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ORIGINAL PAPER

Characterization of *Soybean mosaic virus* resistance derived from inverted repeat-SMV-*HC-Pro* genes in multiple soybean cultivars

Le Gao[1] · Xueni Ding[1] · Kai Li[1] · Wenlin Liao[1] · Yongkun Zhong[1] · Rui Ren[1] · Zhitao Liu[1] · Karthikeyan Adhimoolam[1] · Haijian Zhi[1]

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Abstract

Key message ***Soybean mosaic virus*** **resistance was significantly improved in multiple soybean cultivars through genetic transformation induced by inverted repeat-SMV-*HC-Pro*** **genes based on RNAi and post-transcriptional gene silencing.**

Abstract Here, we demonstrate *Soybean mosaic virus* (SMV) resistance in transgenic soybean plants. Transformation of five soybean genotypes with a construct containing inverted repeat-SMV-*HC-Pro* genes-induced high-level SMV resistance. Through leaf-painting assays, polymerase chain reaction (PCR) verification and LibertyLink® strip detection, 105 T_0 and 1059 T_1 plants were confirmed as transgene-positive. Southern blotting confirmed insertion of the T-DNA into the genomic DNA and revealed a low-copy integration pattern. Most T_0 plants were fertile and transmitted the exogenous genes to their progenies (ratios of 3:1 or 15:1). In the T_1 generation, virus resistance was evaluated visually after inoculation with SMV (strain SC3) and 441 plants were highly resistant (HR). SMV disease rating was classified on a scale with 0 = symptomless and 4 = mosaic symptoms with severe leaf curl. In the positive T_1 plants, the disease rating on average was 1.42 (range 0.45–2.14) versus 3.2 (range 2–4) for the nontransformed plants. With the T_2 generation, 75 transgene-positive plants were inoculated with SC3, and 57 HR plants were identified. Virus-induced seed coat mottling was eliminated in the resistant lines. Analysis of SMV levels in the plants was performed using quantitative real-time PCR and double-antibody sandwich enzyme-linked immunosorbent assays; the results revealed no virus or a gradual reduction over time in the viral content, thereby supporting the visual examination results. This is the first report demonstrating pathogen-derived resistance to SMV induced by inverted repeat-SMV-*HC-Pro* genes in multiple soybean cultivars. Our findings contribute positively to the study of transgenic SMV-resistance using RNA interference.

Introduction

With its high protein and oil content, soybean [*Glycine max* (L.) Merr.], one of the world's most important crops, has been a vital part of the human diet over the five thousand years that it has been cultivated in China (Li et al. 2010; Song et al. 2013). *Soybean mosaic virus* (SMV; genus *Potyvirus*, family *Potyviridae*) is the most prevalent and destructive viral pathogen in soybean-growing areas of China. SMV originates from SMV-infected seeds. Such seeds are the primary inoculum source, and are transmitted by more than 30 different aphid species in a nonpersistent manner that can lead to the secondary spread of infection within and among soybean fields (Hill et al. 1980; Lucas and Hill 1980; Halbert et al. 1981; Steinlage et al. 2002). Symptoms caused by SMV include mosaic, chlorosis, curl, and necrosis on soybean leaves, and the virus also induces discoloration of soybean seeds (hilum bleeding or seed coat mottling), which severely damages the commercial value of the seeds (Steinlage et al. 2002; Zhang et al. 2011; Kim

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✉ Haijian Zhi
zhj@njau.edu.cn

[1] National Center for Soybean Improvement, National Key Laboratory for Crop Genetics and Germplasm Enhancement, Key Laboratory of Biology and Genetic Improvement of Soybean, Ministry of Agriculture, Nanjing Agricultural University, Weigang 1, Nanjing 210095, People's Republic of China

et al. 2013). Infection with SMV usually results in significant yield losses and seed quality deterioration (Wang et al. 2001, 2011; Yang et al. 2013). Yield losses have been reported to range from 35 to 50 % under natural conditions in the field (Ross 1977) and from 50 to 100 % during severe outbreaks (Liao et al. 2002). In addition, more severe yield losses can occur when SMV interacts synergistically with *Bean pod mottle virus* (BPMV; genus *Comovirus*, family *Comoviridae*) (Ross 1968; Calvert and Ghabrial 1983; Anjos et al. 1992; Reddy et al. 2001). The occurrence of SMV impedes improvements in the production and productivity of soybeans and the increasing impact of SMV necessitates introduction of SMV-resistance into soybean crops in China.

Breeding disease-resistant cultivars using naturally occurring host plant resistance is the most common approach for controlling the spread of disease and destruction of crops. In attempts to manage SMV disease, three independent single-dominant SMV resistance (*R*) gene loci (*Rsv1*, *Rsv3* and *Rsv4*) identified in soybean cultivars PI 96983, L29, and V94-5152 have been mapped to soybean chromosomes 13, 14, and 2 (MLG-F, B2, and D1b), respectively (Yu et al. 1994; Hayes et al. 2000; Hayes and Saghai Maroof 2000; Gore et al. 2002; Jeong and Saghai Maroof 2004; Saghai Maroof et al. 2010). However, numerous SMV isolates exist and these have been classified into seven strains (G1-G7) in the United States (Cho and Goodman 1979, 1982) and 21 strains (SC1-SC21) in China. (Wang et al. 2003, 2005; Guo et al. 2005; Zhan et al. 2006; Li et al. 2010). The limited resistance spectrum or the late susceptibility of the *R* gene loci and the complexity of the virus (Hayes et al. 2000; Jagtap et al. 2011; Kim et al. 2013) make it hard to introduce broad-spectrum resistance to SMV strains and isolates into adapted soybean cultivars through traditional plant breeding programs. Moreover, the *Rsv* resistance-breaking SMV strains and isolates that emerge frequently can be attributed to interactions between the virus and its host, mutations in the viral genes, and the strong directional selection pressure created by the widespread use of *Rsv*-resistant cultivars (Steinlage et al. 2002; Choi et al. 2005; Koo et al. 2005; Gagarinova et al. 2008). Nevertheless, breeding SMV-resistant cultivars by traditional methods is a labor-intensive and time-consuming process, and is always accompanied by the generation of undesirable traits (Furutani et al. 2006). Hence, new strategies for improving broad-spectrum and persistent resistance to SMV in soybean are urgently needed.

Thanks to the rapid development of molecular biotechnology, genetic transformation is recognized as an effective approach for soybean improvement since transgenic soybean plants were developed (Hinchee et al. 1988; McCabe et al. 1988). In addition, successful development of transgenic virus-resistant soybean plants has been shown in previous studies through use of cotyledonary node-*Agrobacterium*-mediated or somatic embryo-particle-bombardment-mediated soybean transformation systems based on the pathogen-derived resistance (PDR) mechanism (Sanford and Johnston 1985). PDR can be categorized into protein-mediated resistance, which occurs mainly through overexpressing the viral coat protein (CP) gene (Abel et al. 1986), and RNA-mediated resistance (RNA silencing) or RNA interference (RNAi), otherwise known as post-transcriptional gene silencing (PTGS) (Voinnet 2001; Wang and Metzlaff 2005). Di et al. (1996) reported that transgenic soybean plants expressing the coat protein precursor (CP-P) gene of BPMV exhibited complete resistance to viral infection; a similar result was obtained by Reddy et al. (2001). High levels of resistance were observed by overexpressing the SMV CP gene in soybean plants (Wang et al. 2001; Furutani et al. 2006, 2007). Zhang et al. (2011), as well as Kim et al. (2013) also achieved transgenic soybean plants with systemic resistance to SMV through transformation with a SMV-specific gene comprising the inverted repeat (IR) of the replicase and CP gene. Transgenic soybean lines containing SbDV-*CP*-specific short interfering RNA (siRNA) were shown to be resistant to *Soybean dwarf virus* (SbDV; genus *Luteovirus*, family *Luteoviridae*) (Tougou et al. 2006, 2007). The studies mentioned above show that genetically engineering soybean plants based on the PDR mechanism can be an effective approach for controlling viral diseases. However, almost all of the genetically modified virus-resistant soybean lines were completely confined to the CP gene and other viral genes and sequences have not been used for transformation to date. Moreover, the viral CP genes used for transformation are considered to induce genetic variation somewhat readily in the transformed plants (Zhang et al. 2011).

Like other *Potyvirus*, SMV possesses a single-stranded, positive-sense RNA genome of ~9.6 kb in length encoding 10 different mature proteins (Gagarinova et al. 2008). Among them, the helper component-protease (HC-Pro) is considered to be the suppressor apparatus for PTGS and induces seed coat mottling by suppressing endogenously silenced chalcone synthase genes (Senda et al. 2004; Lim et al. 2005, 2007). It has also been reported that overexpression of the SMV HC-Pro gene can alter leaf morphology and significantly reduce seed production in transgenic soybean plants (Lim et al. 2007).

In the present study, using RNAi, we produced genetically engineered SMV-resistant soybean plants by introducing a transgene construct of IR-SMV-*HC-Pro* genes via the cotyledonary node-*Agrobacterium*-mediated system. Robust virus resistance in the transformed plants was confirmed by monitoring for the viral symptoms and molecular detections of the viral infection in the T_1 and T_2 generations of five soybean cultivars.

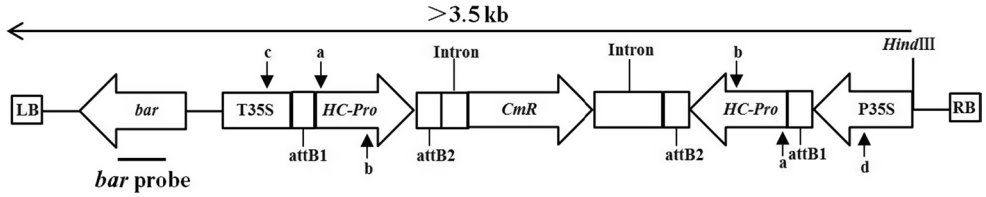

Fig. 1 Schematic representation of the T-DNA region of the recombinant pB7GWIWG2(II)-*HC-Pro* plasmid used for soybean transformation. LB/RB, *left*/*right* border; *bar*, phosphinothricin acetyltransferase gene; P35S/T35S, CaMV 35S promoter/terminator; *CmR*, chloramphenicol resistance gene. *Hind*III recognizes unique restriction enzyme sites within pB7GWIWG2(II)-*HC-Pro*. A *bar* probe specific for the *bar* gene region was used for Southern blot hybridization analysis. **a**, **b**, **c** and **d** indicate the locations of the primers used in this study. **a** and **b** were used to clone the 268-bp HC-Pro gene fragment, **b** and **c** were used to amplify the 435-bp fragment, and **b** and **d** were used to amplify the 449-bp fragment

Materials and methods

Soybean genotypes

Tianlong 1, Huachun 3 and Huachun 6 (Chinese) soybean cultivars and the US cultivars Williams 82 and Jack were used in the *Agrobacterium*-mediated soybean transformation experiments. Mature, dry soybean seeds were surface-sterilized for 16 h in a tightly sealed desiccator containing chlorine gas produced by mixing 3.5 ml of 12 N HCl and 100 ml bleach (5.25 % sodium hypochlorite) as previously described (Di et al. 1996; Paz et al. 2006).

Vector construction and *Agrobacterium tumefaciens* preparation

The 268-bp HC-Pro gene fragment of the SMV SC3 strain was amplified from the corresponding infected leaves by reverse transcriptase polymerase chain reaction. The forward (5′-AGCCACTGATGCAGACAGGATG-3′) and reverse primers (5′-CACGTGCATGGTTTGACACG CA-3′) designed to target the conserved region of the SMV HC-Pro gene were identified by aligning the SMV sequences retrieved from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). According to the manufacturer's manual for the GATEWAY™ system, the HC-Pro gene fragment was subsequently ligated into the entry vector, pDONR™221 (Invitrogen, USA), and finally cloned into the pB7GWIWG2(II) destination vector (Karimi et al. 2002) with the help of BP and LR clonases (Invitrogen, Carlsbad, CA, USA), respectively. The pB7GWIWG2(II) vector contains the phosphinothricin acetyltransferase (*bar*) gene, which confers resistance to the herbicide, phosphinothricin (PPT).

The resulting recombinant pB7GWIWG2(II)-*HC-Pro* construct (Fig. 1) was introduced into the *A. tumefaciens* strain EHA105 by the freeze–thaw method (Hofgen and Willmitzer 1988). A single bacterial colony containing the binary vector was inoculated into liquid YEP medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 100 mg/L spectinomycin and 25 mg/L rifampicin, and grown in a shaker incubator at 200 rpm at 28 °C until the optical density at 600 nm (OD_{600nm}) reached 0.8–1.0. The *A. tumefaciens* cells were pelleted by centrifugation at 4000 rpm for 10 min and subsequently re-suspended in liquid co-cultivation medium containing 0.321 g/L B5 salts with B5 vitamins, 3 % sucrose, 20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 1.67 mg/L *N*-6-benzylaminopurine (BAP), 0.25 mg/L gibberellic acid (GA_3), 3.3 mM L-cysteine, 1.0 mM dithiothreitol, 1.0 mM sodium thiosulfate, and 0.2 mM acetosyringone, pH 5.4. The density of the *A. tumefaciens* cell suspension was adjusted to an OD_{600nm} of 0.6–0.8 before inoculation.

Soybean transformation

The cotyledonary node-*Agrobacterium*-mediated soybean transformation system used in this study was developed on the basis of the procedure described by Hinchee et al. (1988) and Song et al. (2013). Disinfected seeds were placed with their hila proximal to the germination medium containing 3.21 g/L B5 salts with B5 vitamins, 2 % sucrose, 3 mM MES, 0.3 % Phytagel, pH 5.8, and were germinated overnight in an incubator at 25 °C in the dark. On day 2, every one of the imbibed soybean seeds was subjected to coat removal, after which they were split evenly into two explants containing the cotyledons and hypocotyls by a longitudinal cut along the hilum. The cotyledonary-node region of the explants was cut three to four times with the scalpel blade perpendicular to the hypocotyl, and approximately 50 of the wounded explants were immersed in 40 ml of the *A. tumefaciens* suspension for 30 min at room temperature with occasional agitation. After inoculation, the explants were placed with their adaxial side (flat side) oriented upwards on solid co-cultivation medium containing 0.5 % Noble agar (BD-Difco™, cat #214230, Sparks, MD, USA) lined with one to two pieces of sterile filter paper, and incubated at 25 °C in the dark for 3–5 days. After

co-cultivation, the explants were embedded with acropetal tissue upwards in shoot induction medium (SIM) containing 3.21 g/L B5 salts with B5 vitamins, 3 % sucrose, 3 mM MES, 0.8 % agar, 1.67 mg/L BAP, 100 mg/L cefotaxime, 250 mg/L timentin, pH 5.6, and then maintained in a tissue culture chamber at 25 °C with an 18 h photoperiod for 2 weeks. Explants were subcultured into fresh SIM containing 5 mg/L glufosinate for an additional 2 weeks. After shoot induction, the callus/shoot pads without cotyledons were moved to shoot elongation medium (SEM) comprising 4.43 g/L MS salts with B5 vitamins, 3 % sucrose, 3 mM MES, 0.8 % agar, 50 mg/L L-asparagine, 50 mg/L L-glutamine, 0.1 mg/L indole-3-acetic acid, 0.5 mg/L GA_3, 1 mg/L zeatin riboside, 100 mg/L cefotaxime, 250 mg/L timentin, and 5 mg/L glufosinate, pH 5.6. Explants were transferred to fresh SEM with 2-weekly subculture intervals until the elongated shoots reached 3–5 cm in length. Buds suitable for rooting were excised and transferred to rooting medium containing 2.22 g/L MS salts with B5 vitamins, 2 % sucrose, 3 mM MES, 0.3 % Phytagel, 100 mg/L cefotaxime, 0.1 mg/L indole-3-butyric acid, pH 5.6. Two weeks later, the rooted plantlets were rinsed with water to wash off the medium and individually transplanted in plastic pots containing moistened nutrition soil mixed with vermiculite and then covered with a plastic bag for acclimatization in a growth chamber at 25 °C with an 18 h photoperiod for 1–2 weeks. Robustly regenerated seedlings with developed roots were transferred to a greenhouse and prepared for further examination.

Identification of transgenic soybean plants

Putative transformants were confirmed to have been transformed with the IR-sequence of the SMV HC-Pro gene by using a leaf-painting assay, polymerase chain reaction (PCR) verification and LibertyLink® strip detection (QuickStix™ Kit purchased from EnviroLogix Inc., cat #AS 013 LS, Portland, ME, USA). Only plants verified by all three of the identification methods (i.e., unaffected by herbicide treatment, producing PCR amplicons of the correct size, and generating two red lines on a LibertyLink® strip) were considered positive.

The leaf-painting assay was used to estimate herbicide tolerance in the plants. At least three leaves were randomly selected from each plant, and half the upper surface of each leaf was painted with 200 mg/L of PPT containing 0.1 % Tween-20 using a brush, while the other half marked with a black line was the blank control. Treatment responses in the leaves were scored 3–5 days after PPT application. To confirm introduction of the silencing transgene constructs in the plants, PCR was performed to screen for the presence of the 435-bp fragment containing the partial 35S terminator and HC-Pro gene, and the 449-bp fragment containing the HC-Pro gene and partial 35S promoter. Total genomic DNA from the leaves (100 mg fresh weight) of the PPT-resistant plants and the corresponding nontransformed plants was extracted using a DNA isolation kit (Tiangen, Beijing, China). Forward 5′-GCTCAACACATGAGCGAAAC-3′ and reverse primers 5′-CACGTGCATGGTTTGACACGCA-3′ were used to amplify the 435-bp fragment, while the 449-bp fragment was amplified with 5′-CACGTGCATGGTTTGACACGCA-3′ (forward) and 5′-GACGCACAATCCCACTATCC-3′ (reverse) primers. PCR amplification was conducted according to the manufacturer's directions with 2 × Taq PCR MasterMix (Tiangen, China). PCR mixtures (in 25-μl final volumes) contained 1 μl of template DNA (<1 μg), 1 μl of each primer (10 μM), 12.5 μl of 2 × MasterMix, and 9.5 μl of sterilized ddH_2O. A thermal cycler (Perkin Elmer, Waltham, MA, USA) was employed and the conditions set at one cycle at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min with a 4 °C hold. The amplified products were separated by electrophoresis on a 1 % agarose gel and visualized using a gel imaging system. LibertyLink® strip detection was carried out using a QuickStix™ Kit (EnviroLogix Inc., USA) to confirm expression of the *bar* gene, namely the phosphinothricin acetyltransferase (PAT) protein. Leaf tissue was collected and ground-up fully in the bottom of a tapered tube by pestle rotation, 0.5 mL of extraction buffer was added, and a strip was inserted into the tube. Ten minutes later, strips containing only the control line were negative for PAT/*bar* protein expression, while those with two lines (control line and test line) were positive for PAT/*bar* protein expression. Southern blot hybridizations were conducted for further confirmation (see below).

Southern blot hybridization analysis

Total genomic DNA from the leaves of selected T_1 plants (PPT-resistant, both PCR and LibertyLink® strip positive) and the corresponding nontransformed soybean plants was isolated using the cetyltrimethylammonium bromide method. Approximately 30 μg of high molecular weight DNA was digested completely with *Hind*III (Thermo, Waltham, MA, USA), an enzyme that cleaves once in the T-DNA region (Fig. 1). The digested DNA was separated by electrophoresis in a 0.8 % agarose gel and blotted onto a Hybond-N^+ nylon membrane (Amersham, Buckinghamshire, UK). The 538-bp PCR-generated fragment (amplified with 5′-GAGAATTAAGGGAGTCACGTTATG-3′ and 5′-CGTTGCGTGCCTTCCAG-3′ primers) containing the *bar* gene coding region of pB7GWIWG2(II) was labeled with Digoxigenin (DIG)-high prime (Roche, USA) and used as the *bar* probe (Fig. 1). Prehybridization, hybridization, membrane washing and signal detection were

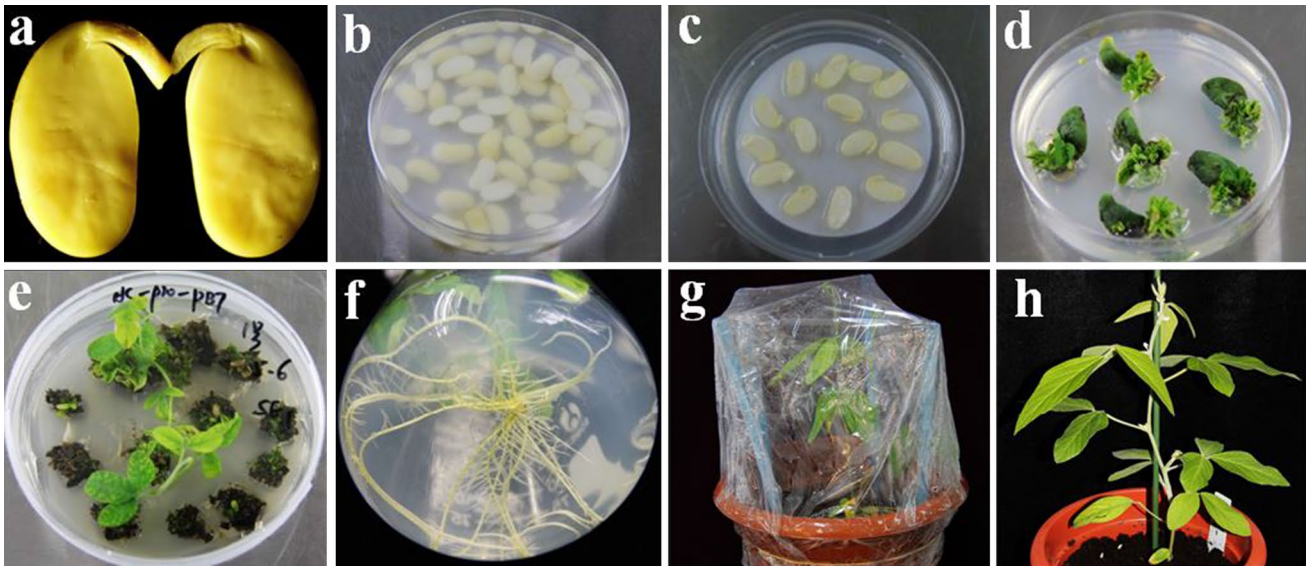

Fig. 2 Cotyledonary node-*Agrobacterium*-mediated soybean transformation system. **a** Two identical explants were generated from one sterilized soybean seed. **b** Wounded explants were inoculated into *A. tumefaciens* suspensions for 30 min. **c** Explants were incubated on solid co-cultivation medium with the adaxial side oriented upwards after inoculation. **d** Multiple buds emerged on the shoot induction medium under 2-week selection with glufosinate (5 mg/L). **e** Shoots became elongated on shoot elongation medium containing 5 mg/L glufosinate. **f** Elongated shoots formed developed roots after 2-week culture in rooting medium without glufosinate. **g** Rooted shoots were transferred to soil and covered with a plastic bag for acclimatization. **h** The surviving plantlets were maintained in a greenhouse

performed according to the instructions of the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Indianapolis, IN, USA).

Virus inoculation and resistance assessment

Diseased leaves collected from the SC3-infected soybean cultivar Nannong 1138-2 (highly susceptible to SMV) were homogenized using a sterilized, ice-chilled mortar and pestle in moderate 0.01 M sodium phosphate buffer (a mixture of sodium phosphate and potassium phosphate, approximately 3–5 ml/g leaf tissue, pH 7.2), with the addition of a small amount of carborundum powder (600 mesh) as an abrasive. Mechanical inoculation of the transgenic plants was carried out by gently rubbing the newly expanded unifoliate leaves with the SMV inoculum (using a paintbrush), and the corresponding nontransformed plants inoculated with SC3 or 0.01 M phosphate-buffered saline were used as the controls. Leaves were rinsed with tap water after the inoculations and the plants were regularly sprayed with pesticides to prevent cross-infection via aphids.

All the positive T_1 plants of the five genotypes were evaluated for virus resistance after inoculation with the SMV SC3 strain in an insect-proof greenhouse. For the T_2 generation, T_1 plants confirmed by the Southern blot described above were selected to generate the T_2 progenies for further evaluation. The responses of the T_1 and T_2 plants inoculated with SMV strain SC3 were visually observed on the fully expanded first (V_1), second (V_2), third (V_3) and fourth (V_4) trifoliate leaves, and were classified as the following four types: HR (highly resistant to SMV and lacking any viral symptoms during the investigations); DR (delayed resistance to SMV with viral symptoms appearing at an early stage and disappearing at later stages); MR (mild resistance to SMV with delayed appearance of viral symptoms or symptoms lighter than those of the susceptible controls); S (susceptible to SMV with the viral symptoms identical to those of the susceptible controls at all the four stages). Also, the SMV disease rating for the fully developed top three leaves (uppermost leaf, top 2nd leaf and top 3rd leaf) on the plants was investigated in the T_1 generation with the average disease rating of these leaves representing the disease rating of the plant. Based on the disease severity of the leaves, the SMV disease rating was classified at five levels: 0 (symptomless); 1 (mild mosaic symptoms); 2 (mosaic symptoms); 3 (mosaic symptoms with shrinking leaves); 4 (mosaic symptoms with severe leaf curl).

Quantitative real-time PCR (qRT-PCR) analysis

Virus accumulation in SMV-inoculated T_1 soybean plants (identified by Southern blotting) was detected by qRT-PCR analysis of the CP gene of SMV strain SC3 using the following primers: 5′-CAGATGGGCGTGGTTATGA-3′ (forward) and 5′-ACAATGGGTTTCAGCGGATA-3′ (reverse). *GmTubulin* (Gene ID: 547844), used as an internal control,

Table 1 Results from screening positive plants from five soybean genotypes in T_0 and T_1 generations

Genotype	No. of infected explants	No. of regenerated plants	No. of positive T_0 plants[a]	No. of generated T_1 plants	No. of positive T_1 plants[a]
Tianlong 1	1969	118	55	637	411
Huachun 3	326	32	10	328	202
Huachun 6	1396	62	33	511	359
Williams 82	712	17	5	90	60
Jack	156	4	2	39	27
Total	4559	233	105	1605	1059

[a] All of the positive plants were confirmed by leaf-painting assay, PCR verification and LibertyLink® strip detection

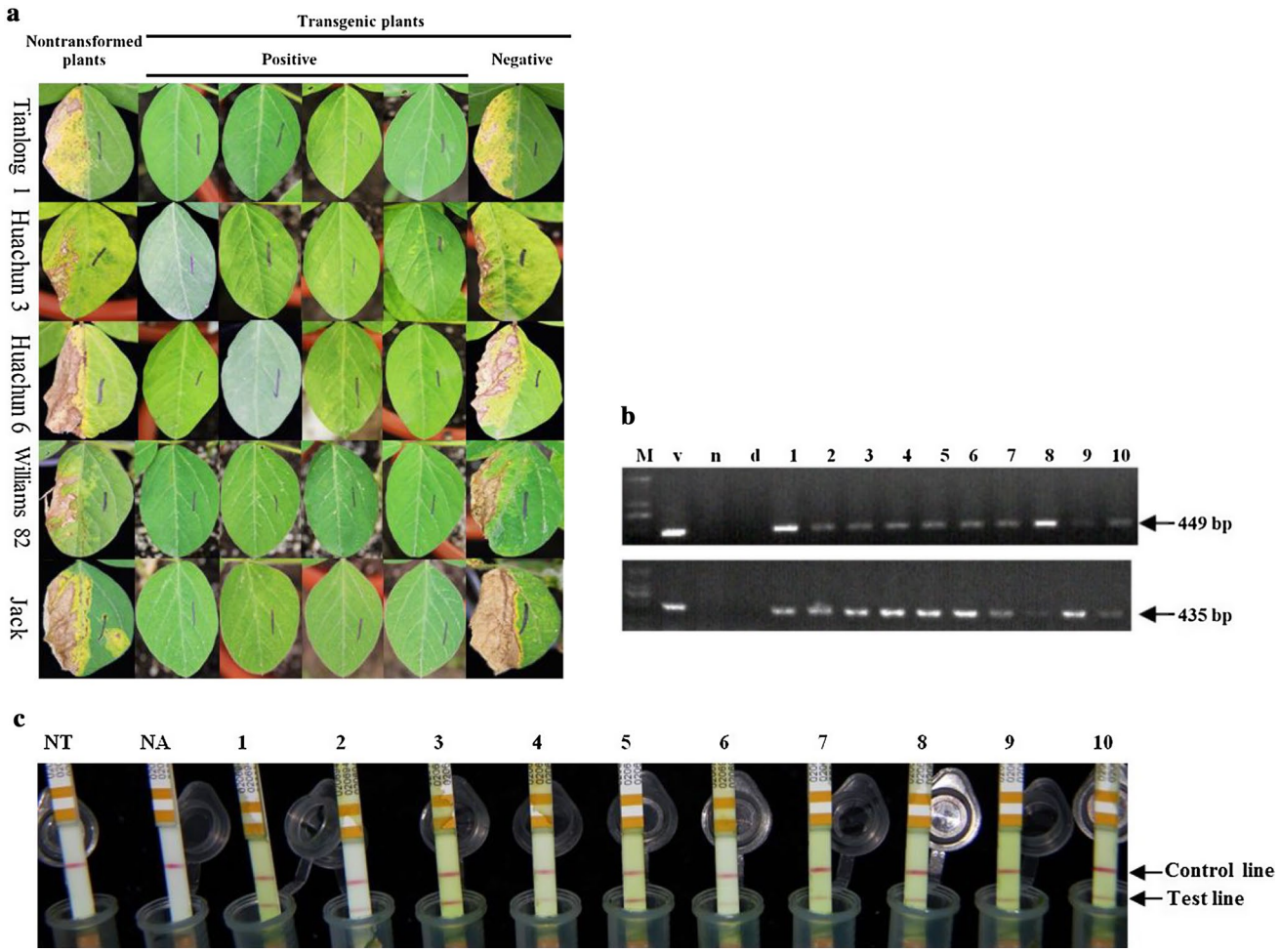

Fig. 3 Confirmation of transgene-positive plants. **a** A leaf-painting assay was used to test for herbicide resistance in the putative transformants. Half of the leaf was painted with 200 mg/L phosphinothricin (PPT) and the other half marked with a black line was the no treatment control. **b** PCR verification was carried out to detect the 449-bp and 435-bp fragments in the genomic DNA of PPT-resistant plants. *M* marker D2000, *v* vector positive control, *n* nontransformed plant negative control, *d* ddH_2O blank control, *1–2*, *3–4*, *5–6*, *7–8* and *9–10* represent the positive transgenic Tianlong 1, Huachun 3, Huachun 6, Williams 82 and Jack plants, respectively. **c** LibertyLink® strip detection was performed to analyze *bar* expression at the translational level. *NT* nontransformed plant, *NA* negative transgenic plant, *1–2*, *3–4*, *5–6*, *7–8* and *9–10* represent the positive transgenic plants from Tianlong 1, Huachun 3, Huachun 6, Williams 82 and Jack, respectively

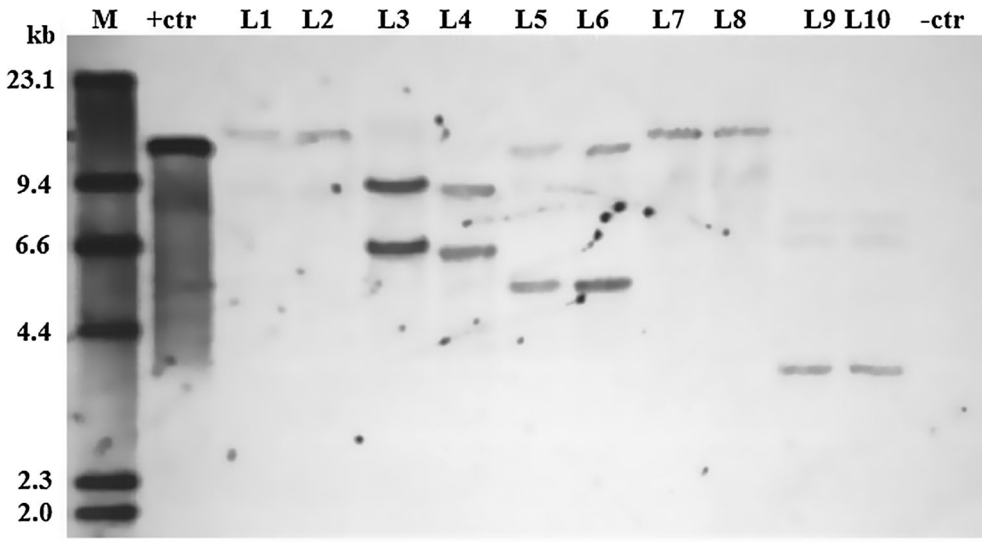

Fig. 4 Southern blot analysis of transgenic T_1 soybean plants. Total genomic DNA (~30 μg) was digested with *Hind*III, which recognizes a single site within the T-DNA region (Fig. 1), and hybridized with the *bar* probe labeled with DIG. M, DNA molecular size markers are shown on the left; +ctr, pB7GWIWG2(II)-*HC-Pro* vector DNA was used as a positive control. *L1–L10* represent T_1 Tianlong 1 (76–18, 76–24), Huachun 3 (11–4, 11–20), Huachun 6 (45–9, 45–21), Williams 82 (10–9, 10–22) and Jack (3–18, 3–23) soybean plants, respectively. −ctr, mixture of genomic DNA sampled from the five genotypes of nontransformed soybean plants was used as the negative controls

was amplified with the following primers: 5′-GGAGTTCACAGAGGCAGAG-3′ (forward) and 5′-CACTTACGCATCACATAGCA-3′ (reverse). Samples were independently collected from the leaves of the T_1 plants and from nontransformed soybeans at 15 and 30 days post-inoculation (dpi). RNA molecules were extracted from finely ground leaf tissue using an RNA Simple Total RNA Kit (Tiangen, China), and first-strand cDNA was synthesized using a PrimeScript® RT Master Mix (Takara, Otsu, Japan). SYBR® Premix Ex Taq™ (Takara, Japan) was used for qRT-PCR and the reactions were analyzed on a LightCycler® 480 II instrument (Roche, USA) according to the manufacturer's protocols. The qRT-PCR mixture in a 20-μl final volume contained 2 μl of template cDNA, 0.4 μl of each primer (10 μM), 10 μl of 2 × SYBR® Premix Ex Taq™, and 7.2 μl of sterilized ddH_2O. The amplification program was set to 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 55 °C for 30 s and 72 °C for 30 s. Samples were analyzed in triplicate. Transcript levels were calculated using the relative quantification ($2^{-\Delta\Delta Ct}$) method, and the data were compared with internal controls.

Serological determination

A double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was employed to assess any changes in the SMV content in the transgenic T_0/T_1 lines and the positive controls after inoculation with the SMV SC3 strain. The kits (complete with anti-SMV antibodies) used for this purpose were purchased from ACD Inc., Fayetteville, AR, USA (cat #V094-R1) and the manufacturer's instructions for use were followed. Five transgenic T_0 lines and eight T_1 lines (confirmed by Southern blotting) were selected for testing. To ensure the reproducibility of the DAS-ELISA results, the transgenic samples were collected from five randomly selected T_1 plants and at least four T_2 plants in each of the selected T_0 and T_1 lines, respectively. The five genotypes of SMV-inoculated and mock-inoculated (inoculated with buffer) nontransformed plants were used as the positive and negative controls, respectively. Positive and negative control samples were independently collected from three plants of each genotype. The raw DAS-ELISA readings from the transgenic and positive samples were averaged and converted to multiples of the negative controls (averaged reading of the negative samples) to represent the SMV-resistance levels of the selected transgenic lines and the nontransformed plants of the five genotypes, respectively. Lines with relative values greater than 2.0 were considered susceptible to SMV.

Statistical analysis

To determine the inheritance patterns of the transgenes, Chi square (χ^2) analyses were conducted using SAS (SAS Institute v. 9.2) for the T_1 segregating progenies (positive: negative) to test goodness-of-fit of the segregation ratios of 3:1, 15:1, and 1:1 with a P value greater than 0.05; these ratios are suggestive of a single functional locus,

Table 2 Segregation analysis of the T_1 progenies of five genotypes

Genotype	T_0 plant no.	T_1 seg. ratio[a]	Best fit	*P*-value	Genotype	T_0 plant no.	T_1 seg. ratio[a]	Best fit	*P*-value	Genotype	T_0 plant no.	T_1 seg. ratio[a]	Best fit	*P*-value
Tianlong 1	9	1:4	1:1	0.1797		22	0:4	NT		Huachun 6	20	3:3	1:1	1.0000
	23	24:15	1:1	0.1495		24	3:0	NT			39	6:10	1:1	0.3173
	58	2:3	1:1	0.6547		30	1:2	NT			55	12:7	1:1	0.2513
	68	3:5	1:1	0.4795		32	1:0	NT			60	4:4	1:1	1.0000
	75	24:16	1:1	0.2059		34	1:0	NT			65	4:6	1:1	0.5271
	86	3:3	1:1	1.0000		38	0:2	NT			9	8:4	3:1	0.5050
	105	5:4	1:1	0.7389		52	3:0	NT			23	4:1	3:1	0.7963
	106	12:7	1:1	0.2513		59	0:2	NT			29	18:10	3:1	0.1904
	12	18:4	3:1	0.4602		62	1:0	NT			41	30:12	3:1	0.5930
	13	4:1	3:1	0.7963		65	1:0	NT			48	18:7	3:1	0.7290
	74	58:25	3:1	0.2813		67	0:3	NT			58	39:10	3:1	0.4579
	76	44:17	3:1	0.6048		71	2:2	NT			64	8:4	3:1	0.5050
	77	27:4	3:1	0.1198		72	2:0	NT			28	5:0	15:1	0.5637
	90	4:1	3:1	0.7963		84	1:0	NT			45	61:4	15:1	0.9745
	93	4:2	3:1	0.6374		85	4:0	NT			50	34:3	15:1	0.6406
	94	5:2	3:1	0.8273		88	0:1	NT			51	29:3	15:1	0.4652
	99	23:4	3:1	0.2216		91	1:0	NT			2	0:18	Other	
	101	25:10	3:1	0.6256		96	4:0	NT			43	65:39	Other	
	103	4:2	3:1	0.6374		98	1:0	NT			4	0:1	NT	
	104	22:4	3:1	0.2575		121	0:2	NT			18	3:1	NT	
	18	22:2	15:1	0.6733	Huachun 3	7	6:7	1:1	0.7815		32	2:0	NT	
	35	7:1	15:1	0.4652		16	12:13	1:1	0.8415		36	0:1	NT	
	48	9:1	15:1	0.6242		30	4:4	1:1	1.0000		42	2:0	NT	
	83	5:0	15:1	0.5637		2	37:10	3:1	0.5555		54	0:4	NT	
	95	13:1	15:1	0.8902		6	15:6	3:1	0.7055		56	2:0	NT	
	120	13:0	15:1	0.3519		19	14:5	3:1	0.8946		63	2:0	NT	
	19	0:5	Other			23	69:28	3:1	0.3792	Williams 82	12	7:11	1:1	0.3458
	31	1:32	Other			11	40:6	15:1	0.0507		10	42:17	3:1	0.4987
	50	0:6	Other			5	4:23	Other			14	11:2	3:1	0.4233
	100	0:9	Other			25	0:19	Other		Jack	3	27:10	3:1	0.7758
	119	0:15	Other			3	0:1	NT			1	0:2	NT	
	11	3:0	NT			9	1:0	NT						
	16	0:1	NT			12	0:4	NT						

NT Not tested

[a] T_1 seg. ratio indicates T_1 segregation ratio (positive: negative)

Table 3 Classification of the response types of positive T_1 plants to SMV strain SC3 at the V_1–V_4 stage

Genotype[e]	Numbers of positive T_1 plants evaluated	HR[a]	DR[b]	MR[c]	S[d]
Tianlong 1	411	47.2 % (194)	26.0 % (107)	24.3 % (100)	2.4 % (10)
Huachun 3	202	35.6 % (72)	0 (0)	43.1 % (87)	21.3 % (43)
Huachun 6	359	33.7 % (121)	2.51 % (9)	35.1 % (126)	28.7 % (103)
Williams 82	60	70.0 % (42)	0 (0)	16.7 % (10)	13.3 % (8)
Jack	27	44.4 % (12)	0 (0)	33.3 % (9)	22.2 % (6)
Total	1059	41.6 % (441)	11.0 % (116)	31.4 % (332)	16.1 % (170)

[a] Highly resistant to SMV, indicating no visible symptoms (i.e., mosaic, chlorosis, curl and necrosis) appeared on soybean leaves at all the four stages

[b] Delayed resistance to SMV, indicating symptoms appeared at an early stage and disappeared at later stages

[c] Mild resistance to SMV, indicating delayed appearance of symptoms or symptoms lighter than those of the susceptible controls

[d] Susceptible to SMV, indicating plants were as symptomatic as the susceptible controls at all the four stages

[e] 20 nontransformed plants of each genotype were evaluated and they were all susceptible at the V_1-V_4 stage

two independent loci and abnormal segregation patterns, respectively (Olhoft et al. 2003). Lines containing more than five T_1 plants derived from self-pollinated positive T_0 plants were tested with the above ratios using the Statistical Analysis System package (SAS Institute v. 9.2). The T_0 lines containing a limited number of T_1 plants (<5) were classified as "Not Tested" so as to avoid any deviations or inaccuracies. Moreover, the T_0 lines tested were classified as "Other" if they did not meet any of the ratios above, and if the lines met more than one ratio, the best fit ratio was selected according to the *P* value.

Results

Generation of transgenic soybean plants containing the IR-sequence

The procedure of soybean transformation is illustrated in Fig. 2. Every soybean seed was separated into two identical explants (Fig. 2a). After inoculation (Fig. 2b), the explants were incubated for co-cultivation (Fig. 2c). Multiple buds were observed (Fig. 2d) after 2-week selection in SIM with glufosinate (5 mg/L). During SEM supplementation with 5 mg/L glufosinate, the majority of the induced shoots were necrotic and few of them elongated beyond 3–5 cm in length (Fig. 2e). When the rooted plantlets (Fig. 2f) had been acclimatized for robustness (Fig. 2g), they were transferred to a greenhouse for further analysis, self-pollination and seed setting (Fig. 2h). In this study, almost all the transgenic T_0 plants recovered via the transformation strategy flowered normally and produced fertile and viable seeds.

Among the 233 regenerated plants from the 4559 infected explants, 105 positive T_0 plants were obtained and subsequently a total number of 1059 positive plants were detected in their T_1 progenies (Table 1). As shown in Fig. 3a, the half-leaf tested on each one of the nontransformed and negative transgenic plants exhibited wilting or yellowing caused by damage from 200 mg/L PPT; however, the half-leaf tested on each the transformation-positive plants was just as healthy as the untreated half. Agarose gel electrophoresis of the PCR products revealed the presence of the expected amplicons only in the positive plants (Fig. 3b). With the LibertyLink® strip detection analysis, the appearance of two red lines simultaneously in a sample (Fig. 3c) indicated that the coding product (PAT) of the *bar* gene was detectable at the translational level.

Genomic DNA from 10 T_1 plants was used for Southern analysis. As shown in Fig. 4, the T_1 soybean plants originating from the same T_0 line exhibited the same integration pattern of T-DNA in the plant genome, and the sizes of all the bands were greater than the expected size of 3.5 kb, which is larger than the fragment between the left border and the unique HindIII site near to the right border (Fig. 1). In contrast, no hybridization signal was detected in the nontransformed plants. The transgene copy numbers were low in every plant. Indeed, L1–L2 (76-18 and 76-24 of Tianlong 1) and L7–L8 (10-9 and 10-22 of Williams 82) contained a single insertion, L3–L4 (11-4 and 11-20 of Huachun 3) and L5–L6 (45-9 and 45-21 of Huachun 6) contained two copies, and L9–L10 (3-18 and 3-23 of Jack) contained three copies (Fig. 4).

Segregation analysis of the T_1 progenies

A total of 1605 T_1 plants from the five cultivars were obtained (Table 1) and the segregation of 63 T_0 lines containing more than five T_1 plants was tested (Table 2), while

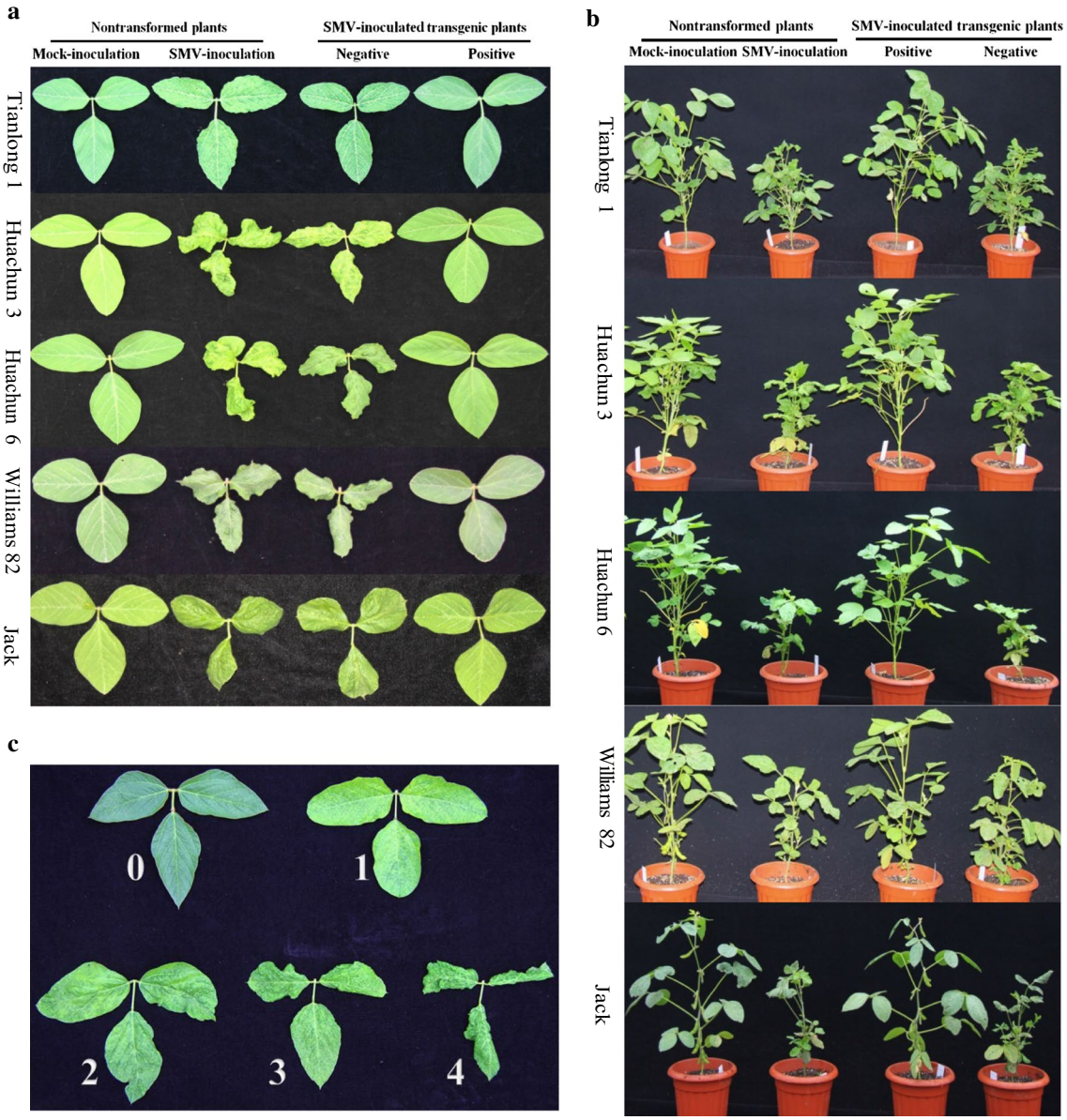

Fig. 5 Responses of the five genotypes of nontransformed and resistant T_1 soybean plants after challenge with SMV strain SC3 and disease rating classification. **a** Symptoms on the V_4 leaves of SMV-infected nontransformed and resistant T_1 plants. Mock-inoculated nontransformed plants were used as the blank controls. **b** Responses of the nontransformed and resistant T_1 plants 8 weeks after mechanical inoculation with SMV strain SC3. Mock-inoculated nontransformed plants were used as the blank controls. **c** The disease rating was classified at five (0–4) levels according to the infection severity in the leaves of the SC3-infected nontransformed Huachun 3 plants

the other 34 lines were not tested because of the limited numbers of T_1 plants. As shown in Table 2, 26 and 11 T_0 lines transmitted the exogenous genes to their T_1 progenies in 3:1 and 15:1 ratios. Additionally, the *P* values were indicative of a significant fit for a Mendelian pattern of inheritance ($P > 0.05$, Table 2). Furthermore, 26 lines transmitted the transgene to the next generation in abnormal segregation ratios (1:1 and "Other", Table 2). Moreover, of the 63 T_0 lines that were evaluated, six showed a lack of T-DNA delivery to the T_1 generation (Table 2).

Transgenic plants displayed robust SMV-resistance in the T_1 and T_2 generations

For the T_1 progenies, the data for the various response types are outlined in Table 3. SMV resistance was significantly enhanced in all the five cultivars and every genotype produced some HR transgenic T_1 plants (Table 3). Of the 1059 positive T_1 plants that were evaluated, 441 HR plants were identified and the ratio reached 41.6 %. In contrast, the number of S plants was only 170 and the

Table 4 Calculation of the average disease rating of five genotypes in the T_1 generation

Genotype	Transgenic T_1 plants		Nontransformed plants	
	Numbers of positive T_1 plants evaluated[a]	Average disease rating[b]	Numbers of plants evaluated[c]	Average disease rating[d]
Tianlong 1	411	0.45	20	3
Huachun 3	202	2.14	20	4
Huachun 6	359	1.82	20	4
Williams 82	60	1.33	20	2
Jack	27	1.36	20	3
Total	1059	1.42	100	3.2

[a] Disease rating of each positive T_1 plant was calculated by averaging the disease ratings of the top three leaves

[b] Disease rating of each genotype was calculated by averaging all the disease ratings of its T_1 progenies

[c,d] Disease ratings of nontransformed plants and each genotype was calculated using the above-mentioned methods

Table 5 Classification of the response types of positive T_2 plants to SMV strain SC3 at the V_1–V_4 stage

Genotype[e]	T_1 line no.	Numbers of positive T_2 plants evaluated	HR[a]	DR[b]	MR[c]	S[d]
Tianlong 1	76–18	11	6	2	3	0
	76–24	9	8	1	0	0
Huachun 3	11–20	15	14	0	1	0
Huachun 6	45–9	5	4	1	0	0
	45–21	6	4	1	1	0
Williams 82	10–9	10	7	3	0	0
	10–22	14	10	4	0	0
Jack	3–23	5	4	0	1	0
Total	–	75	57 (76.0 %)	12 (16.0 %)	6 (8.0 %)	0 (0 %)

[a] Highly resistant to SMV, indicating no visible symptoms (i.e., mosaic, chlorosis, curl and necrosis) appeared on soybean leaves at all the four stages

[b] Delayed resistance to SMV, indicating symptoms appeared at an early stage and disappeared at later stages

[c] Mild resistance to SMV, indicating delayed appearance of symptoms or symptoms lighter than those of the susceptible controls

[d] Susceptible to SMV, indicating plants were as symptomatic as the susceptible controls at all the four stages

[e] 20 nontransformed plants of each genotype were evaluated and they were all susceptible at the V_1–V_4 stage

ratio was low at 16.1 % (Table 3). Among them, Williams 82 had the highest ratio (70.0 %) of HR plants. It is worth mentioning also that the HR ratio (47.2 %) of Tianlong 1 was not as high as Williams 82, which had the lowest ratio (2.4 %, only 10 of 411 plants) of S plants (Table 3). However, as shown in Table 3, most genotypes produced a lot of low resistance plants (DR and MR), which in total comprised the highest proportion (42.4 %) of the plants. S plants of most genotypes, especially Huachun 3 and 6 (Table 3) were also easily found, and possibly resulted from a higher susceptibility to SMV than that observed in the other plants. The appearance of the resistant T_1 plants of the five soybean cultivars was obvious on the leaves and the whole plants (Fig. 5a, b). After challenge with SMV strain SC3, the nontransformed and negative transgenic T_1 soybean plants showed a typical mosaic pattern and serious leaf curl on V_4 leaves; however, in common with the mock controls, the positive plants remained healthy (Fig. 5a). SMV also severely affected soybean plants at the adult stage. Nontransformed and negative transgenic T_1 plants were all badly dwarfed and even fell over 8 weeks after SMV infection; nevertheless, the SMV-resistant ones always showed healthy growth and development (Fig. 5b). Subsequently, the SMV disease rating was classified at five (0–4) levels (Fig. 5c). As Table 4 shows, the 1059 transgenic T_1 plants from the five genotypes showed considerable decreases in their SMV disease ratings with an average of 1.42 (range from 0.45 to 2.14) compared with 3.2 (range from 2 to 4) for the nontransformed plants. In particular, Tianlong 1 had an average SMV disease rating of 0.45, a value far lower than its controls (Table 4).

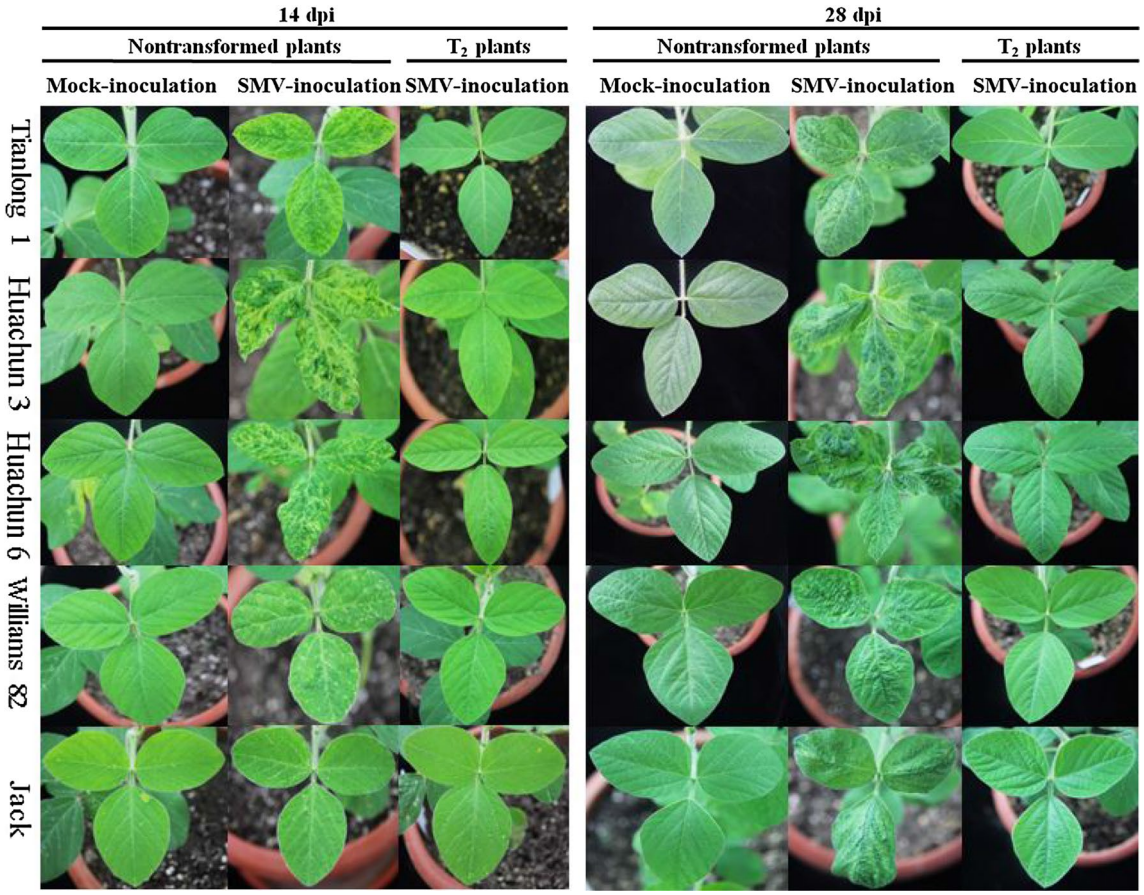

Fig. 6 Appearance of the leaves of five genotypes of nontransformed and resistant T_2 soybean plants at 14 and 28 days post-inoculation (dpi) with the SMV strain SC3. Mock-inoculated nontransformed plants were used as the blank controls

The SMV symptoms were investigated further in the T_2 generation derived from eight selected T_1 plants (confirmed by Southern blotting, Fig. 4). In total, 75 virus-inoculated T_2 plants were evaluated, of which 57 HR plants were identified; no S plants were found at the V_1–V_4 stage in all the five cultivars (Table 5). Photos of T_2 plant leaves were taken at 14 and 28 dpi with the SMV strain SC3 including the SMV-infected nontransformed controls. Compared with the virus-infected controls, the resistant T_2 plants showed no visible symptoms and normal leaves the same as the mock plants were observed (Fig. 6).

To investigate SMV-induced discoloration of the seed coat, the T_3 seeds harvested from the virus-inoculated transgenic lines and the seeds of susceptible nontransformed plants were inspected for mottling damage. Severe seed coat mottling was observed on nontransformed seeds, and dark brown stripes and specks were apparent over the whole seed (Fig. 7). In contrast, no abnormal color was observed on the surface of the T_3 seeds harvested from the resistant lines, and seed coat mottling in these plants was thoroughly eliminated (Fig. 7). Unlike the deformed SMV-infected nontransformed plants producing ~90 % mottled seeds, the SMV-resistant transformants grew normally and produced clean and viable seeds. Moreover, the seed yield of the resistant lines was maintained at a normal level (data not shown).

Molecular analyses of virus content in the transgenic soybeans after SMV infection

Compared with the SMV-infected nontransformed plants, which showed significant increases in virus accumulation from 15 to 30 dpi (Fig. 8), the SMV transcript levels in the transgenic plants decreased markedly and the virus content was considerably lower than that of the SMV-infected nontransformed plants at both 15 and 30 dpi (Fig. 8). Better still, almost all the transgenic T_1 plants harbored negligible amounts of virus 30 days after SMV inoculation (Fig. 8). It is worth noting that despite the increase in SMV RNA in Tianlong 1 (76-18 and 76-24) to a certain extent, the viral content was still apparently lower than that of the controls for both of the two time points (Fig. 8).

Serological determination was applied to the T_1 generation two months after SMV inoculation. Compared with the

nontransformed plants, which are all susceptible to SMV, the viral titers were below the detection limits for all the transgenic T_0 lines (Table 6). For the T_2 generation, seven T_1 lines of the first four cultivars were SMV-negative at 3 weeks post-inoculation (wpi) and no detectable increases in virus were recorded after 2 additional weeks of observation (Table 7). For the Jack line 3–23 (Table 7), although it was mildly positive for SMV at 3 wpi, its OD_{405nm} value was far less than that of the SMV-infected nontransformed (NT) plants and the OD_{405nm} value decreased below 2.0 in the following 2 weeks; this result may be attributed to delayed resistance.

Discussion

Since the production of the first transgenic soybeans (Hinchee et al. 1988; McCabe et al. 1988), cotyledonary node-*Agrobacterium*-mediated and somatic embryo-particle-bombardment-mediated soybean transformation systems have been the most widely and routinely used genetic engineering platforms in recent decades. In terms of its simple manipulation methodology, minimal cost and low-copy integration T-DNA, *Agrobacterium*-meditated transformation is preferred by most researchers for soybean transformation (Yamada et al. 2012). Moreover, the efficiency of *Agrobacterium*-mediated soybean transformation using cotyledonary nodes as the explant material has been greatly improved through the considerable efforts and refined protocols of researchers (Olhoft and Somers 2001; Olhoft et al. 2001, 2003; Paz et al. 2006; Xue et al. 2006; Yamada et al. 2010; Song et al. 2013). Nevertheless, many challenges for genotype- and tissue-specific soybean transformation still remain and soybean is still regarded as recalcitrant to routine transformation (Yamada et al. 2012). In the present research, the average transformation efficiency of the five genotypes was roughly concluded to be about 3 % (data not shown). This value is lower than the efficiency values reported previously by Paz et al. (2006) and Kim et al. (2013) with an average of 3.8 and 5 %, respectively. It may be related to the detection methods used for confirmation of positive transgenic T_0 plants (Fig. 3). As a result of the strict criteria used herein (i.e., plants must be positive by all three identification methods), more than half of the regenerated plants were excluded (Table 1). Although use of such strict criteria possibly leads to a lower transformation efficiency and loss of positive transgenic plants with silenced or fairly weak *bar* expression in the herbicide resistance assay or LibertyLink® strip, the criteria used helped to reduce unnecessary labor in the next generation making this approach an attractive option to the authors of this article.

Chi square analyses were performed on the data from the T_1 generation and some transgenic lines had unexpected segregation ratios such as 1:1 (Table 2). The abnormal segregation events and nontransmission of the T-DNA to the T_1 generation are probably attributable to the chimerism of the primary transformant, nontransformed 'escapes', silenced *bar* expression in the T_1 progenies, unstable integration of T-DNA into the plant genome, and the limited numbers of the T_1 plants (Di et al. 1996; Olhoft et al. 2003). The majority of the lines tested had a Mendelian

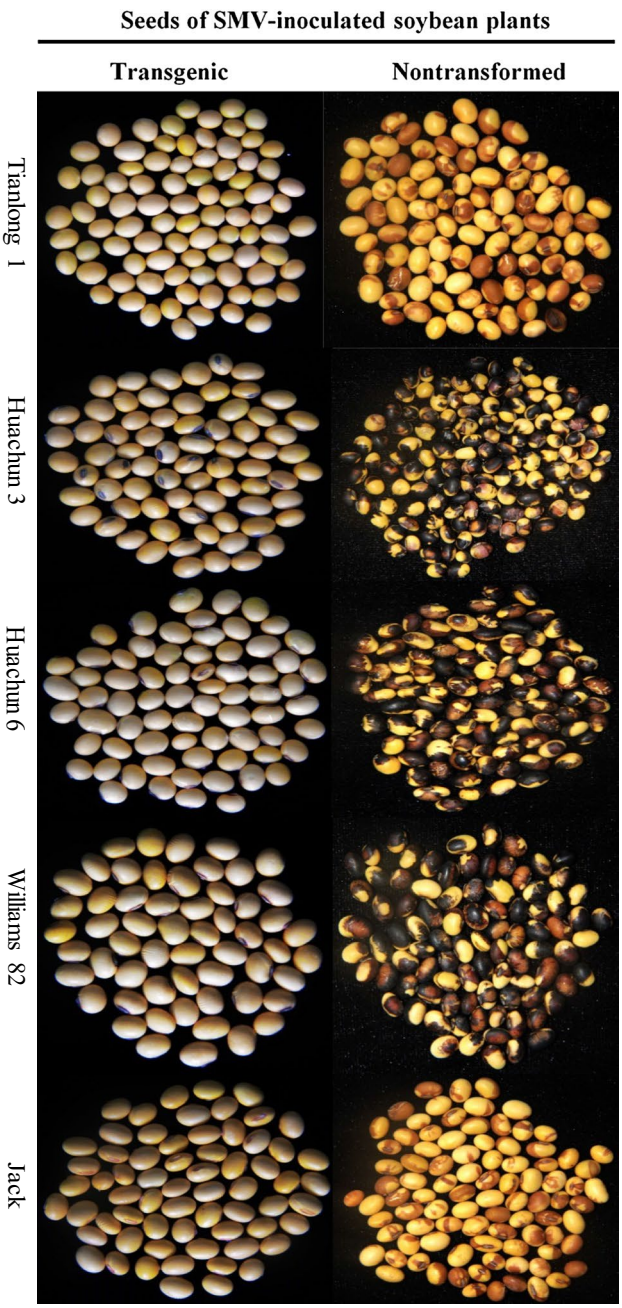

Fig. 7 Virus-induced seed coat mottling was eliminated in transgenic lines. T_3 seeds harvested from the SMV SC3-inoculated resistant lines (*left*) compared with the seeds of the corresponding susceptible nontransformed controls (*right*)

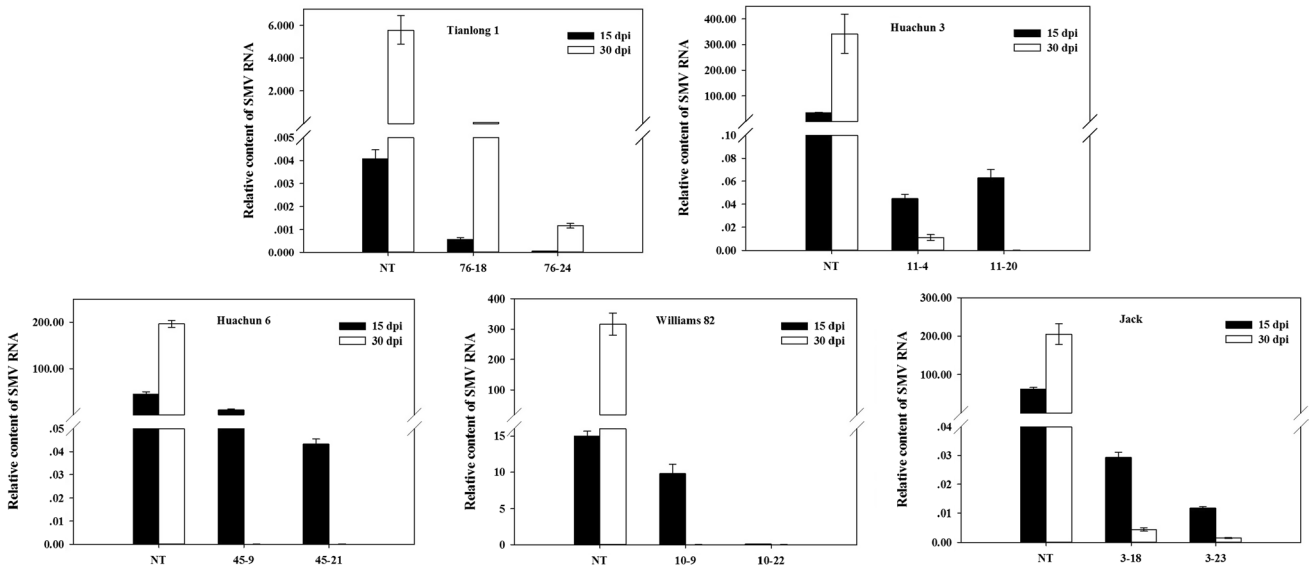

Fig. 8 Virus accumulation in the T_1 generation was detected by qRT-PCR analysis of the CP gene of SMV. *Y*-axes indicate the SMV transcript levels of the five genotypes infected with SMV strain SC3 at different time points (15 dpi and 30 dpi). *X*-axes indicate the transgenic T_1 plants and their related nontransformed (NT) plants. Data are expressed as the means of three biological replicates with error bars indicating the SD

Table 6 DAS-ELISA analyses of five T_0 lines previously inoculated with SMV strain SC3 based on the optical density value (OD_{405nm})

Genotype	Transgenic T_0 lines				Nontransformed plants		
	T_0 line no.	P^a (OD_{405nm})	N^b (OD_{405nm})	*P*/*N*	P^c (OD_{405nm})	N^b (OD_{405nm})	*P*/*N*
Tianlong 1	76	0.112	0.106	1.06 (−)	3.606	0.105	34.24 (+)
Huachun 3	11	0.104	0.104	1.00 (−)	3.533	0.109	32.38 (+)
Huachun 6	45	0.105	0.107	0.99 (−)	3.613	0.111	32.49 (+)
Williams 82	10	0.114	0.106	1.08 (−)	3.594	0.105	34.13 (+)
Jack	3	0.122	0.112	1.09 (−)	3.622	0.116	31.33 (+)

+ positive for SMV, − negative for SMV

[a] OD_{405nm} value of each T_0 line was calculated by averaging the OD_{405nm} values of five SMV-inoculated T_1 plants (transgenic samples) randomly selected in the line

[b] OD_{405nm} value of each negative control was calculated by averaging the OD_{405nm} values of three mock-inoculated nontransformed plants (negative samples)

[c] OD_{405nm} value of each positive control was calculated by averaging the OD_{405nm} values of three SMV-inoculated nontransformed plants (positive samples)

pattern of inheritance with the expected ratios of 3:1 or 15:1 ($P > 0.05$, Table 2). The segregation ratios of Tianlong 1 (line 76), Huachun 3 (line 11), Huachun 6 (line 45) and Williams 82 (line 10) were consistent with the copy numbers detected by Southern blotting (Fig. 4). For Jack (line 3), three copies were detected (Fig. 4); however, it segregated as the 3:1 ratio suggesting the three insertions are closely linked on the same chromosome and inherited as a single functional locus.

It is known that PDR can confer effective resistance against plant viruses in crops including protein-mediated and RNA-mediated resistance, and several studies have reported successful transgenic SMV-derived resistance in soybeans during the past decade (Wang et al. 2001; Furutani et al. 2006, 2007; Zhang et al. 2011; Kim et al. 2013). Wang et al. (2001) reported that two HR transgenic lines (3-24 and 7b-11) were obtained via transformation with the SMV CP gene and its 3′-UTR. However, although the 3-24 line had the highest number of CP gene transcripts and accumulated the highest level of CP, there was no detectable CP protein in the 7b-11 line containing the lowest number of CP gene transcripts. Also, Furutani et al. (2006) obtained three transgenic lines (Nos. 55, 86, and 118) all of which exhibited high resistance to SMV

Table 7 Presence of virus in SMV-inoculated eight T_1 lines at 3 and 5 weeks post-inoculation (wpi) as measured using DAS-ELISA

Genotype	T_1 line no.[a]	3 wpi	5 wpi
Tianlong 1	NT[b]	>10 (+)	>10 (+)
	76–18	0.90 (−)	1.06 (−)
	76–24	0.89 (−)	1.04 (−)
Huachun 3	NT	>10 (+)	>10 (+)
	11–20	1.08 (−)	0.92 (−)
Huachun 6	NT	>10 (+)	>10 (+)
	45–9	1.11 (−)	1.02 (−)
	45–21	1.20 (−)	0.96 (−)
Williams 82	NT	>10 (+)	>10 (+)
	10–9	0.80 (−)	0.78 (−)
	10–22	0.84 (−)	0.74 (−)
Jack	NT	>10 (+)	>10 (+)
	3–23	3.45 (+)	1.17 (−)

NT Nontransformed plants, + positive for SMV, − negative for SMV

[a] OD_{405nm} value of each T_1 line was calculated by averaging the OD_{405nm} values of at least four SMV-inoculated T_2 plants (transgenic samples) randomly selected in the line

[b] OD_{405nm} value of each positive control was calculated by averaging the OD_{405nm} values of three SMV-inoculated nontransformed plants (positive samples), and OD_{405nm} value of each negative control was calculated by averaging the OD_{405nm} values of three mock-inoculated nontransformed plants (negative samples)

infection. Similarly, the CP protein was detected in line Nos. 86 and 118 but not in line No. 55. In the follow-up study on line No. 55, Furutani et al. (2007) demonstrated a strong positive relationship between SMV resistance and the occurrence of siRNAs prior to the inoculation, and the resistance mediated by RNA was maintained even in the T_5 generation. Although Wang et al. (2001) and Furutani et al. (2006) produced SMV CP gene transformants, both studies reported high levels of resistance to SMV in their transgenic soybean plants and no detectable transgene expression. The above findings indicate that RNA-mediated resistance to SMV is easily induced, and that the high levels of resistance conferred almost approach immunity. In our strategy, a hairpin construct made from conserved SMV HC-Pro gene fragments was engineered into five soybean cultivars, and this directly induced RNA-mediated resistance via RNAi. Additionally, the numbers of HR transgenic soybean lines obtained in our study are larger than those reported previously, while the cultivars we transformed were also broader. Zhang et al. (2011) reported that the siRNA levels in transgenic soybean plants increased dramatically after viral infection. Hence, we consider RNA-mediated resistance caused by siRNAs to be more targeted to viruses and more easily triggered compared to protein-mediated resistance. As described above, many of the transgenic plants had DR or MR to SMV (Table 3), and developmental resistance in the transgenic plants was indicated by the gradually attenuating virus content levels (Fig. 8). Additionally, dissipation of the SMV symptoms was also observed after the V_4 stage in some of the transgenic plants considered to be susceptible previously. We speculate that this observation could result from variability in the presence, accumulation, and activity of siRNAs in the different leaf stages, the levels of which became enhanced as the plants grew (Furutani et al. 2007).

HC-Pro, a well-known inhibitor of PTGS, acts by reducing cellular siRNA accumulation and induces seed coat mottling (Senda et al. 2004; Lim et al. 2005, 2007). Kim et al. (2013) produced SMV-resistant plants containing the SMV-CP gene via RNAi; however, most of the transgenic lines were susceptible to SMV, possibly resulting from the SMV HC-Pro gene product blocking the production of siRNAs to suppress RNAi. Thus, the IR-sequence of the SMV HC-Pro gene used in our study is more effective than the SMV CP gene, and SMV RNA silencing will be triggered by direct targeting of the HC-Pro gene coding region in the transgenic soybean plants. Lim et al. (2007) reported that transgenic soybean lines expressing SMV HC-Pro altered the SMV symptoms in a dose-dependent manner. In contrast to the transgenic plants expressing low levels of SMV HC-Pro, the ones with high-level expression showed the most severe symptoms initially, but these symptoms were attenuated in younger leaves. However, all the HC-Pro-transgenic soybean lines obtained by Lim et al. (2007) were susceptible to SMV infection, and the vegetative and reproductive phenotypes of the lines were altered leading to significant seed reductions. In contrast, the transgenic plants, especially the HR lines produced using our RNAi strategy, exhibited robust viral resistance. Moreover, all the transgenic plants containing the harmless 268-bp fragments of SMV HC-Pro gene grew normally, flowered, and set seeds. Furthermore, virus-induced seed coat mottling was prevented and none of the T_3 seeds harvested from the resistant lines were distinguishable from those of the mock-inoculated plants (Fig. 7). These results may be also attributed to silencing the viral HC-Pro gene via our transformation strategy.

Further studies will concentrate on selecting homozygous lines with stable SMV resistance to eliminate the potential effects of hemizygous plants in RNA-mediated resistance. To assess the resistance spectrum of the transgene construct, inoculations with other SMV strains will be performed on advanced generations. Agronomic trait evaluation of transgenic plants and nutritional component detection of the transgenic seeds will be necessary in subsequent field research so as to investigate whether the original traits are altered in the transgenic soybeans. To the best of our knowledge, this is the first report to demonstrate

that PDR to SMV is induced by IR-SMV-*HC-Pro* genes in multiple soybean cultivars.

Author contribution statement HZ conceived the research and oversaw the project. LG designed the study and wrote the manuscript. LG and XD performed construction of RNAi vector, soybean transformation and confirmation of putative transformants. LG, XD, KL, WL, YZ, RR, ZL, and KA conducted SMV inoculations and resistance evaluation, and managed field work. LG performed Southern blot hybridization, qRT-PCR, and DAS-ELISA. LG and KL analyzed the data. All authors critically revised and approved the final manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard The experiments were performed in compliance with the current laws of China.

References

Abel 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(4751):738–743

Anjos RJ, Jarlfors U, Ghabrial SA (1992) Soybean mosaic potyvirus enhances the titer of two comoviruses in dually infected soybean plants. Phytopathology 82(10):1022–1027

Calvert LA, Ghabrial SA (1983) Enhancement by soybean mosaic virus of bean pod mottle virus titer in doubly infected soybeans. Phytopathology 73(7):992–997

Cho EK, Goodman RM (1979) Strains of soybean mosaic virus: classification based on virulence in resistant soybean cultivars. Phytopathology 69(5):467–470

Cho EK, Goodman RM (1982) Evaluation of resistance in soybeans to soybean mosaic virus strains. Crop Sci 22(6):1133–1136

Choi BK, Koo JM, Ahn HJ, Yum HJ, Choi CW, Ryu KH, Chen P, Tolin SA (2005) Emergence of *Rsv*-resistance breaking soybean mosaic virus isolates from Korean soybean cultivars. Virus Res 112(1):42–51

Di R, Purcell V, Collins GB, Ghabrial SA (1996) Production of transgenic soybean lines expressing the bean pod mottle virus coat protein precursor gene. Plant Cell Rep 15(10):746–750

Furutani N, Hidaka S, Kosaka Y, Shizukawa Y, Kanematsu S (2006) Coat protein gene-mediated resistance to soybean mosaic virus in transgenic soybean. Breed Sci 56(2):119–124

Furutani N, Yamagishi N, Hidaka S, Shizukawa Y, Kanemastu S, Kosaka Y (2007) Soybean mosaic virus resistance in transgenic soybean caused by post-transcriptional gene silencing. Breed Sci 57(2):123–128

Gagarinova AG, Babu M, Poysa V, Hill JH, Wang A (2008) Identification and molecular characterization of two naturally occurring soybean mosaic virus isolates that are closely related but differ in their ability to overcome *Rsv4* resistance. Virus Res 138(1):50–56

Gore MA, Hayes AJ, Jeong SC, Yue YG, Buss GR, Saghai Maroof MA (2002) Mapping tightly linked genes controlling potyvirus infection at the *Rsv1* and *Rpv1* region in soybean. Genome 45(3):592–599

Guo DQ, Zhi HJ, Wang YW, Gai JY, Zhou XA, Yang CL, Li K, Li HC (2005) Identification and distribution of soybean mosaic virus strains in Middle and Northern Huang Huai Region of China. Chin J Oil Crop Sci 27(4):64–68

Halbert SE, Irwin ME, Goodman RM (1981) Alate aphid (Homoptera: Aphididae) species and their relative importance as field vectors of soybean mosaic virus. Ann Appl Biol 97(1):1–9

Hayes AJ, Saghai Maroof MA (2000) Targeted resistance gene mapping in soybean using modified AFLPs. Theor Appl Genet 100(8):1279–1283

Hayes AJ, Ma GR, Buss GR, Saghai Maroof MA (2000) Molecular marker mapping of *Rsv4*, a gene conferring resistance to all known strains of soybean mosaic virus. Crop Sci 40(5):1434–1437

Hill JH, Lucas BS, Benner HI, Tachibana H, Hammond RB, Pedigo LP (1980) Factors associated with the epidemiology of soybean mosaic virus in Iowa. Phytopathology 70(6):536–540

Hinchee MAW, Connor-Ward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, Fischhoff DA, Re DB, Fraley RT, Horsch RB (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. Nat Biotechnol 6(8):915–922

Hofgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res 16(20):9877

Jagtap UB, Gurav RG, Bapat VA (2011) Role of RNA interference in plant improvement. Naturwissenschaften 98(6):473–492

Jeong SC, Saghai Maroof MA (2004) Detection and genotyping of SNPs tightly linked to two disease resistance loci, *Rsv1* and *Rsv3*, of soybean. Plant Breed 123(4):305–310

Karimi M, Inzé D, Depicker A (2002) GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci 7(5):193–195

Kim HJ, Kim MJ, Pak JH, Jung HW, Choi HK, Lee YH, Baek IY, Ko JM, Jeong SC, Pack IS, Ryu KH, Chung YS (2013) Characterization of SMV resistance of soybean produced by genetic transformation of SMV-*CP* gene in RNAi. Plant Biotechnol Rep 7(4):425–433

Koo JM, Choi BK, Ahn HJ, Yum HJ, Choi CW (2005) First report of an *Rsv* resistance-breaking isolate of soybean mosaic virus in Korea. Plant Pathol 54(4):573

Li K, Yang QH, Zhi HJ, Gai JY (2010) Identification and distribution of soybean mosaic virus strains in Southern China. Plant Dis 94(3):351–357

Liao L, Chen P, Buss GR, Yang Q, Tolin SA (2002) Inheritance and allelism of resistance to soybean mosaic virus in Zao18 soybean from China. J Hered 93(6):447–452

Lim HS, Ko TS, Lambert KN, Kim HG, Korban SS, Hartman GL, Domier LL (2005) Soybean mosaic virus helper component-protease enhances somatic embryo production and stabilizes transgene expression in soybean. Plant Physiol Bioch 43(10):1014–1021

Lim HS, Ko TS, Hobbs HA, Lambert KN, Yu JM, McCoppin NK, Korban SS, Hartman GL, Domier LL (2007) Soybean mosaic virus helper component-protease alters leaf morphology and reduces seed production in transgenic soybean plants. Phytopathology 97(3):366–372

Lucas BS, Hill JH (1980) Characteristics of the transmission of three soybean mosaic virus isolates by *Myzus persicae* and *Rhopalosiphum maidis*. J Phytopathol 99(1):47–53

McCabe DE, Swain WF, Martinell BJ, Christou P (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. Nat Biotechnol 6(8):923–926

Olhoft PM, Somers DA (2001) L-Cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. Plant Cell Rep 20(8):706–711

Olhoft PM, Lin K, Galbraith J, Nielsen NC, Somers DA (2001) The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary-node cells. Plant Cell Rep 20(8):731–737

Olhoft PM, Flagel LE, Donovan CM, Somers DA (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. Planta 216(5):723–735

Paz MM, Martinez JC, Kalvig AB, Fonger TM, Wang K (2006) Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. Plant Cell Rep 25(3):206–213

Reddy MSS, Ghabrial SA, Redmond CT, Dinkins RD, Collins GB (2001) Resistance to bean pod mottle virus in transgenic soybean lines expressing the capsid polyprotein. Phytopathology 91(9):831–838

Ross JP (1968) Effect of single and double infections of soybean mosaic and bean pod mottle viruses on soybean yield and seed characters. Plant Dis Rep 52(5):344–348

Ross JP (1977) Effect of aphid-transmitted soybean mosaic virus on yields of closely related resistant and susceptible soybean lines. Crop Sci 17(6):869–872

Saghai Maroof MA, Tucker DM, Skoneczka JA, Bowman BC, Tripathy S, Tolin SA (2010) Fine mapping and candidate gene discovery of the soybean mosaic virus resistance gene, *Rsv4*. Plant Genome 3(1):14–22

Sanford JC, Johnston SA (1985) The concept of parasite-derived resistance—Deriving resistance genes from the parasite's own genome. J Theor Biol 113(2):395–405

Senda M, Masuta C, Ohnishi S, Goto K, Kasai A, Sano T, Hong JS, MacFarlane S (2004) Patterning of virus-infected *Glycine max* seed coat is associated with suppression of endogenous silencing of chalcone synthase genes. Plant Cell 16(4):807–818

Song ZY, Tian JL, Fu WZ, Li L, Lu LH, Zhou L, Shan ZH, Tang GX, Shou HX (2013) Screening Chinese soybean genotypes for *Agrobacterium*-mediated genetic transformation suitability. J Zhejiang Univ-Sci B (Biomed Biotechnol) 14(4):289–298

Steinlage TA, Hill JH, Nutter FW Jr (2002) Temporal and spatial spread of soybean mosaic virus (SMV) in soybeans transformed with the coat protein gene of SMV. Phytopathology 92(5):478–486

Tougou M, Furutani N, Yamagishi N, Shizukawa Y, Takahata Y, Hidaka S (2006) Development of resistant transgenic soybeans with inverted repeat-coat protein genes of soybean dwarf virus. Plant Cell Rep 25(11):1213–1218

Tougou M, Yamagishi N, Furutani N, Shizukawa Y, Takahata Y, Hidaka S (2007) Soybean dwarf virus-resistant transgenic soybeans with the sense coat protein gene. Plant Cell Rep 26(11):1967–1975

Voinnet O (2001) RNA silencing as a plant immune system against viruses. Trends Genet 17(8):449–459

Wang MB, Metzlaff M (2005) RNA silencing and antiviral defense in plants. Curr Opin Plant Biol 8(2):216–222

Wang XY, Eggenberger AL, Nutter FW Jr, Hill JH (2001) Pathogen-derived transgenic resistance to soybean mosaic virus in soybean. Mol Breed 8(2):119–127

Wang XQ, Gai JY, Pu ZQ (2003) Classification and distribution of strain groups of soybean mosaic virus in middle and lower Huang-Huai and Changjiang valleys. Soybean Sci 22(2):102–107

Wang YW, Zhi HJ, Guo DQ, Gai JY, Chen QS, Li K, Li HC (2005) Classification and distribution of strain groups of soybean mosaic virus in Northern China spring planting soybean region. Soybean Sci 24(4):263–268

Wang DG, Ma Y, Yang YQ, Liu N, Li CY, Song YP, Zhi HJ (2011) Fine mapping and analyses of R_{SC8} resistance candidate genes to soybean mosaic virus in soybean. Theor Appl Genet 122(3):555–565

Xue RG, Xie HF, Zhang B (2006) A multi-needle-assisted transformation of soybean cotyledonary node cells. Biotechnol Lett 28(19):1551–1557

Yamada T, Watanabe S, Arai M, Harada K, Kitamura K (2010) Cotyledonary node pre-wounding with a micro-brush increased frequency of *Agrobacterium*-mediated transformation in soybean. Plant Biotechnol 27(2):217–220

Yamada T, Takagi K, Ishimoto M (2012) Recent advances in soybean transformation and their application to molecular breeding and genomic analysis. Breed Sci 61(5):480–494

Yang YQ, Zheng GJ, Han L, Wang DG, Yang XF, Yuan Y, Huang SH, Zhi HJ (2013) Genetic analysis and mapping of genes for resistance to multiple strains of soybean mosaic virus in a single resistant soybean accession PI 96983. Theor Appl Genet 126(7):1783–1791

Yu YG, Saghai Maroof MA, Buss GR, Maughan PJ, Tolin SA (1994) RFLP and microsatellite mapping of a gene for soybean mosaic virus resistance. Phytopathology 84(1):60–64

Zhan Y, Zhi HJ, Yu DY, Gai JY (2006) Identification and distribution of SMV strains in Huang-Huai Valleys. Sci Agric Sin 39(10):2009–2015

Zhang XC, Sato S, Ye XH, Dorrance AE, Morris TJ, Clemente TE, Qu F (2011) Robust RNAi-based resistance to mixed infection of three viruses in soybean plants expressing separate short hairpins from a single transgene. Phytopathology 101(11):1264–1269