Screening Isolates of Soybean mosaic virus for Infectivity in a Model Plant, *Nicotiana benthamiana*

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Abstract

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Soybean mosaic virus (SMV), belonging to the genus *Potyvirus* of the family *Potyviridae*, has a relatively narrow host range almost exclusively confined to leguminous hosts. While disease management through genetic transformation can be an effective approach, soybean remains recalcitrant to routine genetic transformation. In this context, it is important to identify new hosts for SMV that can be used to develop effective transgenic resistance strategies. Transformation in *Nicotiana benthamiana* is simple and highly efficient; hence, here we demonstrate the infectivity of SMV strain SC7 in *N. benthamiana* plants. To identify an SMV strain infectious in *N. benthamiana*, we mechanically inoculated *N. benthamiana* plants with 37 isolates from 21 (SC1 to SC21) SMV strains. Plants

inoculated with isolates of strain SC7 produced mosaic symptoms on leaves. However, *N. benthamiana* plants inoculated with the 20 other SMV strains showed no visible symptoms. Furthermore, soybean cv. Nannong 1138-2 inoculated with sap prepared from symptomatic *N. benthamiana* leaves showed typical SMV mosaic symptoms 2 weeks after inoculation. In addition, SMV was detected in symptomatic *N. benthamiana* and soybean leaves by RT-PCR, DAS-ELISA, and further identified by sequencing. Together, the results indicate that *N. benthamiana* plants could support multiplication of SMV strain SC7. The findings of this study would be useful for the investigation of SMV resistance using the model plant *N. benthamiana*.

Soybean mosaic virus (SMV) is a threat to soybean production in most soybean [*Glycine max* (L.) Merr.] growing provinces of China. Infection by SMV usually results in severe yield losses and seed quality reductions. It has been reported that yield losses usually range from 8 to 50% under natural field conditions (14). SMV is a member of the genus *Potyvirus* in the family *Potyviridae* and it has been classified into 21 strains (SC1 to SC21) on the basis of responses and pathogenicity of SMV isolates on the uniform differentials system in China (13,19,29,31,33). Because of the occurrence of SMV, improvement in production and productivity of soybean is becoming difficult and the disease is a major problem in China.

The increasing impact of SMV on soybean emphasizes the need to introduce SMV resistance into soybean in China. Genetically engineering soybean plants for resistance to viral pathogens has shown significant progress, which has paved the way for improving resistance to SMV in soybean. The most frequently employed methods for plant transformation are cotyledonary node-*Agrobacterium*-mediated and somatic embryo-particle-bombardment-mediated transformation (32). There have been some efforts to improve virus resistance in soybean by transgenic approaches. Overexpression of the SMV coat protein (CP) gene and the 3'-untranslated region (UTR) resulted in high levels of resistance to SMV in transgenic soybean plants (10,11,30). In addition, Di et al. (8) reported that transgenic soybean plants expressing the coat protein precursor (CP-P) gene of *Bean pod mottle virus* (BPMV) were resistant to BPMV, while two of the five R₀ lines lost the CP-P gene in R₁ progenies, which could be due to the chimeric primary transformant or the unstable integration of the CP-P gene in the R₀ plants.

Various methods have been used to improve soybean transformation. The preferred and highly reproducible transformation method uses cotyledonary nodes as the explant material for *Agrobacterium*-mediated gene transfer with hygromycin B selection (24). The addition of thiol compounds to the cocultivation medium and the use of immature cotyledons also has improved soybean transformation efficiency (22–25). Thus, new methods have been developed for more efficient soybean transformation. There still remain, however, many challenges for genotype- and tissue-specific independent transformation of soybean (32).

In general, soybean genes were often evaluated for their function in heterogeneous plants such as *Arabidopsis thaliana* or tobacco (*Nicotiana* spp.) because soybean has remained recalcitrant to routine transformation. Furthermore, *N. benthamiana* is an ideal model plant for the investigation of the molecular basis of virus resistance genes and it has been successfully utilized in studying resistance genes to different plant viruses over the past 25 years (5,12). Abel et al. (1) first developed transgenic *N. tabacum* resistance to *Tobacco mosaic virus* (TMV) through transferring TMV CP gene into tobacco. Prins et al. (26) obtained transgenic plants exhibiting high levels of resistance to *Groundnut ring spot virus*, *Tomato chlorotic spot virus*, and *Tomato spotted wilt virus* (TSWV) by transferring a vector comprising three viral nucleoprotein (N) gene sequences into *N. tabacum*. Fuchs et al. (9) first reported that the transgenic *N. benthamiana* plants containing CP gene constructs of *Watermelon mosaic virus-2*, *Cucumber mosaic virus*, and *Zucchini yellow mosaic virus* were resistant to these three viruses based on extensive field trials. Jan et al. (17) transformed the full-length CP gene of *Turnip mosaic virus*, which was linked to N gene segments of TSWV, into *N. benthamiana*, resulting in resistance to both of the viruses.

However, the host range of SMV is so narrow that the virus is almost completely confined to leguminous plants, such as soybean (*G. max*), common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), and pea (*Pisum sativum*) (3,4,16). In addition, some *Chenopodium* species, *Senna occidentalis*, and *Pinellia ternata*

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(Araceae) could be infected by SMV through mechanical or natural inoculation (2,4,6,27,28). Nevertheless, there were no detailed reports of SMV infecting *Nicotiana* species naturally or artificially. Thus, the present study is aiming to infect *N. benthamiana* plants by SMV and results from these studies will be useful in SMV transgenic research.

Materials and Methods

Plant materials and SMV isolates. Soybean cultivar Nannong 1138-2 (highly susceptible to SMV), *N. benthamiana*, and 37 isolates of 21 SMV strains (SC1 to SC21; Table 1) were provided by the National Center for Soybean Improvement, Nanjing Agricultural University. Isolates were maintained in the susceptible Nannong 1138-2 and used to inoculate the *N. benthamiana* plants.

Virus inoculation. *N. benthamiana* seeds were germinated in vitro on 1/2 MS medium (21) at 25°C under 16-h photoperiod in a growth chamber. After germination, seedlings were individually transplanted to plastic cups containing moistened nutrition soil mixed with vermiculite, and inoculated with SMV at the 5 to 6 leaf stage. The inocula were prepared from soybean plants infected with the 37 SMV isolates, and *N. benthamiana* seedlings were inoculated as described by Li et al. (19). Plants inoculated with 0.01 M phosphate buffer were used as negative controls. All inoculated *N. benthamiana* plants were maintained in growth chambers with a 16-h photoperiod at 25°C. Afterward, inoculated plants were observed at 5-day intervals for 4 weeks for virus symptoms. Four weeks after inoculations when the mosaic symptoms were clearly visible on leaves of *N. benthamiana* plants, symptomatic and asymptomatic leaves were collected and used for reverse transcription (RT)-PCR, double antibody sandwich (DAS)-ELISA, and back-inoculation.

RT-PCR analysis. Total RNAs were extracted from all the samples (SMV-inoculated *N. benthamiana* plants), negative controls (mock-inoculated *N. benthamiana* plants), and positive controls (leaves of Nannong 1138-2 infected with SMV strain SC7) using a total RNA isolation kit (Tiangen, Beijing, China). The quality of the RNA was checked on 1.5% agarose gel. The extracted RNA was converted into cDNA using PrimeScript RT Master MixKit (Takara) according to the manufacturer's instructions. The first strand cDNA was used as a template for RT-PCR with the help of SMV CP gene-specific primers (expected amplicon size 1,058 bp). The forward (5'-ATGCTCAGACAAGTGAGCT-3') and reverse primers (5'-CTCCCTGCCATTTCATAAAC-3') were designed based on the conserved region of SMV CP gene identified by aligning SMV sequences retrieved from the National Center for Biotechnology Information (NCBI) database. PCR amplification was conducted based on the instructions of 2xTaq PCR MasterMix (Tiangen, China). A volume of 25 µl of PCR mixture contained 1 µl of template cDNA (<1 µg), 1 µl of each primer (10 µM), 12.5 µl of 2xMasterMix, and 9.5 µl of sterilized ddH₂O. A thermal cycler (Perkin Elmer, Waltham, MA) was used and the conditions set at one cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for

30 s, and 72°C for 1 min; one cycle of a final extension at 72°C for 5 min; and a 4°C hold. The amplified products were separated by electrophoresis on a 1% agarose gel and photographed with a gel imaging system.

DAS-ELISA. Mock-inoculated and SMV-inoculated *N. benthamiana* plants were assayed for SMV infection by DAS-ELISA using kits purchased from ACD Inc. (cat #V094-R1, Beijing, China) and following the manufacturer's instructions. To ensure reproducibility of DAS-ELISA, sample was collected from all the plants in every group for testing. The raw DAS-ELISA readings of each sample were averaged and converted to multiples of the negative controls (mock-inoculated plants). Samples with a relative ratio greater than 2.0 were considered positive for SMV.

Back-inoculation and sequencing. Inocula were prepared from symptomatic leaves of *N. benthamiana* plants (both RT-PCR and DAS-ELISA positive) and inoculated back to Nannong 1138-2 seedlings. Mock-inoculated plants were used as negative controls. After 2 weeks, when symptoms were clearly seen on the leaves, RT-PCR and DAS-ELISA (described earlier) were performed to confirm the presence of SMV. For further confirmation, CP gene fragments were purified by AxyPrep DNA Gel Extraction Kit (Axygen) and cloned into pMD 19-T vectors (Takara) according to the manufacturers' instructions and sequenced in Invitrogen Ltd., Shanghai, China.

Results

Symptoms on inoculated *N. benthamiana* plants. Only *N. benthamiana* plants inoculated with one strain, namely SC7 (isolates S9 and S10), showed distinct mosaic symptoms in systemic leaves. Yellow mosaic and light green mottled areas appeared on the leaves of *N. benthamiana* plants infected with this strain (Fig. 1). In contrast, *N. benthamiana* plants inoculated with the other 20 SMV strains were symptomless, and appeared healthy and similar to mock-inoculated controls (Table 1). To corroborate the results, the experiments were repeated and similar results were obtained.

Fig. 1. SMV-like mosaic symptoms occurred on the leaves of *Nicotiana benthamiana* plants 4 weeks after inoculation. **A**, *N. benthamiana* inoculated with phosphate buffer as the negative control. **B**, Uninoculated upper leaves were symptomatic 4 weeks after the inoculation of isolate S9 of SMV strain SC7. **C**, Uninoculated upper leaves were symptomatic 4 weeks after the inoculation of isolate S10 of SMV strain SC7.

Table 1. SMV isolates used in study and the symptom appeared on *Nicotiana benthamiana*

Strains	Isolates	Symptom on <i>N. benthamiana</i> ^a	Strains	Isolates	Symptom on <i>N. benthamiana</i> ^a	Strains	Isolates	Symptom on <i>N. benthamiana</i> ^a
SC1	S1	—/—	SC10	S15	—/—	SC17	S29	—/—
SC2	S2	—/—		S16	—/—	SC18	S30	—/—
SC3	S3	—/—	SC11	S17	—/—		S31	—/—
SC4	S4	—/—		S18	—/—	SC19	S32	—/—
	S5	—/—	SC12	S19	—/—		S33	—/—
SC5	S6	—/—		S20	—/—	SC20	S34	—/—
SC6	S7	—/—	SC13	S21	—/—		S35	—/—
	S8	—/—		S22	—/—	SC21	S36	—/—
SC7	S9	—/M	SC14	S23	—/—		S37	—/—
	S10	—/M		S24	—/—			
SC8	S11	—/—	SC15	S25	—/—			
	S12	—/—		S26	—/—			
SC9	S13	—/—	SC16	S27	—/—			
	S14	—/—		S28	—/—			

^a Symptoms on inoculated primary leaves/Symptom on uninoculated upper leaves; —: symptomless; M: mosaic.

Detection of SMV in inoculated plants. The presence of viral RNA in inoculated *N. benthamiana* plants was studied by RT-PCR using CP gene primers. Agarose gel electrophoresis of PCR products revealed the presence of the 1,058-bp CP amplicon only in *N. benthamiana* plants inoculated with two strain SC7 isolates (Fig. 2). No viral RNA was detected from the symptomless plants that were inoculated with the other 20 SMV strains. To confirm presence of SMV, the leaf samples were analyzed by DAS-ELISA, which showed that SMV was detected in *N. benthamiana* plants inoculated with SC7-S9 and SC7-S10 (Table 2). However, the SMV titers were below the detection limits for all the other SMV isolates.

Back-inoculation of soybean cv. Nannong 1138-2 and sequence analyses. Soybean cv. Nannong 1138-2 inoculated with sap prepared from symptomatic leaves of *N. benthamiana* plants developed typical mosaic symptoms on the trifoliate leaves two weeks after inoculation (Fig. 3). The accumulation of the viral RNA was confirmed by RT-PCR followed by DAS-ELISA (Fig. 4 and Table 3). Meanwhile, BLAST analyses showed that CP gene sequences (795 nucleotides in length) of SC7-S9 and SC7-S10 were the same and both of them had 97% nucleotide identities to SMV strain G3 (FJ640978) in the CP gene region. The detection

of SMV in soybean plants after inoculation from symptomatic leaves of *N. benthamiana* and BLAST analyses both indicated that *N. benthamiana* plants had been infected by SMV through artificial inoculation.

Discussion

Since the production of the first transgenic soybean plants (15,20), a variety of methods for generating transgenic virus resistance have been developed for soybean. However, almost all of the SMV-resistant transgenic soybean lines have been developed using the SMV CP gene. The observed resistance was attributed to overexpression of the SMV CP or RNA interference (10,11,18,30). Other SMV genes or sequences have not been transformed. At present, transformation efficiency of soybean is still low. The transgenic resistance for plant viruses has been successfully developed using *Nicotiana* species with high rates of success. Especially, *N. benthamiana* has become the most widely used experimental host in plant viruses due mainly to the susceptibility to a wide variety of plant viruses and can be genetically transformed with high regeneration frequency (5,12). However, the host range of SMV is limited and there were no detailed reports of SMV infecting *Nicotiana* species naturally or artificially. Thus, it is almost impossible to evaluate resistance candidate genes to SMV through transgenic *N. benthamiana* plants. It is worth mentioning that Christie and Crawford (7) as well as Almeida et al. (2) reported infectivity of SMV isolates on the *N. benthamiana*. However, the reports were not detailed and there were no further analyses of the SMV-inoculated *N. benthamiana* plants, and the strains/isolates used in study were also limited. In our study, we used

Fig. 2. Detection of SMV CP gene in SMV-inoculated *Nicotiana benthamiana* plants by RT-PCR. M, DNA marker DL5000 (Takara). +, positive control for SMV (Nannong 1138-2 infected with SMV strain SC7). -, negative control of mock-inoculated *N. benthamiana* plants. w, *N. benthamiana* plants without inoculation was the blank control. 1 and 2, *N. benthamiana* plants infected with isolate SC7-S9 and SC7-S10, respectively.

Fig. 3. Soybean cv. Nannong 1138-2 showed typical mosaic symptom 2 weeks after back-inoculation. **A**, Mock-inoculated Nannong 1138-2 as the negative control. **B**, Back-inoculation with symptomatic leaves of *N. benthamiana* plants infected with SC7-S9. **C**, Back-inoculation with the symptomatic leaves of *N. benthamiana* plants infected with SC7-S10.

Table 2. Serological determinations of DAS-ELISA of SMV-inoculated *Nicotiana benthamiana* plants based on the optical density (OD_{405nm})^a

Strains	Isolates	P ^b (OD _{405nm})	P/N ^c	Strains	Isolates	P ^b (OD _{405nm})	P/N ^c
SC1	S1	0.118	1.00 (-)	SC13	S20	0.121	1.03 (-)
SC2	S2	0.115	0.98 (-)		S21	0.115	0.97 (-)
SC3	S3	0.116	0.98 (-)		S22	0.114	0.97 (-)
SC4	S4	0.113	0.96 (-)	SC14	S23	0.128	1.08 (-)
	S5	0.118	1.00 (-)		S24	0.113	0.96 (-)
SC5	S6	0.115	0.97 (-)	SC15	S25	0.113	0.96 (-)
SC6	S7	0.139	1.18 (-)		S26	0.081	0.69 (-)
	S8	0.116	0.98 (-)	SC16	S27	0.132	1.12 (-)
SC7	S9	3.353	28.42 (+)		S28	0.112	0.95 (-)
	S10	3.380	28.64 (+)	SC17	S29	0.117	0.99 (-)
SC8	S11	0.116	0.98 (-)	SC18	S30	0.113	0.96 (-)
	S12	0.115	0.98 (-)		S31	0.113	0.96 (-)
SC9	S13	0.114	0.97 (-)	SC19	S32	0.117	0.99 (-)
	S14	0.116	0.98 (-)		S33	0.115	0.97 (-)
SC10	S15	0.125	1.06 (-)	SC20	S34	0.115	0.97 (-)
	S16	0.110	0.93 (-)		S35	0.115	0.97 (-)
SC11	S17	0.113	0.96 (-)	SC21	S36	0.109	0.92 (-)
	S18	0.113	0.96 (-)		S37	0.116	0.98 (-)
SC12	S19	0.122	1.03 (-)				

^a (+): positive for SMV; (-): negative for SMV.

^b OD_{405nm} value of each sample was calculated by averaging the three readings of the plate.

^c OD_{405nm} value of negative control was calculated by averaging the three readings of the plate and it is 0.118.

Fig. 4. Detection of SMV CP gene in back-inoculated soybean cv. Nannong 1138-2 by RT-PCR. M, DNA marker 2000 (Tiangen, China). 1 to 4, Nannong 1138-2 inoculated back with symptomatic leaves of *N. benthamiana* plants infected with the isolate of SC7-S9. 5 to 8, Nannong 1138-2 inoculated back with symptomatic leaves of *N. benthamiana* plants infected with the isolate of SC7-S10.

Table 3. DAS-ELISA analyses of the back-inoculated soybean based on the optical density value (OD_{405nm})^a

Back inoculation	SC7-S9		SC7-S10	
	P ^b (D _{405nm})	P/N ^c	P ^b (D _{405nm})	P/N ^c
1	3.719	22.54 (+)	3.056	18.52 (+)
2	3.664	22.21 (+)	3.677	22.28 (+)
3	3.220	19.52 (+)	3.714	22.51 (+)
4	3.388	20.53 (+)	3.653	22.14 (+)

^a (+): positive for SMV.

^b OD_{405nm} value of each back-inoculation was calculated by averaging the three readings of the plate.

^c OD_{405nm} value of negative control was calculated by averaging the three readings of the plate and it is 0.165.

37 isolates of 21 SMV strains (SC1 to SC21) and proved *N. benthamiana* to be infected by visual symptoms, RT-PCR, DAS-ELISA, and back-inoculation, as well as by sequencing of CP gene of SMV strain SC7.

In this study, 37 isolates of 21 SMV strains were used to inoculate the *N. benthamiana* plants. Interestingly, among them only two isolates S9 and S10 of SMV strain SC7 induced mosaic symptoms on *N. benthamiana* leaves. The remaining isolates did not infect *N. benthamiana*. As is known, leguminous plants are the main host of SMV, but the virus also infects some other plants such as *Pinellia ternata*, *Senna occidentalis*, and some *Chenopodium* species naturally or artificially (2,4,6). Previously, Chen et al. (6) reported that *P. ternata* plants could be infected by SMV isolate P-1 naturally. However, *P. ternata* remained symptomless and no virus was detectable when inoculated with SMV isolate HH5. On the other hand, although isolate P-1 could infect soybean and *P. ternata*, it failed to infect *N. benthamiana*, *N. tabacum*, *Chenopodium amaranticola*, and other plants. As well, some *Chenopodium* species could be infected by SMV artificially (4,6).

Results from the study clearly show that *N. benthamiana* is a host for SMV strain SC7, and that the SC7 strain is different from the other strains tested here. Therefore, investigating the factors affecting host range of SC7 and studying the similarity of nucleotide sequences of other SC7 isolates will be useful to understand the evolutionary and taxonomic implications between SC7 and other SMV strains. It is worth mentioning that as more and more SMV SC7 isolates are sequenced, the complete genome sequences and partial gene sequences can be used to study the phylogenetic relationship and molecular variability of the isolates. The outcomes of these studies will certainly provide new insights into the evolutionary process of SMV in relation to its natural host range.

In conclusion, to the best of our knowledge, this is the first detailed report of SMV infecting *N. benthamiana*. Results from this research will enable significant progress in the investigation of the molecular basis of SMV transgenic resistance using the model plant *N. benthamiana*.

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