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Article

BnSGS3 Has Differential Effects on the Accumulation of CMV, ORMV and TuMV in Oilseed Rape

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Abstract: Virus diseases greatly affect oilseed rape (Brassica napus) production. Investigating antiviral genes may lead to the development of disease-resistant varieties of oilseed rape. In this study, we examined the effects of the suppressor of gene silencing 3 in Brassica napus (BnSGS3, a putative antiviral gene) with different genus viruses by constructing BnSGS3-overexpressing (BnSGS3-Ov) and BnSGS3-silenced (BnSGS3-Si) oilseed rape (cv. Zhongshuang No. 6) plants. These three viruses are Oilseed rape mosaic virus (ORMV), Turnip mosaic virus (TuMV) and Cucumber mosaic virus (CMV). The native BnSGS3 expressed in all examined tissues with the highest expression in siliques. All three viruses induced BnSGS3 expression, but ORMV induced a dramatic increase in the BnSGS3-Ov plants, followed by TuMV and CMV. Upon inoculation with three different viruses, transcript abundance of BnSGS3 gene follows: BnSGS3-Ov > non-transgenic plants > BnSGS3-Si. The accumulation quantities of ORMV and TuMV exhibited a similar trend. However, CMV accumulation showed an opposite trend where virus accumulations were negatively correlated with BnSGS3 expression. The results suggest that BnSGS3 selectively inhibits CMV accumulation but promotes ORMV and TuMV accumulation. BnSGS3 should be used in different ways (up- and down-regulation) for breeding virus-resistant oilseed rape varieties.
1. Introduction

Virus disease, next to white mold caused by *Sclerotinia sclerotiorum*, is one of the most important yield-limiting diseases of oilseed rape production in China [1]. *Turnip mosaic virus* (TuMV, genus *Potyvirus*, family *Potyviridae*), *Cucumber mosaic virus* (CMV, genus *Cucumovirus*, family *Bromoviridae*) and *Oilseed rape mosaic virus* (ORMV, genus *Tobamovirus*, family *Tobamoviridae*) are the main viruses that infect oilseed rape in China [2]. However, to date, no oilseed rape varieties have been bred that are highly resistant to these virus diseases.

RNA silencing, including posttranscriptional gene silencing (PTGS), is an important mechanism of antiviral defense in plants [3–5]. In recent years, many studies in the mechanism and relevant antiviral plant genes are reported to search for new methods and resources in disease resistance breeding [4,6–12]. *Suppressor of gene silencing 3* (*SGS3*) has antiviral activity [4,13,14] and specifically exists in plant functioning as a RNA-binding protein [15]. It has been shown in *Arabidopsis* that *SGS3* is required for PTGS and natural virus resistance [4]. In the cytoplasm of plant cells, SGS3 stabilizes and recruits the viral single-stranded RNAs (ssRNAs) transcripts to RNA-dependent RNA polymerase 6 (RDR6) and RDR6 converts ssRNAs into double-stranded RNAs (dsRNAs) in SGS3/RDR6 bodies [16]. Next, Dicer-like 4 (DCL4) digests the dsRNAs into 21–24 nt primary small interfering RNAs (siRNAs) assisted by double-stranded-RNA-binding protein 4 (DRB4) and part of the dsRNAs are processed into secondary siRNAs by DCL2. These two classes of siRNAs are protected from degradation aided by HUA ENHANCER 1 (HEN1) and then are combined onto the Argonaute 1/2 (AGO1/AGO2) protein and RNA-induced silencing complexes (RISCs) to silence the complementary viral RNAs [3–5,9,15,17–19]. Furthermore, SGS3 can also function in controlling other kinds of RNA silencing such as those induced by sense transgenes (S-PTGS) [20] or DNA virus-induced gene silencing (VIGS) [21].

CMV, TuMV and *Turnip vein-cleaning virus* (TVCV) have different degrees of inhibitory effects on PTGS [4]. CMV can only partially inhibit PTGS and produces only a little amount of CMV virus RNA in *Arabidopsis* L1 plants, which is significantly lower than that in *sgs3* mutants. On the contrary, TuMV and TVCV completely inhibit PTGS. As a result, these two viruses accumulated to equally high levels in the *sgs3* mutants and L1 plants [4]. These results indicate that the PTGS-mediated resistance efficiency on virus infection is related to the inhibitory capability of virus to PTGS.

SGS3 is an essential component of PTGS and closely associated with plant virus resistance. So far, however, the function of *SGS3* in oilseed rape has not been reported. Little is known about the expression of *SGS3* in oilseed rape or the interaction between major viruses of oilseed rape and SGS3. In this study, we investigated the expression pattern of the *suppressor of gene silencing 3* in *Brassica napus* (designated *BnSGS3*, accession number KP292910) in various oilseed rape tissues. Furthermore, we generated transgenic oilseed rape plants with overexpressed (*BnSGS3*-Ov) and silenced (*BnSGS3*-Si) levels of *BnSGS3*. Using these transgenic plants upon inoculation with three different viruses (ORMV, TuMV and CMV), our results reveal that CMV accumulation was negatively correlated with expression levels of *BnSGS3*, whereas accumulations of both TuMV and ORMV were positively correlated with expression levels.
levels of BnSGS3, suggesting that BnSGS3 selectively inhibits CMV accumulation, but promotes TuMV and ORMV accumulation.

2. Results

2.1. Sequence Comparison and Phylogenetic Analysis of SGS3 Genes in Different Hosts

According to report, SGS3 contains three conservative functional domains: The zinc finger (ZF) domain, the rice gene X and SGS3 (XS) domain and the coiled-coil (CC) domain [16,22–24]. The XS domain is a single-stranded RNA-binding domain [22], while the CC domain is related to homodimer formation and the movement of SGS3/RDR6-bodies [16,24]. The N-terminal ZF domain of potato SGS3 was recently shown to control SGS3 location and be the main determinant of the interaction with the viral genome-linked protein (VPg) of Potato virus A (PVA) [13]. In this study, a cDNA encoding a homolog of AtSGS3 was cloned from Brassica napus cv. Zhonghuang No.6. The entire open reading frame (ORF) of the cloned cDNA encodes a protein of 607 amino acid residues that exhibit 72.6% identity with AtSGS3 (AF239719). Similar to AtSGS3, the protein sequence contains three conserved domains: ZF domain, XS domain and CC domain, which shared, respectively, 93.3%, 78.3% and 73.3% identity with these domains of AtSGS3. Thus, the cloned cDNA was designated as BnSGS3 (GenBank accession no. KP292910).

Analysis of phylogenetic relationships would help provide initial insights into degree of structural and functional conservation. Several plant SGS3 genes, initiated from Arabidopsis thaliana (AtSGS3) [4], Solanum lycopersicum (SlSGS3) [25], Oryza sativa (OsSGS3) [26], and Nicotiana tabacum (NtSGS3) [27] were implicated in plant viral resistance. Based on alignments, neighbor-joining tree of SGS3 nucleotide sequences was generated (Figure 1). Results showed that there were three distinct clusters of these SGS3 genes. The homology was 55.1%–81.0% between BnSGS3 and other SGS3 genes. BnSGS3 was more closely related to AtSGS3 but distantly to those of N. tabacum, S. lycopersicum and O. sativa. BnSGS3 and AtSGS3 belonged to one cluster and they shared the highest homology of 81.0%. The difference between them might relate to the potentially resistance to different viruses.

![Figure 1](image_url)  
**Figure 1.** Phylogenetic analysis of the suppressor of gene silencing 3 in Brassica napus (BnSGS3) and other published SGS3 genes involved in plant viral resistance based on the entire open reading frame (ORF) nucleotide sequence. The neighbor-joining tree was generated with the DNAMAN 6.0.4 and Mega 5.0. The significance of the branching order was assessed by bootstrap resampling of 1000 replicates. Values are indicated at the forks. The scale bar corresponds to a 5% difference.
2.2. BnSGS3 Expression in Transgenic and Non-Transgenic Plants of Oilseed Rape

Quantitative RT-PCR (qRT-PCR) was used to detect the expression levels of endogenous BnSGS3 in different tissues of oilseed rape (B. napus cv. Zhongshuang No. 6). When the expression level of BnSGS3 in roots was designated to be 1, the relative expression level in each tissue from high to low followed: Siliques (2.81), flowers (2.05), leaves (1.68), stems (1.64) and roots. The relative expression of BnSGS3 in roots was only 35% of that in siliques (Figure 2).

Figure 2. Relative expression levels of BnSGS3 in different tissues of oilseed rape.

To further functionally characterize BnSGS3, we produced transgenic oilseed plants. BnSGS3 overexpression vector (BnSGS3-Ov) containing a Cauliflower mosaic virus (CaMV) 35S enhancer, double CaMV 35S promoters, an Ω sequence (to improve protein translation), the BnSGS3 cDNA, and a CaMV Nos terminator within the T-DNA region (Supplementary Figure S1A). BnSGS3 silenced vector (BnSGS3-Si) containing the CaMV 35S promoter, BnSGS3 sense and antisense repetitive sequences, a Pdk intron and an OCS terminator (Supplementary Figure S1B). These two vectors, each with the pSoup helper plasmid were transformed into the Agrobacterium tumefaciens GV3101, which was in turn used to transform oilseed rape cv. Zhongshuang No.6.

A total of 58 transgenic oilseed rape plants were confirmed by PCR, including 33 BnSGS3-Ov and 25 BnSGS3-Si transgenic plants. The relative transcript levels of BnSGS3 in randomly selected 22 plants were determined by qRT-PCR. The results showed that the relative transcript levels of BnSGS3 among transgenic BnSGS3-Ov and BnSGS3-Si plants were 144%–581% and 13%–71% of the non-transgenic plants, respectively, indicating that BnSGS3 expression increased in the BnSGS3-Ov plants but was suppressed in the BnSGS3-Si plants (Figure 3).

Figure 3. Relative expression levels of BnSGS3 in single BnSGS3-overexpressing (BnSGS3-Ov) oilseed rape plants (1–11), non-transgenic oilseed rape plant (12) and single BnSGS3-silenced (BnSGS3-Si) oilseed rape plants (13–23).
2.3. Dynamics of BnSGS3 Expression in T<sub>0</sub> Generation Transgenic and Non-Transgenic Plants of Oilseed Rape after Viral Infection

To test the effects of BnSGS3 expression and the accumulation of viruses, each 50 ng of purified ORMV (Tobamovirus, subgroup III strain), TuMV (Potyvirus, MB cluster strain) and CMV (Cucumovirus, subgroup I strain) viruses were, respectively, inoculated onto five T<sub>0</sub> generation transgenic and non-transgenic plants at the four-leaf-stage. Analyses of the changes in expression of BnSGS3 after inoculation showed that all three viruses induced the expression of BnSGS3 in both transgenic and non-transgenic oilseed rape. Generally, the expression of BnSGS3 first increased and then dropped (Figure 4). In all three viruses’ inoculation treatments, transcript levels of BnSGS3 in overexpressing T<sub>0</sub> generation plants were significantly greater than those in the non-transgenic and BnSGS3 silenced plants while expression in the non-transgenic plants was slightly higher than that in the silenced plants. In BnSGS3-overexpressing plants upon virus inoculation, three viruses showed different induction on BnSGS3 expression, most efficiently by ORMV followed by TuMV and CMV, while the inductive effect of CMV on BnSGS3 expression was slightly stronger than those by ORMV and TuMV in BnSGS3-Si transgenic and non-transgenic plants. These results suggest that in both transgenic and non-transgenic plants of oilseed rape, all three viruses ORMV, TuMV and CMV were able to induce expression of BnSGS3, but induction capability is different.

![Figure 4. Dynamic expression of BnSGS3 induced by (A) Oilseed rape mosaic virus (ORMV); (B) Turnip mosaic virus (TuMV) and (C) Cucumber mosaic virus (CMV) in the transgenic and non-transgenic plants. The expression level of BnSGS3 in non-transgenic plants uninoculated was designated to be 1.](https://example.com/image)

2.4. Effect of BnSGS3 on the Accumulation of ORMV, TuMV and CMV

A diluted concentration gradient of the plasmid containing viral CP gene fragments was subjected to qRT-PCR amplification. The Ct values of a gradient of viral plasmid concentrations were used for
the Y-axis, and logarithm (log10) values of the copy number (copies/μL) were used for the X-axis to construct a standard curve (Supplementary Figure S2). The results showed that the amplification efficiency (E) of ORMV, TuMV, and CMV viral plasmids was 86.2%, 88.7% and 84.9%, respectively, and R² was greater than 0.99, indicating that there was a good linear relationship between Ct values and the logarithm of the copy numbers and the standard curves were appropriate for calculation of virus quantity.

To investigate whether the BnSGS3 expression effect the viral accumulation, five T₀ generation transgenic and non-transgenic plants were inoculated with 50 ng of the purified ORMV, TuMV and CMV particles at the four-leaf-stage. The viral accumulation was measured in 0–24 days of post-inoculation (dpi) at intervals of three days. After inoculation of BnSGS3-Ov, oilseed rape plants with ORMV and TuMV, virus levels first increased and then decreased, reaching peak values of 8.05 × 10⁴ copies and 4.47 × 10⁴ copies at 12 dpi, respectively. In non-transgenic oilseed rape, the virus levels initially increased, reaching peak values of 3.52 × 10⁴ copies and 2.77 × 10⁴ copies at 15 and 12 dpi, respectively, followed by a decrease. However, in BnSGS3-Si oilseed rape inoculated with ORMV and TuMV, the virus levels reached peak values of 1.82 × 10⁴ copies and 1.14 × 10⁴ copies at 18 and 15 dpi, respectively. These results demonstrate that the quantities of ORMV and TuMV exhibited a trend: BnSGS3-Ov plants > non-transgenic plants > BnSGS3-Si plants (Figure 5A,B), consistent with BnSGS3 expression levels in these type plants.

The replication rate of CMV in BnSGS3-Ov plants was small and its accumulation levels were low, reaching a peak of 1.50 × 10³ copies at 24 dpi, and the amplitude of variation of qRT-PCR throughout the entire test time course was small. In inoculated non-transgenic and BnSGS3-Si plants, increase of CMV levels was relatively quick, reaching a peak of 1.48 × 10⁴ copies and 2.34 × 10⁴ copies at 3 dpi, respectively. The levels fluctuated a bit, but the changes were small (Figure 5C). In non-transgenic oilseed rape, CMV levels were much higher with 1.5 × 10³ copies than that of ORMV at 3 dpi and afterwards virus quantities were ORMV > TuMV > CMV. The general trend of CMV accumulation was the BnSGS3-Si > non-transgenic plants > BnSGS3-Ov, in contrary to both the induction expression trend of BnSGS3 and the accumulation trends of ORMV and TuMV. Furthermore, virus accumulation of ORMV and TuMV in the BnSGS3-Ov are drastically higher than that of CMV at 6 dpi and afterwards.

Figure 5. Cont.
Figure 5. Accumulation of ORMV (A); TuMV (B) and CMV (C) in the transgenic and non-transgenic oilseed rape plants.

2.5. Correlation between BnSGS3 Expression and Virus Accumulation

SPSS17.0 statistical software was used to analyze the relationship between virus accumulation and BnSGS3 expression levels in transgenic and non-transgenic oilseed rape. There was a positive and significant ($p < 0.01$ or $0.05$) linear correlation between the accumulation of ORMV and TuMV (in transgenic and non-transgenic plants) and BnSGS3 expression (Figure 6A–F). However, the relationship between CMV accumulation and BnSGS3 expression in transgenic and non-transgenic oilseed rape exhibited a negative and significant ($p < 0.01$ or $0.05$) linear correlation (Figure 6G–I). These results indicated that the expression of BnSGS3 promoted the accumulation of ORMV and TuMV but suppressed CMV accumulation.

Figure 6. Cont.
Figure 6. Relationship between the accumulation level of ORMV (A–C), TuMV (D–F) or CMV (G–I) and the expression quantity of BnSGS3 in BnSGS3-Ov (A, D, G), non-transgenic (B, E, H) and BnSGS3-Si (C, F, I) plants.

3. Discussion

In this study, we first reported the oilseed rape SGS3 gene (BnSGS3) and determined the relationship between the BnSGS3 expression and the virus accumulation of ORMV, TuMV and CMV, which are the main viruses that infect oilseed rape. For ORMV and TuMV, after inoculation of transgenic and non-transgenic oilseed rape plants, differences in the induced expression of BnSGS3 were significant. The higher the level of BnSGS3 expression was, the higher the corresponding accumulation of ORMV and TuMV was. Furthermore, in BnSGS3-Si plants, the expression of BnSGS3 was low, and the virus accumulation was also low. Correlation analysis showed that there was a positive linear correlation between the two (Figure 6A–F). Similar phenomenon was observed in PVA and Soybean mosaic virus (SMV). A study of the interaction between PVA VPg protein and potato SGS3 (StSGS3) revealed that the accumulation of PVA in StSGS3-overexpressing plants was higher than in StSGS3-silenced plants [13]. Likewise, there is a lower level of SMV strain G7 RNA in the Glycine max SGS3 (GmSGS3)-silenced
soybean than non-silenced plants. The resulting severity of lethal systemic hypersensitive response (LSHR) in GmSGS3-silenced soybean was also alleviated [14].

In our research, we found that the accumulation of ORMV and TuMV increased accompanied by the up-regulated expression of BnSGS3, suggesting that these two viruses can successfully inhibit RNA silencing in plants [28]. So far, many viruses have been reported to have this function [29–33], which may be relevant to the virus suppressors of RNA silencing (VSRs). Among these VSRs, HC-Pro protein encoded by potyviruses (TuMV also encodes this HC-Pro) is a highly effective VSR that not only inhibits silencing but can also reverse an already established RNA silencing [34,35]. The expression of HC-Pro does not inhibit the production of a systemic silencing signal but prevents the accumulation of the siRNAs [36]. In Tobamovirus (ORMV is included in this category), both P126 of Tobacco mosaic virus (TMV) [5] and 130k of Tomato mosaic virus (ToMV) [37] can function as VSRs to prevent RNA silencing. Therefore, BnSGS3 may have a role in ORMV and TuMV accumulation effect through closely linked to VSRs. Since SGS3 stabilizes the viral ssRNA transcripts toRDR6 [16], it is possible that these two viruses’ VSRs directly or indirectly interact with BnSGS3 and recruit BnSGS3 to protect viral RNA from degradation [13,15,21,25,26,38]. Alternatively, the VSRs prevent BnSGS3 from binding and accessing their common substrate RNAs, which lead to inhibition of RNA silencing [15]. Another possibility is that the accumulation differences may be induced by interference in the amplification step of RNA silencing via a yet-unidentified mechanism [13]. Many studies have reported that sense-mediated RNA silencing may interlink with other RNA quality control systems [11,16,39]. Therefore, it is possible that TuMV and ORMV encoding VSRs interact with BnSGS3 in both RNA silencing and other interlinked RNA quality control systems, which also need BnSGS3 to inhibit RNA silencing.

By contrast, the accumulation level of CMV in different oilseed rape plants was differed from ORMV and TuMV. The peak value of CMV accumulation in various plants exhibited the trend BnSGS3-Si (2.34 × 10^4) > non-transgenic oilseed rape (1.48 × 10^4) > BnSGS3-Ov (1.50 × 10^3; Figure 5C). The opposite trend was observed for CMV-induced BnSGS3 expression. Therefore, the higher the level of BnSGS3 expression was, the lower the level of CMV was. Correlation analysis revealed a negative linear correlation between the two factors (Figure 6G–I). Previous studies also showed 5-fold over accumulation of CMV RNA in sgs3 mutant [4] and CMV 2b deficient mutant failed to infect wild-type Arabidopsis but was highly virulent in sgs3 mutant [40], which suggested that the CMV 2b protein did not target the silencing mechanism in the same way with HC-Pro [30,31]. During infection, CMV 2b directly interacted with AGO1 and specifically inhibited its slicing activity [41–44]. Since AGO1 was dispensable for RDR-dependent production of CMV secondary siRNAs, which revealed that the antiviral pathway might differ from the trans-acting siRNAs (tasiRNAs) pathway [5]. Thinking of our results, excepting BnSGS3, there may also be one or more RNA silencing genes, such as RDR6 or AGO1, essential for silencing amplification, contribute to the biosynthesis of CMV secondary siRNAs. In a word, the CMV accumulation effect suggests that there is a work way differs from TuMV and ORMV, and the CMV accumulation strategy has some intimate relationships with BnSGS3, RDR6 and AGO1, but the exact mechanism needs to be further studied.

In summary, the effects of BnSGS3 expression on the accumulation of ORMV, TuMV and CMV in oilseed rape are different. BnSGS3 may promote ORMV and TuMV accumulation, but inhibit CMV
accumulation, suggesting different strategies for use of SGS3 (up- and down-regulation) for breeding resistant oilseed rape against various viral diseases.

4. Materials and Methods

4.1. Plant Materials

Oilseed rape (Brassica napus) cv. Zhongshuang No. 6, which is low susceptible to the viruses, was used for virus inoculation and for the analysis of BnSGS3 expression in this study. Oilseed rape seedlings were grown in the greenhouse with a light/dark cycle of 14/10 h at 22 °C (D)–24 °C (L). The humidity in the greenhouse was 50%–70%.

4.2. Sequence Analysis of BnSGS3 and Vector Construction

The primers used in this study are shown in Table 1. For BnSGS3 cDNA amplification, total RNA of B. napus leaves was extracted by using total RNA kit I (OMEGA, Norcross, GA, USA), and 1 μg RNA was reverse transcribed to cDNA (RevertAid First Strand cDNA Synthesis Kit, Thermo, Waltham, MA, USA). A 1974 bp cDNA containing the entire open reading frame (ORF) of BnSGS3 was PCR amplified from its cDNA with primers Bn-F1 and Bn-R1. Software DNAMAN6.0.4 and Mega5.0 were used for sequence alignments and generated a neighbor-joining tree.

To construct a vector for constitutive expression of BnSGS3, the vector pG4A was generated by inserting a fragment from pTΩ4A containing a CaMV 35S enhancer, double CaMV 35S promoters, and a CaMV Nos terminator into the KpnI-NotI site of pGreen0229 containing a bar gene within the T-DNA region for selecting transgenic plants. A 1848 bp fragment containing the entire ORF segment of BnSGS3 was PCR amplified from its cDNA clone with primers Bn-F2 and Bn-R2, and inserted into the multiple cloning sites (XhoI) of pG4A, creating a BnSGS3 overexpressing vector BnSGS3-Ov (Figure S1A).

Vectors pGreen0229 and pKANNIBAL were used as primary and intermediate vectors to construct the BnSGS3 silenced vector. A 404-bp BnSGS3 sense (amplified by BnRI-C and BnRI-KC, containing XhoI and EcoRI sites) and antisense (amplified by BnRI-A and BnRI-E, containing XbaI and HindIII sites) fragments were inserted into both ends of pKANNIBAL Pdk introns. Then, NotI was used to extract the product containing the CaMV 35S promoter, BnSGS3 sense and antisense repetitive sequence, Pdk intron and OCS terminator from pKANNIBAL, and inserted into the NotI site of pGreen0229, creating a BnSGS3 silenced vector BnSGS3-Si (Figure S1B).

All the inserted sequences were confirmed by enzyme digestion and sequencing. The resulting BnSGS3-Ov and BnSGS3-Si vectors together with the pSoup helper plasmid [45,46] were transformed into the Agrobacterium tumefaciens GV3101 by electroporation for plant transformation.

Table 1. List of primers.

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4.3. Production of BnSGS3-Ov and BnSGS3-Si Brassica napus Transformants

*Brassica napus* cv. Zhongshuang No. 6 was transformed using an improved transformation method based on the method of Liu *et al.* [47]. Rapeseeds were soaked in 5% sodium hypochlorite for 3–5 min and plated onto M0 medium (1/2MS). After 6 days of cultivation in the dark, the hypocotyledonal axis of each seedling was co-cultured with *A. tumefaciens* liquid culture containing the BnSGS3-Ov or BnSGS3-Si plasmids for 30 min. The hypocotyledonal axes were then transferred to M1 medium (MS + 30 g/L sucrose + 18 g/L mannitol + 1 mg/L 2,4-D + 0.3 mg/L kinetin + 100 μM acetosyringone + 8.5 g/L agarose, pH 5.8) and cultured in the dark at 24 °C for 2–3 days, followed by culture on M2 medium (MS + 30 g/L sucrose + 18 g/L mannitol + 1 mg/L 2,4-D + 0.3 mg/L kinetin + 20 mg/L AgNO₃ + 8.5 g/L agarose + 25 mg/L kanamycin + 250 mg/L carbenicillin, pH 5.8) at 24 °C for 3 weeks. Hypocotyl callus was then transferred to M3 medium (MS + 10 g/L glucose + 0.25 g/L xylose + 0.6 g/L MES hydrate + 2 mg/L zeatin + 0.1 mg/L indole-3-acetic acid + 8.5 g/L agarose + 25 mg/L kanamycin + 250 mg/L carbenicillin, pH 5.8) and subcultured once every 3 weeks until the emergence of green shoots, which were cultured on M4 medium (MS + 10 g/L sucrose + 10 g/L agar, pH 5.8) for 3–4 weeks for rooting.
Primers Bar-F and Bar-R (see Table 1) were utilized to identify transgenic plants. Each transgenic plant was used to produce at least three plants, which were used as materials in independent repeated trials. The plants were transplanted into pots at the 3-leaf-stage and grown in the greenhouse as described above.

4.4. Virus Purification and Inoculation

ORMV (Tobamovirus, subgroup III strain), TuMV (Potyvirus, MB cluster strain) and CMV (Cucumovirus, subgroup I strain) were originally isolated from systemically infected oilseed rape (B. napus L. var. oleifera) in Hubei Province, China. Chinese cabbage (Brassica rapa) was used to grow and multiply these viruses by mechanical inoculation [48]. Three to four weeks post-inoculation, young emerging leaves of infected plants were harvested. Virus purification was performed as described by Aguilar et al. [49]. About 50 ng of the purified ORMV, TuMV or CMV particles were mechanically inoculated [48] onto the leaves of transgenic T₀ generation and non-transgenic oilseed rape plants at the 4-leaf-stage. Each virus was inoculated onto five plants of each type, with two independent biological replications. Thirty plants were inoculated per BnSGS3-Ov, BnSGS3-Si and non-transgenic oilseed rape plants.

4.5. Sample Collection and RNA Extraction

To detect differences in BnSGS3 expression in different tissues of oilseed rape and in each transgenic T₀ generation plant, the tender roots, stems, leaves, flowers and siliques of oilseed rape and the tender leaves of each transgenic plant were collected at the same time. After inoculation with one of three types of virus, 0, 3, 6, 9, 12, 15, 18, 21 and 24 days post-inoculation (dpi, as well as the uninoculated control), tender leaves from the upper parts of plants were used to detect virus-induced BnSGS3 expression and the amount of virus accumulation. Total RNA was extracted from the above samples (E.Z.N.A. Total RNA Kit I, OMEGA, Norcross, GA, USA), and 1 µg RNA was reverse transcribed to cDNA (RevertAid First Strand cDNA Synthesis Kit, Thermo, Waltham, MA, USA).

4.6. Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed to detect the expression levels of BnSGS3 in different tissues of non-transgenic oilseed rape plants, single transgenic plants and the virus-inoculated plants. Primers Actin-F and Actin-R were used to amplify the reference β-Actin gene (AF111812), and primers Bn-F and Bn-R were used to amplify BnSGS3 (all primers are listed in Table 1). The relative transcript levels of BnSGS3 \( 2^{-\Delta\Delta Ct} \) were obtained by calculating \( \Delta Ct \) values \( \Delta Ct = Ct(BnSGS3)-Ct(\text{Actin}) \).

qRT-PCR was also used to detect the accumulation of three viruses (by copy number) in the samples. Primers ORMV-F/R, CMV-F/R and TuMV-F/R were used to amplify CP gene fragments of the three viruses via PCR. The PCR products were ligated to pMD-18T, which was transformed into E. coli DH5α, and plasmid DNA was subsequently extracted (TIANprep Mini Plasmid Kit, TIANGEN). The molecular weights of the ORMV, TuMV and CMV plasmids were 1,730,750.4 Da, 1,730,135 Da and 1,723,338.6 Da, respectively. The copy number of 1 µL plasmids was calculated according to Avogadro’s formula “\( 6.022 \times 10^{23} \) molecules/moL”, and the plasmids were diluted to
1.0 \times 10^9 \text{–} 1.0 \times 10^3 \text{ copies/\mu L concentration gradient, with three repeats per gradient, which were simultaneously subjected to qRT-PCR [50,51]. The copy numbers of the three viruses in different samples were automatically calculated based on the sample Ct values and the standard curve.}

Two independent biological replicates and three technical replicates were carried out for the inoculation experiment for each virus, taking the average value of two sets of data. qRT-PCR was performed on the BioRad CFX96 Real-Time System (C1000 Thermal Cycler). The reaction system included 10 µL SYBR Green PCR Master Mix, 0.5 µL of each upstream and downstream primer (10 µmol/L), 1 µL cDNA and 8 µL ddH₂O. The reaction conditions were as follows: 95 °C for 3 min, 95 °C for 10 s, 62 °C for 30 s, 72 °C for 15 s (40 cycles), and 65 °C for 5 s.

4.7. Correlation Analysis of Virus Accumulation vs. BnSGS3 Expression

SPSS17.0 statistical software and Pearson’s two-sided test were used to analyze whether there was a correlation between the accumulation of three types of viruses and BnSGS3 expression levels, as well as the level of significance.

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Author Contributions

L.C., M.H. and S.L. conceived and designed the experiments; Q.C., J.H. and J.W. performed the experiments; and Q.C. and L.C. analyzed the data and wrote the paper. All authors have read and approved the final manuscript.

Conflict of Interests

The authors declare no conflict of interest.

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