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ORIGINAL ARTICLE

Fine-mapping and identification of a novel locus *Rsc15* underlying soybean resistance to *Soybean mosaic virus*

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Abstract

Key message ***Rsc15*, a novel locus underlying soybean resistance to SMV, was fine mapped to a 95-kb region on chromosome 6. The *Rsc15*-mediated resistance is likely attributed to the gene *GmPEX14*, the relative expression of which was highly correlated with the accumulation of H_2O_2 along with the activities of POD and CAT during the early stages of SMV infection in RN-9.**

Abstract *Soybean mosaic virus* (SMV) causes severe yield losses and seed quality deterioration in soybean [*Glycine max* (L.) Merr.] worldwide. A series of single dominant SMV resistance genes have been identified on respective soybean chromosomes 2, 13 and 14, while one novel locus, *Rsc15*, underlying resistance to the virulent SMV strain SC15 from soybean cultivar RN-9 has been recently mapped to a 14.6-cM region on chromosome 6. However, candidate gene has not yet been identified within this region. In the present study, we aimed to fine map the *Rsc15* region and identify candidate gene(s) for this invaluable locus. High-resolution fine-mapping revealed that the *Rsc15* gene was located in a 95-kb genomic region which was flanked by the two simple sequence repeat (SSR) markers SSR_06_17 and BARCSOYSSR_06_0835. Allelic sequence comparison and expression profile analysis of candidate genes inferred that the gene *Glyma.06g182600* (designated as *GmPEX14*) was the best candidate gene attributing for the resistance of *Rsc15*, and that genes encoding receptor-like kinase (RLK) (i.e., *Glyma.06g175100* and *Glyma.06g184400*) and serine/threonine kinase (STK) (i.e., *Glyma.06g182900* and *Glyma.06g183500*) were also potential candidates. High correlations were established between the relative expression level of *GmPEX14* and the hydrogen peroxide (H_2O_2) concentration and activities of catalase (CAT) and peroxidase (POD) during the early stages of SMV-SC15 infection in RN-9. The results of the present study will be useful in marker-assisted breeding for SMV resistance and will lead to further understanding of the molecular mechanisms of host resistance against SMV.

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Introduction

Soybean [*Glycine max* (L.) Merr] is the most important legume crop in the world due to its high protein and oil contents. *Soybean mosaic virus* (SMV; Potyvirus), a seed-borne and aphid-transmitted virus, generally infects soybean varieties, causing a typical mosaic pattern in the leaves, top-necrosis, stunted plant growth and seed mottling. Widespread SMV leads to seed quality deterioration and serious yield losses (up to ~50%) in almost all soybean production regions worldwide (Hill et al. 1987; Wrather et al. 2001a, b; Yang et al. 2014).

Based on the expression of disease symptoms in different soybean cultivars (Conover 1948), SMV isolates

have been grouped into different strains. Seven pathotypic strains (G1–G7) have been identified in the United States and Korea (Cho and Goodman 1979; Cho et al. 1983), while 22 strains (SC1–SC22) have been identified in China (Yang 2002; Wang et al. 2003, 2004; Guo et al. 2005; Zhan et al. 2006; Li et al. 2010; Wang et al. 2014). However, the pathotypic relationships among these groups have not yet been fully established. Due to host–pathogen co-evolution, resistance-breaking virulent strains have been identified in South Korea, China, Canada and Iran (Choi et al. 2005; Guo et al. 2005; Viel et al. 2009; Ahangaran et al. 2013). In China, SC15 is the most virulent strain among the 22 strains infecting all ten soybean differentials. This strain was first identified from the SMV isolates collected from Shandong and Beijing (Guo et al. 2005). Furthermore, the following identification of the isolates from soybean fields in Southern and Northeastern China have revealed that SC15 is one of the most virulent and widespread strains (Li et al. 2010, 2014). The distribution of the SMV SC15 strain in China is summarized in Fig. 1.

Because breeding soybean cultivars with broad-spectrum and durable resistance is the most cost-effective and eco-friendly approaches for SMV management in soybean production, and many attempts have been made to identify and explore diverse SMV resistance genes in soybeans. Marker-assisted selection (MAS) is one of the key methods assisting both traditional breeding practices as well as resistance gene-mediated transgenic breeding approaches.

SMV resistance in soybean has been known for many years, and the inheritance of resistance has been the subject of several studies. The SMV resistance in soybean is controlled mostly by single dominant genes. Thus far, multiple independent resistance genes with different SMV strain specificities have been identified. Three distinct SMV resistance loci ('*Rsv*') were first identified from PI 96983 (*Rsv1*), L29 (*Rsv3*) and V94-5152 (*Rsv4*) in the United States. These three loci, which underlie extreme resistance (ER) to SMV strains G1–G4, G5–G7 and G1–G7, were identified on chromosomes 2, 13 and 14, respectively (Kiihl and Hartwig 1979; Buzzell and Tu 1989; Yu et al. 1994; Hayes

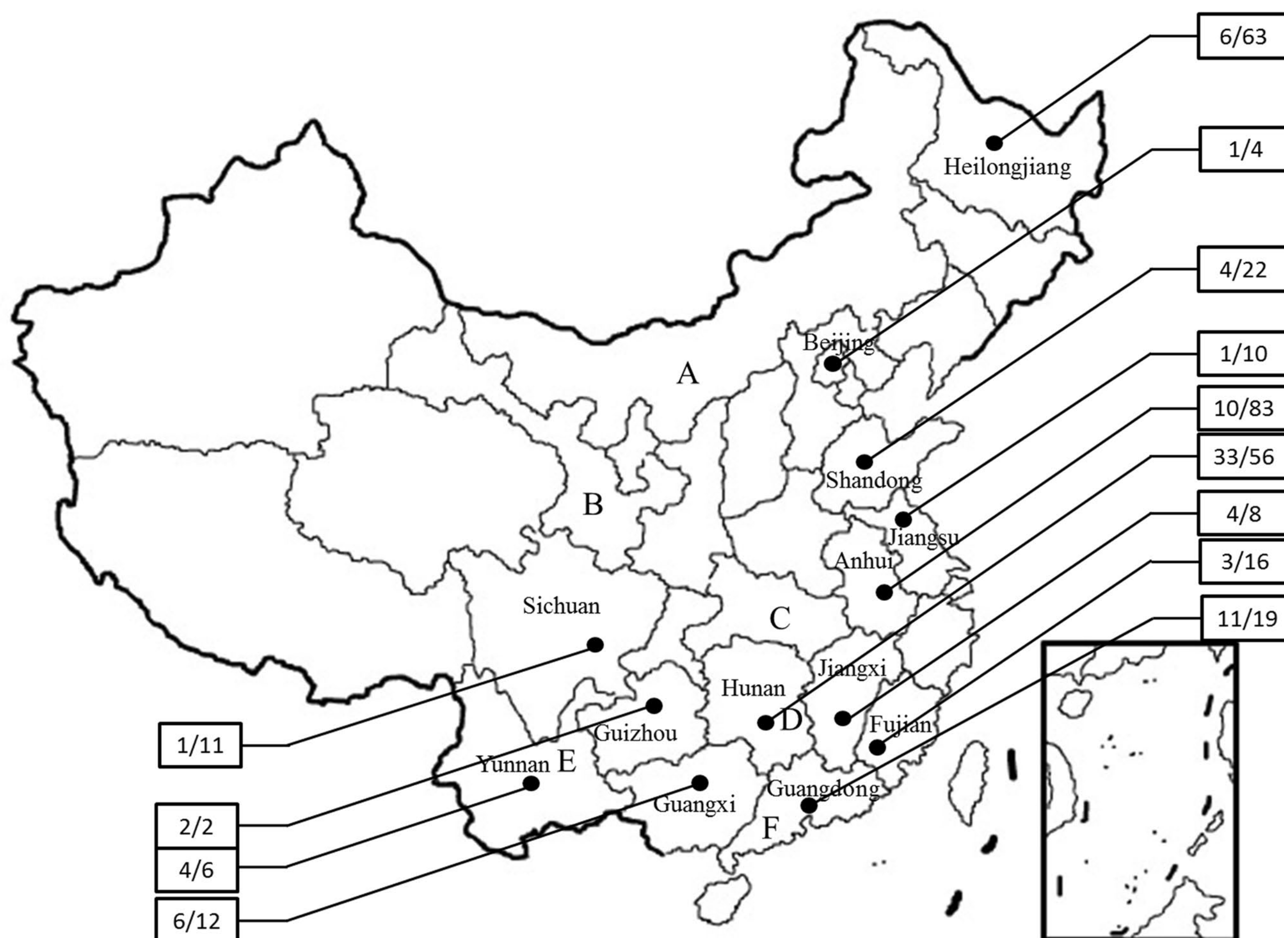

Fig. 1 Summary of the distribution of the SMV strain SC15 in China (Wang et al. 2004, 2014; Guo et al. 2005; Zhan et al. 2006; Li et al. 2010). The fractions in the *boxes* indicate the number of SC15 isolates/the total number of SMV isolates collected in corresponding regions

et al. 2000; Jeong et al. 2002; Gore et al. 2002). In addition, many resistance loci involving the resistance to certain Chinese SMV strains (‘*Rsc*’) from soybean cultivars Kefeng No. 1 (*Rsc7* and *Rsc8*, etc.), Qihuang No. 1 (*Rsc3* and *Rsc14*) and Dabaima (*Rsc4*) were also positioned on chromosome 2, 13 and 14, respectively (Yan et al. 2015; Li et al. 2014; Ma et al. 2011; Zheng et al. 2014; Ma et al. 2010; Li et al. 2012; Wang et al. 2011a, b; Zhao et al. 2016). In a previous study, we screened 957 simple sequence repeat (SSR) markers throughout the soybean genome (Song et al. 2004) using bulked segregant analysis (BSA) of 200 $F_{7:10}$ recombinant inbred lines (RILs). We found that novel resistance locus *Rsc15* was located within a 14.6-cM (~1.6-Mb) genomic interval flanked by Sat_213 and Satt286 on chromosome 6 (Yang and Gai 2011) (Figs. 2a, 3b, c). Although the ‘*Rsv*’ and ‘*Rsc*’ genes are located in the vicinity of each other on the genome, the allelic relationships between these genes remain unclear.

The availability of soybean reference whole genome sequence (WGS) has laid the basis for the development of new markers and identification of candidate genes. With high-density markers developed by Song et al. (2010), many SMV resistance loci have been delimited into meticulous regions, revealing the likely involvement of disease-resistant genes. Researchers discovered that the ER to SMV mediated by *Rsv1*, *Rsv3* and *Rsc4* is likely attributed to one or more members of the NBS-LRR class of disease-resistant genes (Hayes et al. 2004; Shi et al. 2008; Zhang et al. 2012; Yang et al. 2013; Ma et al. 2016; Suh et al. 2011; Wang et al. 2011a; Li et al. 2016). However, *Rsv4*, *Rsc7* and *Rsc8* are not similar to other resistance genes and belong to an original class of disease *R*-genes (Maroof et al. 2010; Ilut et al. 2016; Yan et al. 2015; Wang et al. 2011b; Zhao et al. 2016). Despite studies focussing primarily on resistance gene identification for decades, none of SMV resistance gene has been cloned.

Cloned plant disease-resistance genes share conservative architectural features, and majority of these genes, such as tobacco mosaic virus (TMV) resistance gene *N* in tobacco (Whitham et al. 1994) and a strong candidate gene *3Gg2* for *Rsv1* (Hayes et al. 2004), belong to the NBS-LRR classification. Following NBS-LRR, receptor-like kinases (RLKs) and serine/threonine kinases (STKs) are the second most abundant type of resistance gene classes, such as *Cladosporium fulvum* resistance genes *Cf-9* and *Cf-2* in tomato (Jones et al. 1994; Dixon et al. 1996); *Xanthomonas oryzae* resistance genes *Xa21* and *Xa26* in rice (Song et al. 1995; Sun et al. 2004), *Pseudomonas syringae* resistance gene *Pto* in tomato (Martin et al. 1993), *Puccinia triticina* resistance gene *Lrk10* in wheat (Feuillet et al. 1997) and *Puccinia graminis* resistance gene *Rpg1* in barley (Brueggeman et al. 2002). These conservative architectural features of resistance genes would

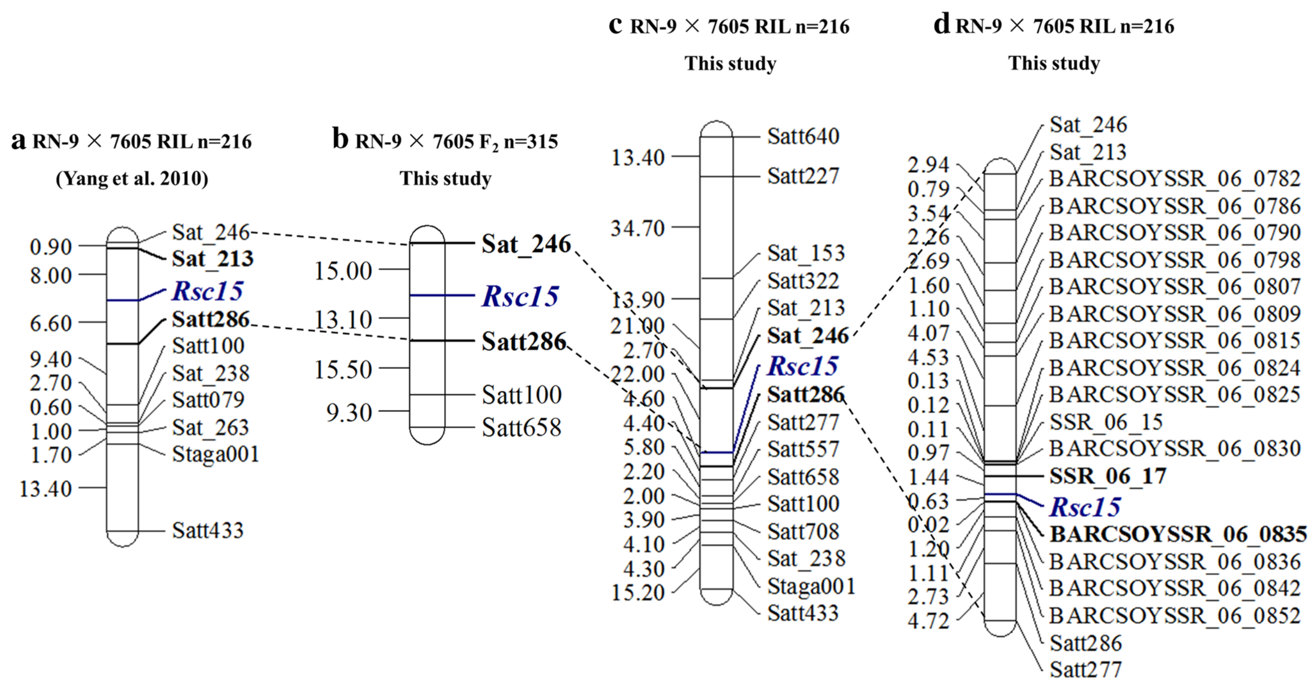

Fig. 2 Genetic linkage map of *Rsc15* with the F_2, RIL population derived from RN-9×7605. **a** Linkage map of *Rsc15* constructed by Yang et al. with the RIL population. **b**, **c** Linkage maps of *Rsc15* constructed with the F_2 and RIL populations in this study. **d** Linkage maps of *Rsc15* using the RIL population genotyped with the additional 17 SSR markers. Genetic distances between the neighboring markers are on the *left* side of linkage groups and the marker names are shown on the *right* side

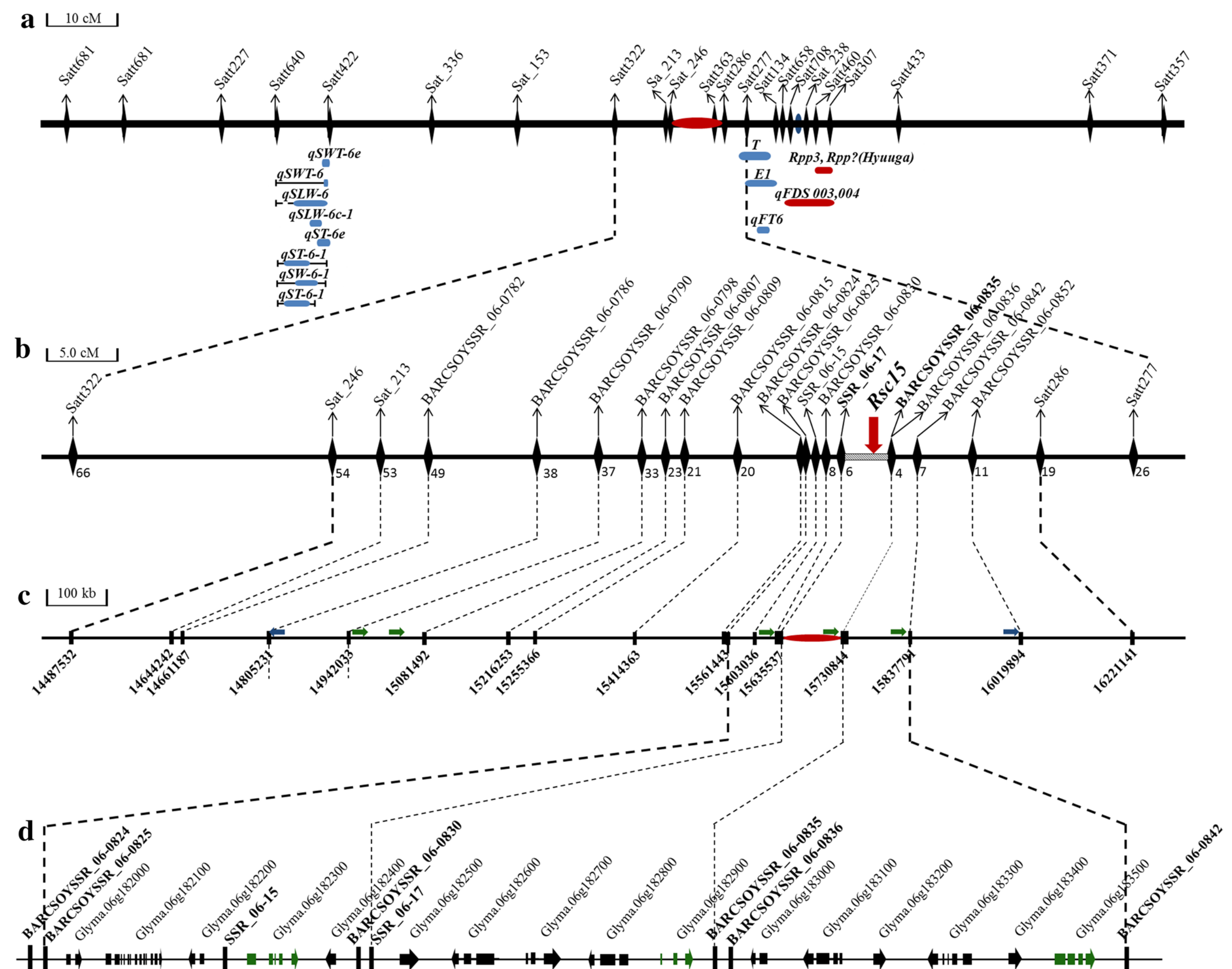

Fig. 3 Genetic and physical maps of SMV resistance locus (*Rsc15*) and transcriptions of candidate resistance genes. **a** Putative position of SMV resistance locus (*Rsc15*) and quantitative trait loci (QTLs) of other traits on chromosome 6. Markers are arranged according to the linkage map of soybean chromosome 6 constructed by Song et al. (2004). The *blue short bars* indicate the putative position of QTLs for seed size traits (Xu et al. 2011), flowering time (*qFT6*) (Zhang et al. 2013) and maturity (*T* and *E1*) (Molnar et al. 2003). The *red short bars* indicate the putative location of QTLs for the resistance of soybean sudden death syndrome (SDS) (*qFDS 003,004*) (Njiti et al. 1998; Abdelmajid et al. 2012) and Asian soybean rust (ASR) [*Rpp3* and *Rpp?* (Hyuuga)] (Hyten et al. 2009; Monteros et al. 2007; Hossain et al. 2015). **b** Genetic maps of SMV resistance locus *Rsc15*. SSR markers were arranged above the *bar* according to the linkage map of the RIL population (Fig. 2d). *Numbers* under the *line* indicate the recombinants detected between the corresponding markers and the resistance gene. **c** Physical map position of *Rsc15*. *Numbers* at the foot of the markers under the line indicate the physical start position of corresponding markers on chromosome 6 according to the Williams 82 reference whole genomic sequence (WGS) (*Glyma* Wm 82.a2.v1 accessible at http://soybase.org/). **d** Transcription models of *Rsc15* candidate genes. *Blue* and *green arrows* indicate the positions of RLK and STK-encoding genes, respectively. Totally, 5 putative genes existed in the target genomic region of *Rsc15* flanked by the two SSR markers SSR_06_17 and BARCSOYSSR_06_0835, according to the soybean reference genome annotation (Glycine max Wm82.a2.v1, accessible at http://soybase.org/, December 2016) (color figure online)

provide references for the identification of SMV resistance genes.

Virus infection of plants is often accompanied by a burst of reactive oxygen species and changes in the levels of endogenous phytohormones. The levels of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are increased with the spontaneous cell death and they activate defense responses in plants (Apel and Hirt 2004; Gadjev et al. 2006; Hernández et al. 2016; Liu et al. 2011). In addition, stress and defense-related proteins, such as pathogenesis-related proteins, peroxidase (POD) and catalase (CAT), are involved in host response to SMV infection (Yang et al. 2011; Liu et al. 2011). The phytohormone salicylic acid (SA) is a signaling molecule of the systemic acquired resistance pathway involved in host response to SMV infection (Sandhu et al. 2009; Hajimorad and Hill 2001), and abscisic acid (ABA)

can also induce *GmPP2C3a* to function as a key regulator of *Rsv3*-mediated ER against SMV (Seo et al. 2014). Exploring the involvement of H_2O_2, POD, CAT and phytohormones in SMV infection would facilitate the clarifying of the molecular mechanisms of host resistance against the SMV in soybean.

Based on the previous study, the objectives of the present study were to: (1) fine map the *Rsc15* gene region on chromosome 6; (2) identify candidate genes for *Rsc15* using sequencing and expression analyses in the presence of SMV; and (3) explore the likely involvement of resistance mediated by *Rsc15*.

Materials and methods

Plant materials and mapping populations

Soybean cultivar RN-9 contains the *Rsc15* gene conferring resistance to SMV strain SC15 (Yang and Gai 2011). The popular cultivars 7605 and Nannong 1138-2 are susceptible to all SMV strains (SC1-SC22). The F_1, F_2 (315 F_2 individuals) and RIL population (comprising 216 $F_{7:11}$ lines) derived from the cross of RN-9×7605 were used to verify the previous results (Table 1), and the RILs were used for the fine-mapping of *Rsc15*. A detailed account of the RIL population is provided in Yang and Gai (2011). The soybean seeds of the two parents along with the F_1, F_2 and RIL populations were planted in plastic pots (20 cm × 20 cm) in aphid-free greenhouses. For each line, a total of 15–20 seeds were planted. All seed materials were obtained from the National Center for Soybean Improvement (NCSI; Nanjing Agricultural University, China).

Inoculation and resistance evaluation

SMV strain SC15, which was previously used by Yang and Gai (2011) to map *Rsc15*, was used in the present study. The SC15 strain was obtained by the NCSI and maintained in leaves of the susceptible cultivar Nannong 1138-2. Young symptomatic leaves were ground using a sterilized mortar and pestle in sodium phosphate-buffered saline (PBS, 0.01 M, pH 7.4) at a ratio of 1:2 (w/v) mixed with a small amount of carborundum powder (600-mesh). Fully expanded primary leaves of soybean plants were mechanically inoculated by gently rubbing with the inoculum using a paintbrush (Li et al. 2010). Immediately after inoculation, tap water was sprayed to the inoculated leaves. Pesticides were regularly sprayed onto the plants to prevent cross-infection via aphids.

All plants of the parents, and the F_1, F_2 and RIL populations were evaluated for virus resistance through weekly visual assessment with detailed scoring in 40 days post-inoculation (dpi). Any inoculated plants that showed a mosaic pattern or chlorotic symptoms in upper leaves were considered as susceptible (S), while those that were symptomless and necrotic were classified as resistant (R) to the virus (Buzzell and Tu 1984; Chen et al. 1991).

DNA isolation, new SSR marker development and genotyping

Young trifoliate leaves from parents and the mapping populations were collected and ground using a mortar and pestle in liquid nitrogen. Total genomic DNA was isolated using a modified cetyltrimethyl ammonium bromide method (Doyle 1990).

A total of 15 SSR markers (i.e., Satt640, Satt227, Sat_153, Satt322, Sat_213, Sat_246, Satt286, Satt277, Satt557, Satt658, Satt100, Satt708, Sat_238, Staga001 and Satt433) on soybean chromosome 6 were selected and used to verify the previous mapping results. For this purpose, the F_2 and RIL populations were used. After verifying the position of *Rsc15*, fine-mapping was performed in the RIL population.

A total of 132 SSR markers flanking the target region, including 108 available SSR markers (Song et al. 2010) and 24 newly developed SSR markers, were used to shorten the *Rsc15* flanking interval. Briefly, for SSR development, the targeted region of the DNA sequence was obtained from the WGS (http://www.phytozome.net/soybean), and SSRs were identified using *SSRHunter* 1.3 software (Li and Wan 2005). The primer pairs were designed using Primer Premier 5.0

Table 1 Reactions of genotypes and progenies when inoculated with SC15

Genotype/progeny	Number of plants			Expected		
	R	S	Total	Ratio	χ^2	*P*
RN-9	21	0	21			
F_1	12	0	12			
F_2	247	68	315	3R:1S	1.7788	0.1823
RILs	109	107	216	1R:1S	0.0046	0.9458
7605	0	17	17			
Nannong 1138-2	0	20	20			

R resistant, *S* susceptible

software (Premier, Palo Alto, CA, USA) (Supplement S1). SSR markers were screened using polymerase chain reaction (PCR), polyacrylamide gel electrophoresis and silver staining following the method used by Wang et al. (2011a).

The phenotypic data (the resistance gene served as a morphological marker) and genotypic data of the F_2 and RIL populations were used to construct linkage maps. Genetic distances between SMV resistance loci and markers were calculated using 'Join Map 4.0′ linkage analysis software (Van Ooijen 2006). MapChart 2.2 (Voorrips 2002) was used for map construction and modification. The markers were assigned to linkage groups (LGs) using a minimum logarithm of likelihood ratio (LOD) score of 3.0 as a criterion to test the linkage.

Collection of various tissues and stress treatments

The roots, stems and leaves were collected from RN-9 plants at the V2 stage; flowers were collected at the R2 stage; and immature pods were collected at the R5 stage. Plant stress treatments were performed at the VC stage. Mock-inoculated (inoculated with PBS) and SMV-infected RN-9 and 7605 leaves were collected at 0, 1, 2, 4, 8, 12 and 24 h post-inoculation (hpi). RN-9 seedlings were used for SA, jasmonic acid (JA), ABA, ethylene (ET) and cold-stress treatments. The seedlings were sprayed with SA (2 mM SA) and JA (100 μM methyl-jasmonate dissolved in 0.01% ethanol), ABA (200 μM ABA) and ET (100 μM 1-aminocyclopropane-1-carboxylic acid). For chilling treatments, the seedlings were placed in a 4 °C growth chamber, and the leaves were harvested at 0, 1, 2, 3, 6, 12, 24, 36, 48, 72 h post-treatment (hpt). Each sample was independently collected with three biological replicates and stored at −80 °C immediately after freezing in liquid nitrogen. Total RNA was extracted from various tissues, and quantitative real-time PCR was conducted.

Candidate gene prediction and quantitative real-time PCR (qRT-PCR)

Putative candidate gene models in the identified flanking region were predicted according to the WGS (https://soybase.org/gb2/gbrowse/gmax2.0/; Glycine max Wm82.a2.v1; verified December, 2016). Gene-specific primers for qRT-PCR were designed from the respective gene transcript sequences (http://www.phytozome.net/soybean) using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al. 2012). The gene *Tubulin* (accession No. AY907703) was used as an internal reference control to normalize the total amount of cDNA in each reaction (Supplement S1). PCR was conducted, and only primers that amplified a single product were selected for qRT-PCR.

Tissues with different treatments were collected, and total RNA was extracted using an RNA Simple Total RNA Kit (Tiangen, Beijing, China). Subsequently, the DNA-free RNA was used for first-strand cDNA synthetization using Oligo (dT) primers and a PrimeScript™ И 1st strand cDNA Synthesis Kit 6210A (Takara, Dalian, China) following the manufacturer's instructions. The qRT-PCR was performed in a 20-μL reaction volume comprising 2.0 μL (approximately 50 ng) of 10× diluted first-strand cDNA, 0.8 μL of each primers (10.0 μM), 10.0 μL of 2× SYBR Green I Master Mix and 6.4 μL of sterile distilled H_2O (Takara, Dalian, China). All reactions were performed in 96-well reaction plates using a LightCycler 480® Real-Time PCR System (Roche, Germany) with three technical replicates. The following PCR conditions were used: 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, and then at 68 °C for 5 min.

The expression of candidate genes was quantified using the relative quantification ($2^{-\Delta\Delta CT}$) method (Wang et al. 2011b). The ratios of relative expression levels between samples infected with SMV and samples inoculated with PBS were regarded as a calibration of the relative expression levels induced by SMV infection alone at different time points.

Sequence alignments of candidate resistance genes

For sequence alignments of candidate genes, gene-specific primers were designed according to the promoter, genomic and transcript sequences of each gene models using Primer Premier 5.0 software (Premier, Palo Alto, CA, USA) (Supplement S1). The fragments for each gene were amplified using the DNA or cDNA of RN-9 and 7605. Purified PCR products were cloned into the *pMD19-T* vector (Takara, Dalian, China) according to the manufacturer's instructions, and sequencing was subsequently performed at Invitrogen Biotechnology Co., Ltd (Shanghai, China). Sequence alignments and polymorphism evaluation were performed using DNAMAN™ (LynnonBiosoft version 8.0, Pointe-Claire, Canada). The sequences were analyzed for open reading frames using GENESCAN (http://genes.mit.edu/GENSCAN.html) and protein domains were annotated using SMART (http://smart.embl-heidelberg.de) and InterProScan (http://www.ebi.ac.uk/InterProScan).

Detection of H_2O_2 and enzyme activity assays

The inoculated leaves of RN-9 and Nannong 1138-2 (inoculated with SC15 and PBS) were collected at 0, 1, 2, 3, 6, 12, 24, 36, 48 and 72 hpi. Subsequently, the samples were ground and suspended in 0.9 mL PBS (pH 7.4) and centrifuged at 4 °C, 2500 rpm for 10 min. The resulting supernatants were collected for H_2O_2 determination and enzyme

activity assays. The H_2O_2 concentration was detected by monitoring the absorbance of the titanium-peroxide complex at 405 nm using the determination kit [A064-1; Nanjing Jiancheng Bioengineering Institute (NJBI), Nanjing], and the CAT and POD activities were assayed using the determination kit (A007-1 and A084-3; NJBI, Nanjing) according to the manufacturer's instructions (Li et al. 2013; Yong et al. 2008).

Statistical analyses

The observed R:S ratios were determined based on the goodness-of-fit for the expected ratios tested using Chi-square (χ^2) analysis with a P value greater than 0.05. The Chi-square (χ^2) test and correlation analyses were both conducted using SAS (SAS Institute v. 9.2).

Results

Response of parents, F_1, F_2 and RILs populations to SC15

Responses of the parents and different populations to SC15 are summarized in Table 1. The results confirmed that RN-9 was R, but that Nannong 1138-2 and 7605 were S to SC15. The F_1 plants generated with RN-9 and 7605 showed the same response to SC15 with RN-9, suggesting the dominant nature of SC15 resistance in RN-9. Through self-pollination of F_1 plants, we generated 315 F_2 plants including 247 R and 68 S plants. The Chi-squared text indicated that this segregation pattern was consistent with a Mendelian segregation ratio of 3:1 (R:S). Thus, SC15 resistance derived from RN-9 is controlled by a single dominant gene. Additionally, the results for the RIL population were also consistent with an expected Mendelian inheritance ratio of 1:1 (R:S). This finding further demonstrated that SC15 resistance was controlled by one dominant gene, consistent with the report of Yang and Gai (2011).

Fine-mapping of *Rsc15* with the RILs

The objective of the present study was to fine map *Rsc15*, the locus previously described by Yang and Gai (2011). The *Rsc15* locus was mapped within a 14.6-cM (~1.6-Mb) genomic region flanked by Sat_213 and Satt286 on chromosome 6, using 200 RILs and a genetic map comprising ten SSRs. To verify the map location of the *Rsc15* gene, 4 and 15 SSR markers were screened in the F_2 and the RIL population (216 lines), respectively. The genotypic data were used for linkage analysis, and the results revealed that the resistance gene was located between Sat_246 and Satt286 (Fig. 2b, c). These results were consistent with those of Yang and Gai (2011).

To fine map *Rsc15*, new markers were added around and within the original map interval using RILs. A total of 108 SSR markers flanking *Rsc15*, developed by Song et al. (2010), were used to screen the parental lines. Among the 108 SSRs tested, 16 markers exhibited polymorphisms between the parents. These markers were used to genotype the RILs, and *Rsc15* was delimited within the interval flanked by BARCSOYSSR_06_0830 and BARCSOYSSR_06_0835. To further narrow down the region, a total of 24 new SSR markers in the targeted region were developed, and two of the markers (SSR_06_15 and SSR_06_17) were polymorphic. RILs were subsequently used for genotyping analysis. The result revealed that *Rsc15* was located within the 95-kb region in the Williams 82 reference genome (Glyma Wm 82.a2.v1), and it was flanked by the two markers SSR_06_17 and BARCSOYSSR_06_0835 at genetic distances of 2.38 and 1.66 cM, respectively (Figs. 2d, 3b, c).

Identification of candidate resistance genes through allelic sequence comparison

To identify the likely candidate genes of *Rsc15*, the target region was examined for predicted genes according to the soybean reference genome annotation database (Glycine max Wm82.a2.v1 accessible at https://soybase.org/gb2/gbrowse/gmax2.0/; December 2016). A total of 136 genes were identified between Sat_246 (physical start position Gm06:14487532) and Satt286 (Gm06: 16221141), but 23 genes were not annotated for the loci (Supplement S2; Fig. 3c). None of the remaining 113 genes was found to belong to NBS-LRR family, while two genes encoded RLK (i.e., *Glyma.06g175100* and *Glyma.06g184400*) and five genes encoded STK (i.e., *Glyma.06g176800*, *Glyma.06g177700*, *Glyma.06g182300*, *Glyma.06g182900* and *Glyma.06g183500*) (Fig. 3c).

In the 95-kb *Rsc15* region, five gene models were predicted (Fig. 3d; Table 2). Gene *Glyma.06g182500* encodes a protein of unknown function (*DUF1645*); *Glyma.06g182600* (designated as *GmPEX14*) encodes a peroxisomal membrane anchor protein; *Glyma.06g182700* encodes a predicted carbonic anhydrase involved in protection against oxidative damage; *Glyma.06g182800* encodes a tetratricopeptide repeat (TPR)-like superfamily protein; and *Glyma.06g182900* encodes an STK (Table 2). Allelic sequence comparison was conducted using the DNA and cDNA sequences from the two parents. The results showed that all of these five candidate genes were conserved in the coding sequence (CDS) with their allelic counterparts, and the three genes (*Glyma.06g182700*, *Glyma.06g182800* and *Glyma.06g182900*) were also conserved in promoter sequences, while a nucleotide insertion/deletion

Table 2 Candidates' annotations in the *Rsc15* region on chromosome 6 from the soybean WGS (Glycine max Wm82.a2.v1, December, 2016)

Gene and marker name	Chromosome location	AA	Arabidopsis homologs	Annotations	Polymorphism
BARCSOYSSR_06_0824	Gm06:15558998..15559065				
BARCSOYSSR_06_0825	Gm06:15561443..15561478				
Glyma.06g182000	Gm06:15571670..15573965	173	AT4G22220.1	Iron-binding protein involved in Fe-S cluster formation	
Glyma.06g182100	Gm06:15581101..15595225	913	AT4G32180.1	Pantothenate kinase PanK and related proteins	
Glyma.06g182200	Gm06:15600149..15602576	354	AT5G62940.1	Zinc finger (zf-Dof)	
SSR_06_15	Gm06:15603036..15603155				
Glyma.06g182300[a]	Gm06:15610782..15625927	321	AT3G48190.1	Non-specific serine/threonine protein kinase/threonine-specific protein kinase	
Glyma.06g182400	Gm06:15628130..15629231	318	AT2G29880.1	Myb/SANT-like DNA-binding domain (Myb_DNA-bind_3)	
BARCSOYSSR_06_0830	Gm06:15632326..15632345				
SSR_06_17	Gm06:15635537..15635794				
Glyma.06g182500[a,b]	Gm06:15642471..15643990	308	AT5G62770.1	Protein of unknown function (DUF1645) (DUF1645)	None
Glyma.06g182600[a,b]	Gm06:15654270..15659464	460	AT5G62810.1	PEROXISOMAL MEMBRANE PROTEIN GMPEX14	1 SNP and 1 InDel
Glyma.06g182700[a,b]	Gm06:15677974..15682343	262	AT5G14740.1	Predicted carbonic anhydrase involved in protection against oxidative damage	None
Glyma.06g182800[a,b]	Gm06:15705290..15709591	754	AT1G27500.1	Tetratricopeptide repeat, kinesin light chain	None
Glyma.06g182900[a,b]	Gm06:15722947..15727315	343	AT1G62400.1	Serine/threonine protein kinase|PROTEIN KINASE 6-LIKE PROTEIN	None
BARCSOYSSR_06_0835	Gm06:15730844..15730903				
BARCSOYSSR_06_0836	Gm06:15732394..15732447				
Glyma.06g183000	Gm06:15755835..15757981	264	AT2G36330.1	Domain of unknown function (DUF588) (DUF588)	
Glyma.06g183100	Gm06:15760149..15766534	635	AT1G27510.1	Domain of unknown function (DUF3506) (DUF3506)	
Glyma.06g183200	Gm06:15763941..15764520	74		–	
Glyma.06g183300[a,b]	Gm06:15770386..15776183	379	AT3G47990.1	Zinc finger, C3HC4 type [RING finger]	None
Glyma.06g183400	Gm06:15779899..15782514	630	AT1G70370.1	BURP domain, polygalacturonase 2	
Glyma.06g183500[a,b]	Gm06:15828917..15834067	387	AT5G50000.1	Serine/threonine protein kinase	8 SNPs
BARCSOYSSR_06_0842	Gm06:15837791..15837810				

[a]Putative gene models selected for further expression analysis

[b]Putative gene models selected for further sequence analysis

mutation (InDel; 8 bp) and a single nucleotide polymorphism (SNP) were detected in the promoter sequence of *Glyma.06g182600* (Supplement S3). Moreover, the CDS of all three STK-encoding genes (*Glyma.06g176800*, *Glyma.06g177700* and *Glyma.06g183300*) were conserved between the two parents (Supplement S3). In contrast, 3 and 6 SNPs were identified in the CDS or promoter of *Glyma.06g175100* and *Glyma.06g183500* between the two parents, respectively. The gene model *Glyma.06g175100* encodes an RLK protein containing one signal peptide, one LRR domain, one low complexity region, one TM region and one STK domain, and only one of the 3 SNP resulted in an R203H AA substitution between the two parents (Supplement S3). The gene *Glyma.06g183500* encodes an STK protein containing one S_TKc domain, and 6 SNPs resulted in a V371A AA substitution between the two parents (Supplement S3). For another RLK-encoding gene, *Glyma.06g184400*, a single base-pair deletion close to the initiation codon delayed the transcription in 7605 (Supplement S3). Full-length PCR products of *Glyma.06g182800*

could not be obtained from the two parents using four pairs of specific primers.

Taken together, among the five genes predicted in the identified interval, *GmPEX14* (*Glyma.06g182600*) was the only gene possessing polymorphisms in the aligned DNA or cDNA sequences. Moreover, the RLK and STK genes (namely, *Glyma.06g175100*, *Glyma.06g183500* and *Glyma.06g184400*), which exhibited different polymorphisms were also potential candidate genes.

Expression analysis of candidate resistance genes using qRT-PCR

Based on architectural features, these five genes predicted in the resistance locus, along with 12 other genes near *Rsc15* (genes encoding RLK, STK or disease-resistance-related protein), were further selected for expression analysis. The results showed that most of these genes could be induced by SMV infection with different magnitudes of relative expression levels (Fig. 4; Supplement S5a). For the five genes located in the *Rsc15* flanking region, only *GmPEX14* (*Glyma.06g182600*) was up-regulated in RN-9 from 1 to 12 hpi, while its expression was significantly suppressed in 7605. The remaining four genes displayed almost the same expression profile in the two parents after inoculation. Other plant immunity-related genes near *Rsc15* could, to some extent, be induced by SMV infection. For instance, *Glyma.06g175700* (E3 ubiquitin ligase) was significantly up-regulated in RN-9 from 4 to 12 hpi; *Glyma.06g181300* (F-box family protein) and *Glyma.06g183300* (zinc finger protein, C3HC4 type) were both significantly up-regulated from 8 to 24 hpi, but there was no significant difference in the expression level of *Glyma.06g185400* (U-box domain containing protein). In summary, the results revealed that 8 of the 17 candidate genes (*Glyma.06g175100*, *Glyma.06g176800*, *Glyma.06g177700*, *Glyma.06g181300*, *Glyma.06g182300*, *Glyma.06g182600*, *Glyma.06g183300* and Glyma.06g183500) showed significant differences in expression profiles between the two parents in response to SMV infection.

Phytohormones, such as SA and ABA, play important roles as signaling molecules of the resistance pathway against SMV (Hajimorad and Hill 2001; Sandhu et al. 2009; Seo et al. 2014; Liu et al. 2011). The expression of candidate genes, including RLK- and STK-encoding genes, and *Glyma.06g182600*, was further examined under various

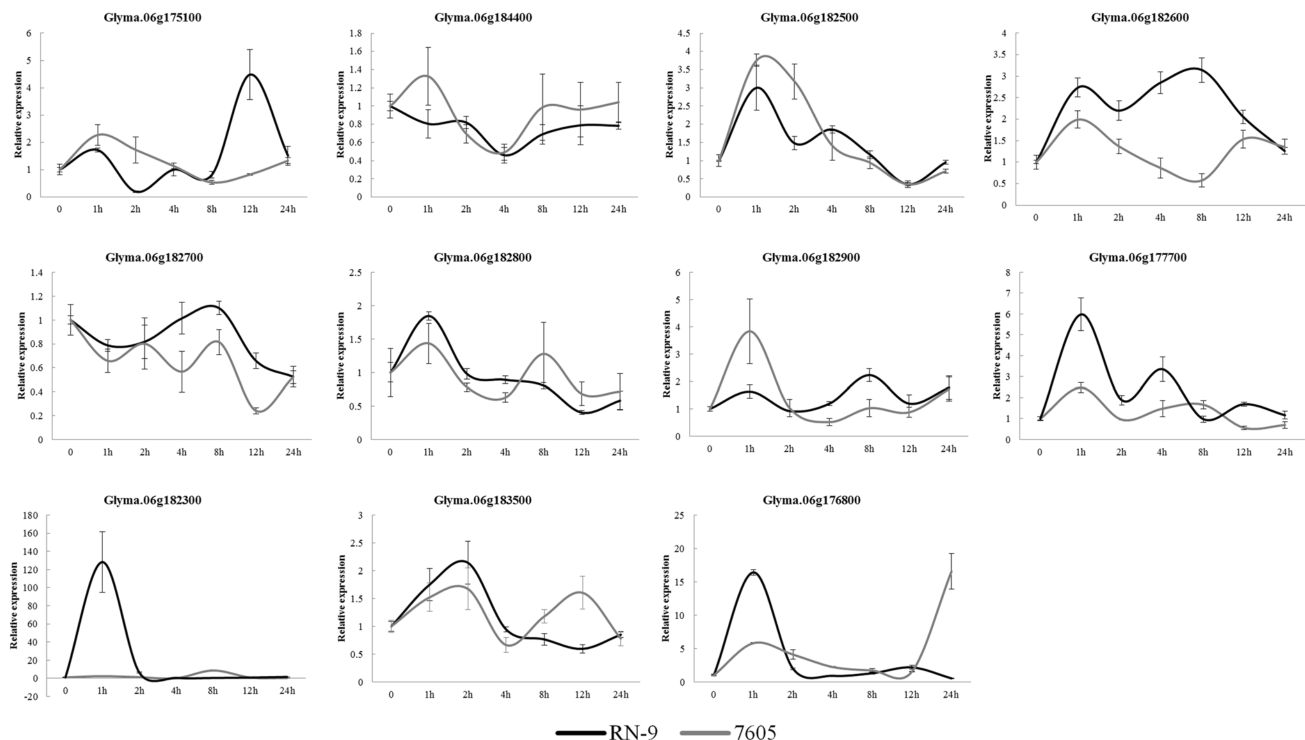

Fig. 4 Expression analyses of 11 of 17 selected candidates by qRT-PCR. **a**, **b** The two RLK-encoding genes (namely *Glyma.06g175100* and *Glyma.06g175100*). **c**–**g** the five genes predicted in the identified interval (*Glyma.06g182500*, *Glyma.06g182600*, *Glyma.06g182700*, *Glyma.06g182800* and *Glyma.06g182900*). **h**–**k** The four STK-encoding genes (*Glyma.06g177700*, *Glyma.06g182300*, *Glyma.06g183500*, *Glyma.06g176800*). *Y*-axes indicate the ratios of relative expression levels between samples infected with SMV and samples inoculated with PBS (phosphate buffer saline); *X*-axes indicate the time points post-inoculation (hpi; 0–24 hpi); data are expressed as means of three biological replicates with *error bars* indicating the standard deviation (SD)

stress treatments, and all eight genes showed significant differences on average (Fig. 5). For example, *Glyma.06g175100* was up-regulated by approximately 20-fold at 12 hpt with SA and ACC, while *Glyma.06g182600* was up-regulated by approximately 2.5-fold from 3 to 48 hpt with ABA. The results showed that these genes responded to the different phytohormones at different time intervals. Further, the expression profiles of these genes were examined in specific tissues (root, stem, leaf, flower and immature pod) (Fig. 6). The results revealed that the expression of these genes in the leaves was generally higher than that in other tissues and that the relative expression of *Glyma.06g182300* was much lower compared with that of the other tested genes.

Overall, expression analysis of candidate resistance genes revealed that 8 of the 17 candidate genes (namely *Glyma.06g175100*, *Glyma.06g176800*, *Glyma.06g177700*, *Glyma.06g181300*, *Glyma.06g182300*, *Glyma.06g182600*, *Glyma.06g183300* and *Glyma.06g183500*) showed significant differences in expression profiles between the two parents under various stress treatments, including SMV infection.

Effects of H_2O_2, CAT and POD on the expression of *GmPEX14* in SMV infection

H_2O_2 detection and enzyme activity assays were conducted, and the results revealed that H_2O_2 concentration and activities of CAT and POD were all involved in SMV infection. Overall, the levels of endogenous H_2O_2 in RN-9 were higher than those in Nannong 1138-2, and they were significantly increased from 2 to 12 hpi in RN-9 infected with SC15 compared with the mock control (Fig. 7a). The CAT activity, an antioxidant enzyme that specifically catalyzes the degradation of H_2O_2, was negatively correlated

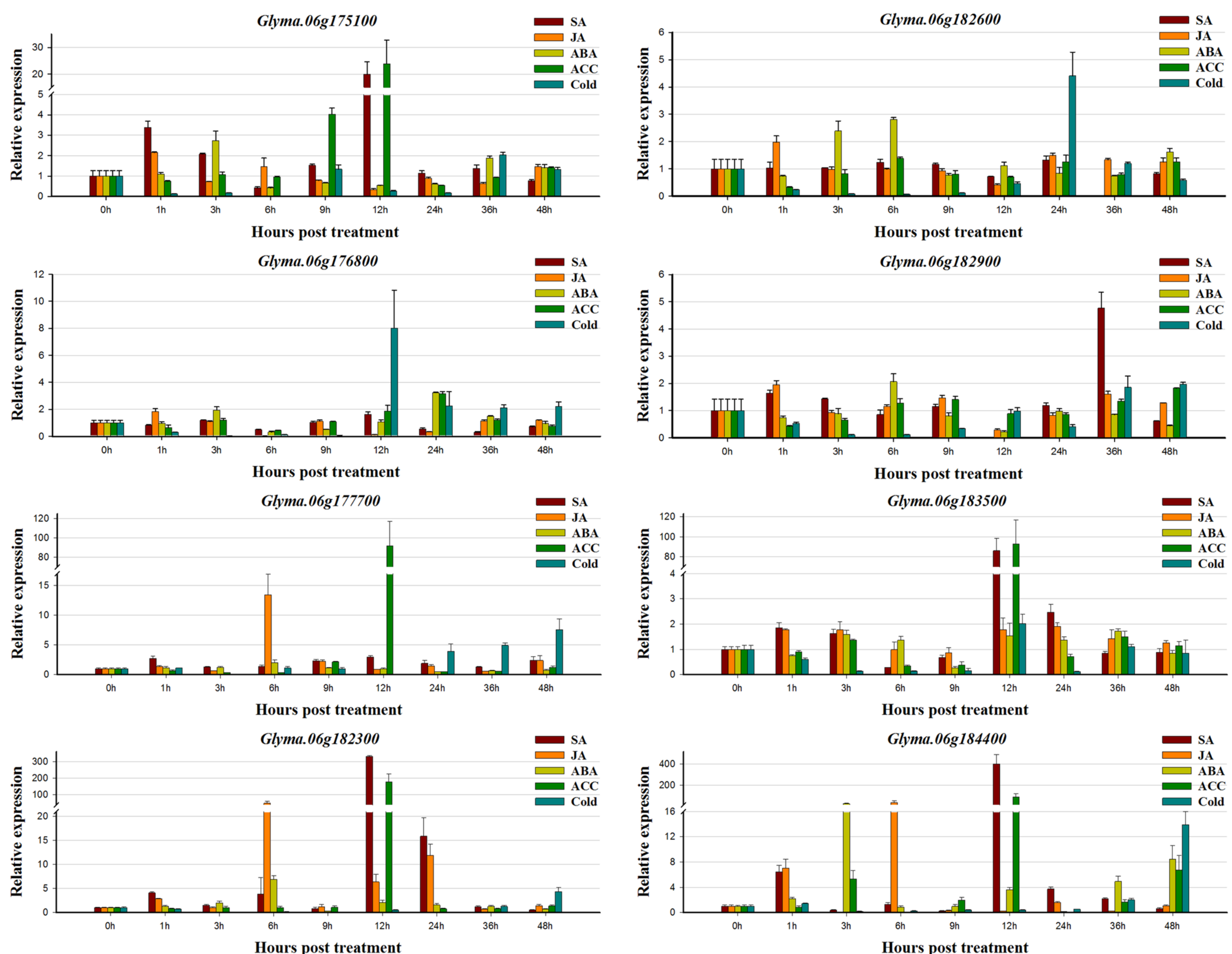

Fig. 5 Expression analyses of the four selected candidates under various stress treatments (SA, JA, ABA, ET and cold) by qRT-PCR. *Y*-axes indicate the ratios of relative expression levels between samples under various stress treatments (SA, JA, ABA, ET and cold) and control at different time points (0–48 hpi); data are expressed as mean of three biological replicates with *error bars* indicating the SD

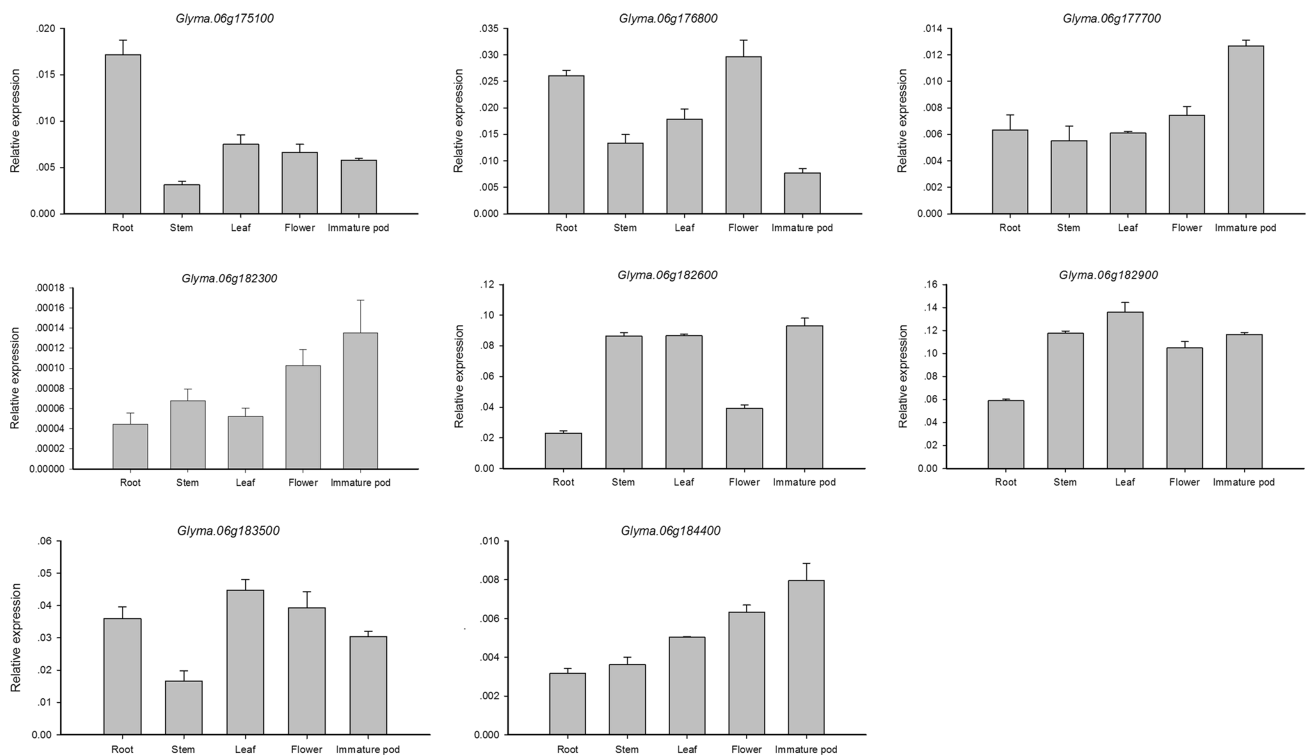

Fig. 6 Expression analysis of the eight selected candidates in different tissues (root, stem, leaf, flower and immature pod) by qRT-PCR. *Y*-axes indicate the relative expression levels in different tissues (root, stem, leaf, flower and immature pod) at different time points (0–9 days); data are expressed as the mean of three biological replicates, with *error bars* indicating the SD

with the levels of endogenous H_2O_2. The CAT activity in Nannong 1138-2 was higher than that of in RN-9, and two peaks were observed in Nannong 1138-2 infected with SC15 at 2 and 24 hpi, while the levels remained relatively stable in the mock control (Fig. 7b). POD is an antioxidant enzyme that promotes the removal of peroxides, including H_2O_2. However, no significant difference in POD activity was detected between Nannong 1138-2 and RN-9 infected with SC15 (Fig. 7c). These results indicated that H_2O_2, CAT and POD might be involved in SMV infection during the early stages (approximately 2–12 hpi).

Because *GmPEX14* was significantly up-regulated from 1 to 12 hpi, accompanied by a burst of H_2O_2 in the SMV-resistant genotype RN-9 (Figs. 5d, 7a), we further investigated the relationship between the expression level of *GmPEX14* and the involvement of the H_2O_2 concentration along with CAT and POD activities in SMV infection (Table 3). High correlations were established between the relative expression of *GmPEX14* and the H_2O_2 concentration along with activities of CAT and POD at the early stages of SMV infection in RN-9 (0–12 hpi) (Fig. 8). However, it was difficult to establish any correlation either during the other time intervals in RN-9 and during the same time interval in the susceptible genotype Nannong 1138-2 (data not shown).

Discussion

Among the 22 SMV strains have been characterized in China, SC15 is the most virulent and widely distributed strain (Fig. 1). The popular soybean cultivars Kefeng No. 1 and Qihuang No. 1 are resistant to most of the Chinese strains, but are susceptible to SC15 (Guo et al. 2005; Li et al. 2010, 2014). Yang and Gai (2011) conducted a large-scale screening for SC15 resistance sources in 205 diverse soybean accessions originating from China and other countries. This screening test showed that ten accessions, including RN9 with ER to SC15, were obtained. Further inheritance analyses and mapping studies indicated that one single dominant gene controls the resistance to SC15 in RN-9, and that this gene was located within the 14.6-cM (~1.6-Mb) genomic region flanked by Sat_213 and Satt286 on chromosome 6 (Yang and Gai 2011). The present study also confirmed these results, consistent with the report by Yang and Gai (2011). Previously, many '*Rsv*' and '*Rsc*' genes for certain SMV strains have been identified on chromosomes 2, 13 and 14, respectively. The *Rsc15* gene is an interesting novel locus underlying resistance gene to the virulent strain SC15 and is the first SMV resistance gene identified on chromosome 6.

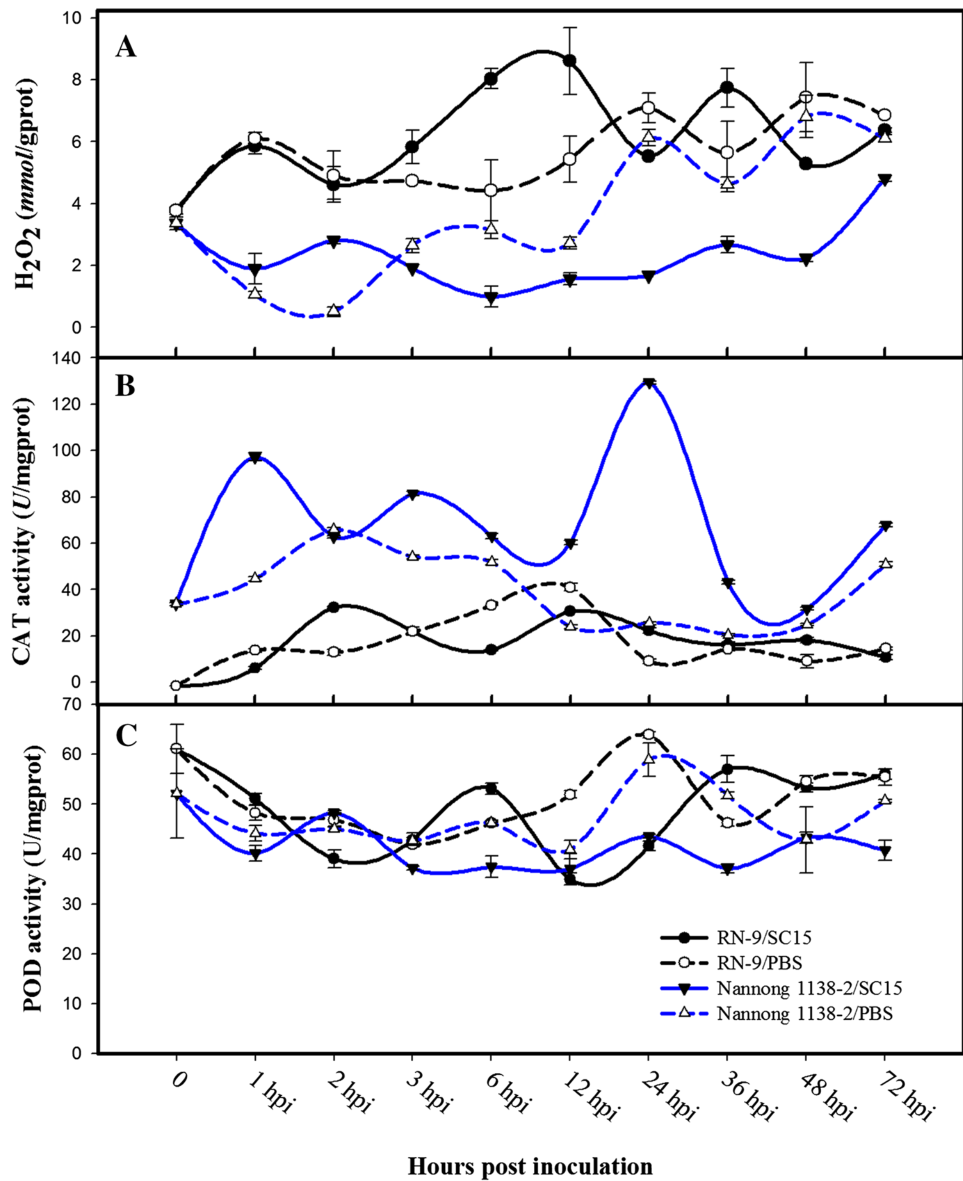

Fig. 7 Detection of the H_2O_2 concentration and CAT and POD activities at various time points. **a** The concentration of endogenous H_2O_2; **b** activity of the antioxidant enzyme CAT; **c** activity of the antioxidant enzyme CAT

Table 3 Relative expression of *GmPEX14*, concentration of H_2O_2 and activities of CAT and POD in RN-9 at different hours post-inoculation (0–12 hpi)

hpi	*GMPEX14*[a]		H_2O_2 (mmol/gprot)[a]		CAT (U/gprot)[a]		POD (U/gprot)[a]	
	Average	SD	Average	SD	Average	SD	Average	SD
0	1.000	0.160	3.770	0.118	-1.588	0.138	61.078	4.927
1	2.733	0.221	5.866	0.273	6.110	0.748	51.078	1.102
2	2.199	0.222	4.620	0.578	32.269	0.293	39.043	1.764
3	2.849	0.249	5.820	0.544	21.967	1.862	42.856	1.403
6	3.144	0.284	8.027	0.317	13.938	0.408	53.154	1.122
12	2.053	0.158	8.608	1.081	30.558	0.158	34.942	1.178

SD standard deviation

[a]Data are expressed as average value of three biological replicates

In the present study, we further fine mapped the *Rsc15* gene and identified the candidate resistance genes. Using SSR markers to perform high-resolution fine-mapping in the modified RIL population, we narrowed the genomic region from 1.6 Mb to 95 kb, which was flanked by the SSR markers SSR_06_17 and BARCSOYSSR_06_0835 (Figs. 2d, 3b,

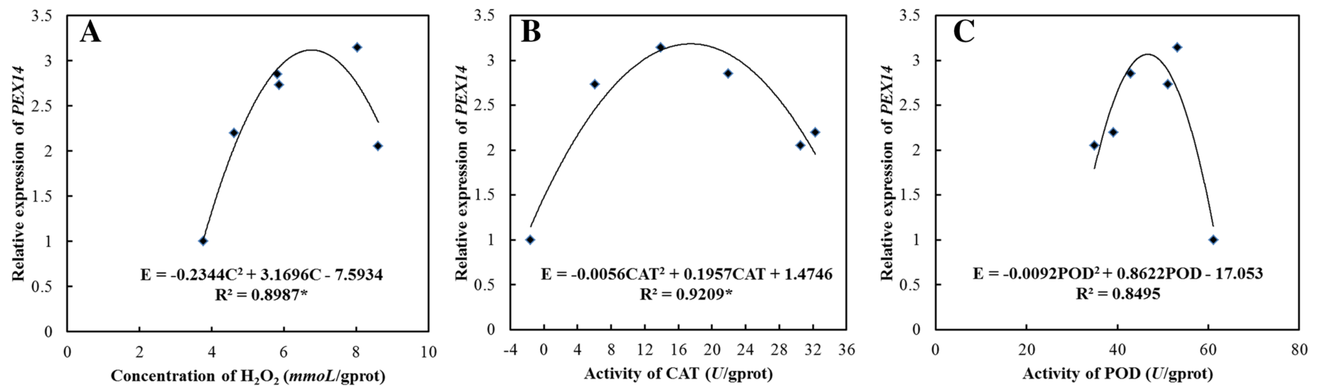

Fig. 8 Correlations between the concentration of H_2O_2, the activities of CAT and POD and the relative expression of *PEX14* in RN-9 at different hours post-inoculation (0–12 hpi). "E" represents the relative expression of *PEX14*; "C" indicates the concentration of H_2O_2; "CAT" and "POD" stands for the activities of CAT and POD, respectively

c). Here, we also identified numerous SSR markers closely linked to *Rsc15*. These markers could be used as effective and consistent tool that enable traditional breeders to tag the genetic loci linked to SMV resistance.

To our knowledge, there are many QTLs for seed size traits (i.e., *qSWT*, *qSLW*, *qSW* and *qST*) (Xu et al. 2011), and flowering time (*qFT6*) (Zhang et al. 2013), and maturity genes (*T* and *E1*) (Molnar et al. 2003) distributed in different regions on chromosome 6. In addition, many QTLs for resistance to sudden death syndrome (SDS) (*qFDS 003, 004*) (Njiti et al. 1998; Abdelmajid et al. 2012), Asian soybean rust (ASR) [*Rpp3* and *Rpp?* (Hyuuga)] (Monteros et al. 2007; Hyten et al. 2009; Hossain et al. 2015), soybean cyst nematode (SCN) (Concibido et al. 2004) and White mold (Huynh et al. 2010) were also located in different regions close to *Rsc15* on chromosome 6 (Fig. 3a). Previously, Kang et al. (2012) reported that 23 NBS-LRR genes are located on this chromosome, and the resistance of *Rpp?* (Hyuuga) is likely attributed to a cluster of NBS-LRR genes (Hossain et al. 2015). Therefore, we examined the genomic region around the putative resistance locus (*Rsc15*), flanked by Sat_246 (Gm06:14487532) and Satt277 (Gm06:17218711), for predicted genes. The results revealed that no genes belonged to the NBS-LRR family, but that three RLK- and six STK-encoding genes shared conservative architectural features with other cloned *R*-genes (Supplement S2). Moreover, these RLK- and STK-encoding genes (namely, *Glyma.06g175100*, *Glyma.06g183500* and *Glyma.06g184400*) showed polymorphisms in the DNA or cDNA sequences, while the other RLK- and STK-encoding genes were all conserved in the CDS and promoter sequences (Table 2; Supplement S3). In addition, two genes (*Glyma.06g175100* and *Glyma.06g183500*) both showed significant differences in expression profiles between the two parents during SMV infection and under various stress treatments. Therefore, these RLK and STK genes were potential candidate genes. These data indicated that *Rsc15* was located in a disease-resistance gene-poor region, and that the resistance associated with this locus might be attributed to an original class of disease-resistant genes, similar to that of *Rsv4*, *Rsc7* and *Rsc8* (Maroof et al. 2010; Ilut et al. 2016; Yan et al. 2015; Wang et al. 2011b; Zhao et al. 2016).

Despite none of candidate genes was annotated to the NBS-LRR resistance gene family (Table 2), *Glyma.06g182600* (*GmPEX14*) was inferred as a strong candidate gene governing the *Rsc15* resistance. Among the five genes predicted in the identified interval, only *GmPEX14* possessed polymorphisms in the DNA/cDNA sequence alignments (1 SNP and 1 InDel in the promoter sequence) (Supplement S3). Meanwhile, *GmPEX14* showed significant differences in expression profiles in response to SMV infection between the two parents (Fig. 4d), and could be induced by JA, ABA and cold treatments (Fig. 5). However, whether the variations in cis-regulatory elements in the promoter sequence (~2000 bp) lead to differences in plant gene expression and regulation should be further explored. To date, no SMV resistance gene has been cloned, and a greater concentration in the delimitation of mapping intervals is required to verify the likely involvement and perform cloning of SMV resistance genes.

The strong candidate gene *Glyma.06g182600* was annotated to encode the peroxisomal membrane protein *PEX14*. *PEX14* is the sole peroxin that has a unique dual function in peroxisome formation and selective degradation (van Zutphen et al. 2008). The formation and selective degradation of peroxisome play a key role in the maintenance of cellular oxidative balance (Nordgren and Fransen 2014). In our study, the relative expression level of *GmPEX14* was highly correlated with the concentration of H_2O_2 and the activities of CAT and POD during the early stages of SMV infection in RN-9 (Fig. 8). As known, H_2O_2 could trigger programmed plant cell death during the hypersensitive

response mediated by *R*-genes (Chen et al. 2017), acting as a signal in the induction of SAR and inducing defense-related genes (Apel and Hirt 2004; Gadjev et al. 2006; Hernández et al. 2016). As well, plant cells contain oxygen radical-detoxifying enzymes, such as CAT, POD and peroxin, that protect them from oxidative damage. Taken together, these results suggest that *PEX14* might confer the resistance to SC15 in RN-9 through the involvement of cellular oxidative balance, in which CAT and POD might also be involved. However, more efforts are necessary to clarify the relationship between the involvement of peroxide and the resistance.

In summary, our research presents evidence that the SSR markers SSR_06_17 and BARCSOYSSR_06_0835 are two markers that delimit a 95-kb region in the reference Williams 82 genome, flanking the *Rsc15* locus. In such a region, the gene *GmPEX14* (*Glyma.06g182600*) is the best candidate gene attributed for the resistance of *Rsc15*, and the four RLK and STK genes (i.e., *Glyma.06g175100*, *Glyma.06g182900*, *Glyma.06g183500* and *Glyma.06g184400*) are also potential candidates. Peroxide, particularly H_2O_2, might be involved in the resistance mediated by *Rsc15* during the early stages of SMV infection. Moreover, the markers that are closely linked to SMV resistance locus *Rsc15*, as determined in this study, will be potentially used for MAS in soybean breeding programs and the pyramiding of resistance genes.

Author contribution statement HZ, KL, QY and RR designed the methods and experiments. RR, SL, JY, TW, HN, AK, YY, and LW conducted SMV inoculations and resistance evaluation. RR, SL and TW performed genotype analysis. RR, SL, and JY performed qRT-PCR and sequencing. RR, SL and YY conducted the H_2O_2 detection and enzymes activity assays. RR and KL analyzed the data. HZ, KL, RR and AK drafted the manuscript. All authors critically reviewed and approved the final manuscript.

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Compliance with ethical standards

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

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

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