Zea mays* L.) inbred line 18-599(red), bred by maize research institute, Sichuan agricultural university, P. R. China, was widely employed to produce elite hybrids, due to its high general combining ability in maize yield, strong resistance to most pathogens and wide adaptability in Southern China (Mu et al., 2003, Pan et al., 2006). However, the material was highly susceptible to sugarcane mosaic virus (SCMV), restricting its cultivation in Northern and Northwestern China.

SCMV is one of the most important pathogens of maize and related crops, causing significant yield losses, chlorosis and stunting (Alegria et al., 2003, Gemechu et al., 2006, Achon et al., 2007). This virus includes many strains and the strain MDB is the most prevalent in P. R. China and many other countries (Chen et al., 2002, Cheng et al., 2002; Jiang and Zhou 2002, Zhong et al., 2005). Because of its transmission in non-persistent manner by aphids (Mohammadi et al., 2006), chemical pesticide for preventing the viral transmission is not efficient enough and would lead to environment pollution. So, it is necessary to control its transmission via genetic transformation for the susceptible materials. To date, these maize materials such as R90, R91 and Zong3 had been transformed with the *CP* gene from SCMV and the transgenic progenies showed resistant to inoculated SCMV to some extent (Murry et al., 1993, Bai et al., 2006). However, to our knowledge, there is no report in the literature on genetic transformation of 18-599(red) for increasing its resistance to SCMV.

In this experiment, the *CP* gene of SCMV-MDB was cloned and introduced into 18-599 (red), the obtained

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Figure 1. The linear map of plasmid p35SCPrBar. *CaMV35S*: 35S promoter from cauliflower mosaic virus; *Bar*: Selective gene from *Streptomyces hygroscopicus*; *Nos*: Terminator from agrobacterium tumefaciens; *SCMV CP*: Coat protein gene cloned from SCMV strain MDB in this study.

transgenic progenies showed resistance to SCMV-MDB to some extent.

MATERIALS AND METHODS

Plant and pathogen sources

Maize inbred line 18-599(red) was obtained from maize research institute, Sichuan agricultural university, P. R. China. The inoculum of SCMV-MDB was extracted from maize inbred line Ye107 infected by SCMV-MDB in field.

Cloning and identification of *SCMV CP* gene

The total RNA was extracted from the maize leaves showing typically dwarf mosaic symptoms (Li et al., 2004). The degraded primers used to amplify *SCMV CP* gene were designed based on the sequences of the coat protein genes from SCMV and maize dwarf mosaic virus (MDMV, relative to SCMV) in GeneBank (http://www.ncbi.nlm.*NIH*.gov).

Forward primer: 5′ AAMGCXATGTCQAAgAAMATGCG 3′;
Reverse primer: 5′ TCTCTYACCAZGA MACTCGC 3′.
(M = G or A; X = C, T, or A; Q = G, T or A; Y = T or C; Z = C or A).

SCMV CP gene was amplified by reverse-transcription polymerase chain reaction (RT-PCR) using SuperScript II reverse transcriptase (GIBCO BRL, USA), separated by electrophoresis on 1% agarose gel and was reclaimed from gel with extraction kit (Takara, Japan). Then the reclaimed fragment was cloned into pGEM-T vectors (Promega, USA), and sequenced at Sangon (Shanghai, P. R. China). Its characteristics were identified by homologous analysis according to the related sequences in GeneBank.

Transformation of *SCMV CP* gene and regeneration of transgenic plants

The *SCMV CP* gene was cloned into the expression plasmid p35SCPrBar in the antisense orientation, under the control of CaMV35S promoter and Nos terminator. *Bar* gene as selective gene was harbored in the vector (Figure 1).

The embryogenic calli induced from the maize inbred line 18-599(red) on N6 medium with 2 mg/l 2,4-D were subjected to particle bombardment with p35SCPrBar. According to the lethal concentration (8 mg/l PPT) determined by the primary experiment using the embryogenic calli of untransformed 18-599(red), the transformed calli were cultured successively on N6 medium with 2 mg/l 2,4-D and added 8, 10 and 15 mg/l antibiotic phosphinothricin (PPT) for selection, each for 21d. Then, the selected resistant calli were placed on the N6 medium with 2 mg/l Kn for shoot induction. Regenerated shoots were transferred to N6 basal medium for rooting. Well developed plantlets were placed on the nutritive soil containing 34% vermiculite and 66% humus for acclimatization, 20 d later, transplanted into field. All the culture media were prepared according to the report by Liu et al. (2007).

Molecular identification

Polymerase chain reaction (PCR) was used to detect primarily the presence of the introduced genes in transformed plants. The total DNA was extracted from transgenic plants by CTAB method (Allen et al., 2006). Referring to the results of *CP* gene sequencing, the primers were designed as follows:

Forward primer: 5′ ATGTCGAAGAAGATGCGCCTG 3′;
Reverse primer: 5′ TCACCACGAGACTCGCAGCAC 3′.

The PCR reaction system and thermal cycle processes were based on the illumination of *Taq* DNA polymerase (Takara, Japan). The PCR products were separated on 1% agarose gel and stained with ethidium bromide (EB). The DNA extracted from untransformed plants 18-599(red) was used as control.

The DNA extracted from PCR-positive transgenic plants was digested with restriction enzyme *Hin*d III for Southern analysis to confirm the presence of *SCMV CP* gene. *SCMV CP* gene probe was amplified from p35SCPrBar and purified via agarose gel electrophoresis and then labeled with ^{32}P-dCTP. The procedure of Southern blotting hybridization could be seen in Sambrook et al. (1989). The DNA extracted from untransformed 18-599(red) was used as control in Southern analysis.

Challenge inoculations

Virus inoculum for resistance analysis to SCMV-MDB was prepared based on the previous report (Lin, 1989). The young leaves of inbred line Ye107 infected by SCMV-MDB were grinded in 0.1 mol/l sodium phosphate buffer (pH 7.0) and were diluted 10-fold in volume with the buffer before inoculation. All the T_1 transgenic progenies and 20 plants of nontransgenic 18-599(red) as control were inoculated with SCMV-MDB. The youngest leaf of each plant was artificially rub-inoculated for 3 times at the 10 leaf stage. 10 d later, these leaves were inoculated once more to avoid disease escapes. The average chlorosis of the functional leaves (ACFL) on a plant, including the leaf on the ear, the 2 leaves above the ear and the 2 leaves below the ear, was evaluated on their resistance to inoculated virus 2 weeks after tasselling as described by Wu et al. (2002). The rating scale of chlorosis on a leaf was determined as follows:

0 - No symptoms
1 - Stippled mottle or light mosaic on leaf
2 - Narrow streak on leaf
3 - Severe mosaic with chlorosis on leaf
4 - Blotches with red streaks on leaf.

The ACFL on a plant = Σ (rating scale of chlorosis on a leaf × the number of leaves on the rating) /5.

RESULTS

Cloning of *SCMV CP* gene and identification of its characteristics

After electrophoresis, obvious band of the RT-PCR pro-

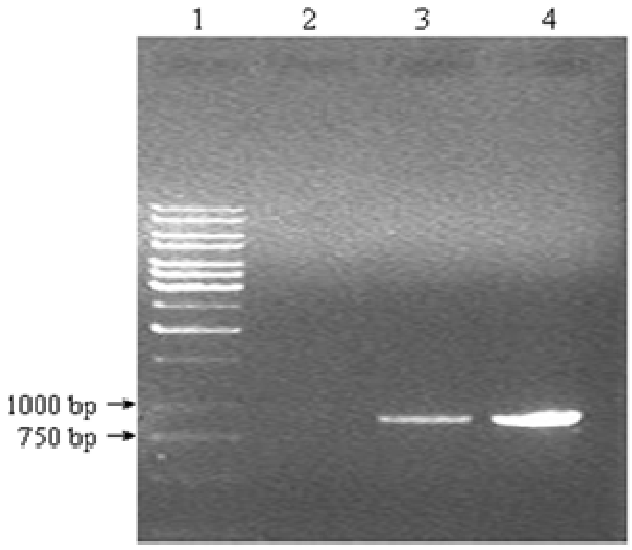

Figure 2. The products of RT-PCR were separated on agarose gel. Lane 1: GeneRuler™ 1000 bp DNA Ladder marker; Lane 2: No amplification product from the young healthy leaf as control; Lanes 3-4: Amplification products from the young leaf showing typical dwarf mosaic symptoms.

ducts from the leaf showing typical dwarf mosaic symptoms could be viewed on agarose gel (Figure 2), with about 900 bp in length. But, no electrophoretic bands were found from the healthy leaf. The sequencing results showed that the cloned fragment is 920 bp, containing an open reading frame (ORF) encoding 219 amino acids and 3′ terminal untranslated region (3′UTR), in agreement with electrophoretic band in length.

To determine the origin of the isolated fragment, the DNA sequence and deduced amino acid sequence of the coding region were compared with the corresponding sequences of MDMV-KS1 (A34978), MDMV-A (A34974) and SCMV-MDB (S77088) from GeneBank. The results indicated that the identities of the DNA sequence were 56, 73 and 98% respectively and the amino acid sequence were 57, 88 and 100% respectively. According to the generally accepted taxonomic criterion (Shukla and Ward, 1988), the cloned fragment was derived from the coat protein gene of SCMV strain MDB.

Transformation of *SCMV CP* gene and regeneration of transgenic plants

After the embryogenic calli from 18-599(red) were subjected to particle bombardment with p35SCPrBar harboring *SCMV CP* gene, 320 calli were selected, showing resistance to the antibiotic PPT. After further regeneration culture, 206 plantlets were obtained (Table 1). Then these plantlets were transferred to flower pots for acclimatization and further transplanted to field, finally, 12 fertile putative transgenic plants were harvested. The transformation frequency was one regenerated plantlet per 3.6 piece of calli.

Molecular identification of T_0 transgenic plants

The results of PCR for the 12 fertile plants demonstrated that the exogenous *SCMV CP* gene was integrated into genomes of 7 T_0 maize plants (partial results shown in Figure 3). The fragments were confirmed to be the expected 920 bp, whereas, no foreign gene was detected in the untransformed control plants.

For Southern analysis, DNA was extracted from 7 PCR-positive plants, digested with restriction enzyme *Hin*dIII and hybridized with labeled *SCMV CP* gene as probe. As a result, all the PCR-positive plants represented some bands, among which lane 3 - 8 displayed very clear, but the 2 bands in lane 2 appeared slightly blur (Figure 4). The band number in lane 7 could not easily be counted, due to the short interval between the bands, thus, the number of *SCMV CP* gene copies in transgenic CP6 plant could not be ascertained exactly. The intensities among the hybridized bands were not identical, for example, in lane 5, the top band is more intense than the other 3 bands, this might be more copies of exogenous gene were integrated into the fragment of the top band than those of the other 3 bands. However, no hybridized evidence was detected in the control plants.

Viral challenge of T_1 transgenic plants

The seeds of each T_0 transgenic plant were harvested individually and sown in the same conditions with the control 18-599(red) and their T_1 progenies were evaluated by artificial inoculation with SCMV-MDB. The results showed that most of the T_1 progeny plants presented resistance to SCMV-MDB, but their resistant abilities were not uniform, among plants from different transgenic lines, or even within the same line (Table 2). For example, some plants derived from transgenic plant CP1, CP3, CP6 and CP7 showed high resistant ability, their ACFL of were recorded as 0 and their phenotypes were quite accordant with those of noninloculated control plants, except for the inoculated portion of the plants (Figure 5). Some plants from CP5 presented high susceptibility, with 2.2 of ACFL. However, all the untransformed 18-599(red) appeared typical mosaic symptoms, with high up to 2.0 of average ACFL.

DISCUSSION

Maize inbred line 18-599(red) possesses numerous merits, including high general combining ability, strong resistance to most pathogens and wide adaptability in southern China, thus, it was widely employed to produce elite

Table 1. Transformation of calli and their regeneration.

No. of bombarded calli	No. of resistant calli	No. of regenerated plantlets	No. of harvested plants
750	320	206	12

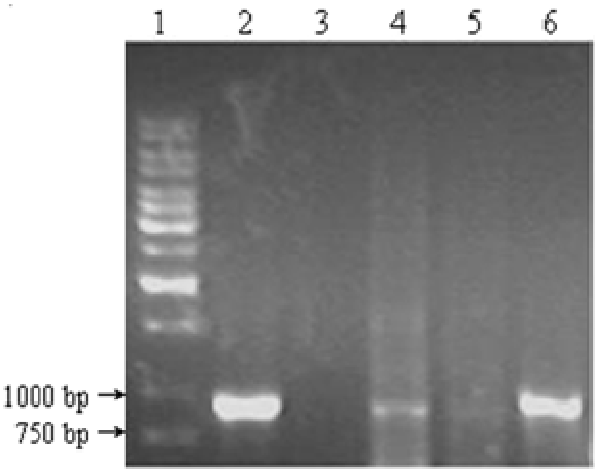

Figure 3. PCR analysis on T_0 transformed regenerated plants. Lane 1: GeneRuler^{TM} 1000 bp DNA ladder; lane 2: plasmid p35SCPrBar; Lane 3: control; Lane 4: CP1; Lane 5: CP10, Lane 6: CP7.

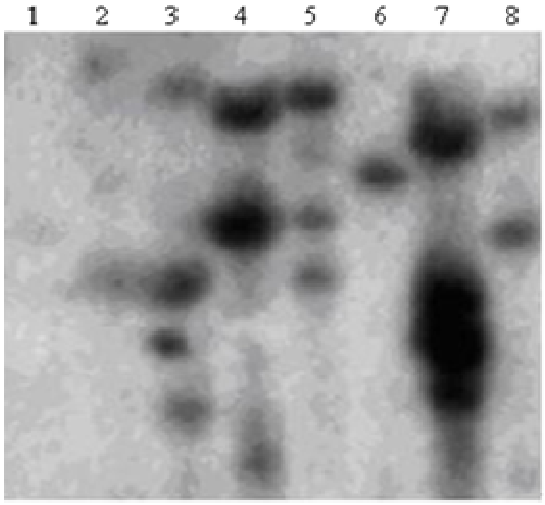

Figure 4. Southern blotting hybridization analysis on T_0 transgenic plants. Lane 1: control; Lane 2: CP1; Lane 3: CP2; Lane 4: CP3; Lane 5: CP4; Lane 6: CP5; Lane 7: CP6; Lane 8: CP7.

hybrids (Mu et al., 2003, Pan et al., 2006). However, the inbred line was high susceptible to sugarcane mosaic virus, restricting their cultivation in Northern and Northwestern China, where SCMV is transmitted extensively (Chen et al., 2002; Jiang and Zhou, 2002; Cheng et al., 2002). Thus, the maize inbred line 18-599(red) was used for genetic operation to improve its resistance against SCMV.

Usually, virus-controlling could be resolved by chemical pesticide, backcross breeding, and genetic transformation. Chemical pesticide is not efficient enough and would lead to environment pollution. Backcross breeding should last very long time and the available resistant resources were limited. Relatively, transgenic approach is a fast and high efficient method, and has been used successfully in many plants such as in rice (*Oryza sativa* L.) (Kouassi et al., 2006) and wheat (*Triticum aestivum* L.) (Yan et al., 2006).

At present, many types of acceptors, including suspension culture cells, immature embryos and embryogenic calli, have been successfully used for transformation in maize (Aulinger et al., 2003; Ahmadabadi et al., 2007). Comparatively, suspension culture cells could be efficiently transformed, but it is time-consuming to prepare well-grown suspension cell lines (Ma et al., 2003). Immature embryos as acceptor could shorten transformation cycle due to without calli induction, yet they could not be propagated before transformation. Embryogenic calli have been successfully used as acceptor for transformation in maize and other plants (Asad et al., 2008; Platisa et al., 2008). In this experiment, embryogenic calli of maize inbred line 18-599(red) were selected for transformation, and the results proved that it could simplify the operation process, propagate the transgenic acceptors before transformation and obtain high transformation rate and high plant regeneration frequency, similar to the results by Binott et al. (2008).

Particle bombardment and *Agrobacterium* mediation were the two most commonly used methods in plant transformation (Vasil and Vasil, 2006; Shirgurkar et al., 2006). Usually, *Agrobacterium*-mediated transformation was widely used in dicotyledonary plants and readily achieved transgenic plants with improved-character, but, it is difficult to be used in monocotyledonary plants including maize, due to their low ability to accept exogenous genes from *Agrobacterium* (Shrawat and Lörz, 2006). In addition, it is difficult to eliminate *Agrobacterium* after transformation. Thus, particle bombardment was chosen for transformation in our experiments, the results showed that the exogenous gene was readily introduced into maize materials by this method, similar to the previous reports (Geng et al., 2005; Wang et al., 2006; Sun et al., 2007).

The *CP* gene from SCMV-MDB was first introduced into sweet corn inbred lines R90 and R91 by Murry et al., (1993) to improve their resistance to virus, including SCMV-MDB, MDMV-A and maize chlorotic mottle virus (MCMV). Afterwards, the *CP* gene and the *NIH* gene from

Table 2. The resistance to SCMV for the progenies of seven T_0 transgenic plants.

Genotypes	Plant number	Minimum of ACFL	Maximum of ACFL	Mean of ACFL
Control	20	1.8	2.2	2.0
CP1	14	0	2.1	0.9
CP2	10	0.2	1.6	0.6
CP3	15	0	1.8	0.7
CP4	5	0.8	1.4	1.2
CP5	8	0.2	2.2	1.4
CP6	11	0	2.0	0.8
CP7	22	0	2.0	0.7

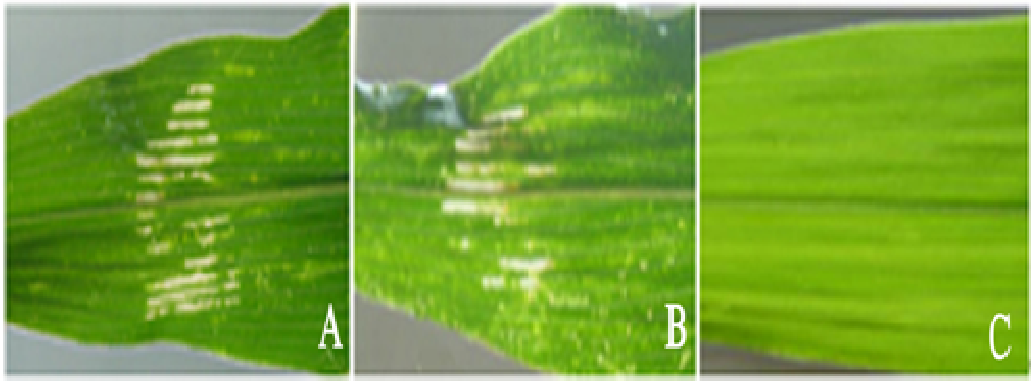

Figure 5. Inoculating identification with SCMV-MDB in T_1 transgenic plants. (A) The leaf from T_1 CP7-3 plant showing resistance to SCMV-MDB after inoculation. (B) The leaf from untransformed plant showing high susceptible to SCMV-MDB after inoculation. (C) The normal plant leaf without being inoculated.

SCMV were transformed into the immature embryos of Zong3 and transgenic plants showing resistant to SCMV were gained by Bai et al. (2006, 2007). According to these reports, the *CP* gene is a beneficial gene to improve the trait of maize against SCMV. Wu et al. (2004) introduced *CPTI* gene into 18-599(red) successfully. Later, Du et al. (2006) transformed *MnSOD* gene into this inbred line and achieve transgenic plants with resistance to oxidative damage. But, to date, no reports existed in the literature on improving the resistance of 18-599(red) to virus. Therefore, the cloned *SCMV CP* gene from SCMV-MDB was transformed into the embyogenic calli of 18-599(red) to increase its resistance to SCMV, the results of PCR and Southern hybridization indicated that *SCMV CP* gene had been integrated into maize genome. Most T_0 transgenic plants appeared some deficient characters, such as lower plant height, smaller ear or less ear grain numbers. Nevertheless, their progenies, the T_1 transgenic plants, recovered to normal thoroughly in plant phenotypes and most of these plants showed much more resistant to SCMV-MDB than control. The results suggested that the transgenic operation did not affect normal agronomic traits of the plants, similar to some previous studies (Du et al., 2006; Sun et al., 2007).

Although all the genes used for increasing maize resistance to SCMV were the *CP* gene from SCMV in our studies and previous experiments by Murry et al. (1993) and Bai et al. (2006), there were different in several aspects among these experiments and the main differrences were listed in Table 3. To be mentioned, the resistant mechanisms for transgenic plants against SCMV were not identical. For Murry et al. (1993), the resistance of virus was achieved by accumulating coat protein in cell to prevent viral decapsidation, thus, the resistance must depend on high-level coat protein expressed by *CP* gene (Powell-Abel et al., 1986; Li and Liang, 2005). While, the resistant mechanism of ours and Bai et al. (2006) was to express antisense RNA for inhibiting the translation of viral mRNA (Lim et al., 1999; Praveen et al., 2005), only less transcripts of antisense *CP* gene in cell could lead to resistance to SCMV (Yang et al., 1996; Hammond and Kamo, 1993).

In summary, the *SCMV CP* gene from SCMV-MDB was cloned and transformed into the elite inbred line 18-599(red) with particle bombardment. The results of PCR and Southern blotting hybridization showed that the *SCMV CP* gene had been integrated into maize genome. The plants regenerated from T_1 transgenic seeds showed

Table 3. The main differences on transformation with *CP* gene of SCMV-MDB in maize.

Genotype	Acceptor	Transformation method	Reference
R90, R91	Suspension culture cells	Particle bombardment, electroporation	Murry et al., (1993)
Zong3	Immature embryos	*Agrobacterium* mediation	Bai et al., (2006)
18-599(red)	Embryogenic calli	Particle bombardment	This study

resistance to SCMV-MDB by artificial inoculation to some extent. Furthermore, the transformation of *SCMV CP* gene did not affect normal agronomic traits. This work lasted only 24 months, and is beneficial for extending the planting regions for the inbred line.

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