Expression of an Antiviral Gene *GmRUN1* from Soybean Is Regulated via Intron-Mediated Enhancement (IME)

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**Abstract:** Most of *R* (resistance) genes encode the protein containing NBS-LRR (nucleotide binding site and leucine-rich repeat) domains. Here, *N. benthamiana* plants were used for transient expression assays at 3–4 weeks of age. We identified a TNL (TIR-NBS-LRR) encoding gene *GmRUN1* that was resistant to both soybean mosaic virus (SMV) and tobacco mosaic virus (TMV). Truncation analysis indicated the importance of all three canonical domains for *GmRUN1*-mediated antiviral activity. Promoter-GUS analysis showed that *GmRUN1* expression is inducible by both salicylic acid (SA) and a transcription factor *GmDREB3* via the cis-elements as-1 and ERE (ethylene response element), which are present in its promoter region. Interestingly, *GmRUN1* gDNA (genomic DNA) shows higher viral resistance than its cDNA (complementary DNA), indicating the existence of intron-mediated enhancement (IME) for *GmRUN1* regulation. We provided evidence that intron2 of *GmRUN1* increased the mRNA level of native gene *GmRUN1*, a soybean antiviral gene *SRC7* and also a reporter gene Luciferase, indicating the general transcriptional enhancement of intron2 in different genes. In summary, we identified an antiviral TNL type soybean gene *GmRUN1*, expression of which was regulated at different layers. The investigation of *GmRUN1* gene regulatory network would help to explore the mechanism underlying soybean-SMV interactions.

**Keywords:** *GmDREB3; GmRUN1; intron-mediated enhancement; resistance gene; salicylic acid; soybean mosaic virus; tobacco mosaic virus; transcriptional regulation*

1. Introduction

Two layers of innate immune systems have evolved to recognize the potential pathogens and initiate an effective defense response. The first type of immune response is initiated by the pattern recognition receptors (PRRs) localized at the plasma membrane [1]. PRR recognizes and responds to evolutionarily conserved pathogen-associated molecular pattern (PAMP), and it is called pattern-triggered immunity (PTI) [2]. Some pathogens secrete virulence effectors to counteract PTI. Plants activate the second type of innate immune system to recognize the virulence effectors, named the effector-triggered immunity (ETI) [3]. ETI is generally mediated by a resistance (*R*) gene and leads to local necrosis of plants to limit the continuous spread of pathogens, namely hypersensitive response (HR) [4]. Nucleotide-binding site (NBS), leucine-rich repeat (LRR)-containing proteins (NLRs) occupy the largest proportion in plant R proteins [5]. *NBS-LRR* genes belong to a large gene family, with hundreds of copies in the genome, and are distributed in obvious uneven clusters [6,7]. To date, many *NBS-LRR* type *R* genes have been cloned from different plant species [8].

Plants NLR proteins belong to signal transduction ATPases with numeric domains (STAND) superfamily [9]. The central NBS domain performs the function of molecular
switch and controls the binding state of ATP/ADP to mediate downstream signal transduction [10, 11]. Leucine-rich repeats (LRRs) are ubiquitously present protein domains involved in mediating protein–protein interactions [12, 13]. Some studies suggest that LRR motifs can give recognition specificity in plant defense response [14]. According to their different N-terminal structures, these NBS-LRR proteins can be further divided into two categories: TIR-NBS-LRR (TNL) proteins with the homologous domain of toll/interleukin-1 receptor (TIR) and non-TNL (nTNL) proteins [15]. Most nTNL type-R proteins have a coiled coil (CC) structure at the N-terminal, commonly known as CC-NBS-LRR (CNL) type-R protein [16, 17]. Generally, a large number of CNL genes are found in all plant genomes, but TNL genes are not identified in monocotyledons [18]. The N-terminal CC or TIR domain can be used as a signal transduction center, which is associated with cellular targets or downstream signaling components of effectors [19].

The expression of plant R gene needs a strict regulation mechanism, and its ability to activate defense signal and trigger immunity depends on its protein level [20]. Over-expression of R gene can lead to autoimmunity and even plant growth retardation [21]. Therefore, the precise regulation of R proteins in plant homeostasis, including transcriptional and translational regulation, is crucial for plant growth and plant disease resistance. The expression of R genes is strictly regulated at multiple steps including transcription, post-transcriptional processing, and transcript turnover [22].

Soybean mosaic virus (SMV) is one of the main members of potyvirus [23]. The infection of SMV causes mosaic, necrosis, and other symptoms in many soybean varieties by means of aphid and seed transmission [24]. Therefore, SMV is a major disease that seriously threatens the yield and quality of soybean [25–28]. SMV genome is a single-stranded sense RNA which encode eleven functional proteins: P1, HC-Pro, P3, PIPO, 6K1, CI, 6k2, Nla-vpg, Nla-Pro, Nlb, and CP [29, 30]. Three independent SMV resistance loci, Rsv1, Rsv3, and Rsv4, were identified from soybean. Rsv1 is located on chromosome 13, which may contain one or more members of the NBS-LRR gene family and is highly resistant to most SMV strains except G7 [31, 32]. Rsv3 locus was located on chromosome 14 and was resistant to strains G5, G6, and G7 [33]. Rsv4 is resistant to strains v94-5152 and encodes a SMV specific dsRNase [34, 35]. In our previous study, we characterized dozens of SMV-responsive NLR genes in the susceptible soybean variety Hefeng25 by transcriptome sequencing [36]. Here, we identified a SMV-resistant soybean gene GmRUN1 encoding a typical TNL protein. GmRUN1 also showed resistance to TMV (tobacco mosaic virus) using transient expression assays in Nicotiana benthamiana, which is the most widely used experimental host in plant virology, due mainly to the large number of diverse plant viruses that can successfully infect it [37]. GmRUN1 genomic DNA (gDNA) is more resistant to these two viruses than its cDNA (complementary DNA), indicating the existence of intron-mediated enhancement (IME) for GmRUN1 regulation. Further analysis showed that intron2 of GmRUN1 was responsible for transcriptional enhancement of GmRUN1. GmRUN1 expression is also inducible by plant hormone SA and a transcription factor GmdREB3.

2. Materials and Methods

2.1. Plant Growth Conditions

Soybean [Glycine max (L.) Merr.] and Nicotiana benthamiana plants were grown in a glasshouse under a 14 h light/10 h dark cycle (24 °C day/22 °C night). Plants were used for transient expression assays at 3–4 weeks of age.

2.2. Construction of Recombinant Vectors

To generate cDNA clones of soybean GmRUN1 and GmdREB3 genes, total RNA was isolated from soybean cv. Hefeng25 leaves using the TRIzol reagent (Invitrogen, Waltham, MA, USA, Cat#15596026), and cDNA was generated using the GoScript reverse-transcription system (Promega, Madison, WI, USA, Cat#A5001) following the manufacturers’ instructions, and the sequences were then amplified from this cDNA using the primers listed in Supplemental Table S1 with PrimeSTAR® GXL DNA Polymerase (Takara,
Kusatsu, Japan, Cat\#R050A). For full-length \textit{GmRUN1} gene, PCR product was cloned into the binary vector pBI121 digested by \textit{SmaI} and \textit{SacI} using the In-Fusion HD cloning kit (Takara, Cat\#639650). For \textit{GmDREB3} gene, PCR product was cloned into the TA cloning vector using the pMD19-T vector cloning kit (Takara, Cat\#639650), and then recombinated with the pMD1-T7 vector digested by \textit{BamHI} and \textit{XhoI} using the ClonExpress Ultra one-step cloning kit (Vazyme, Nanjing, China, Cat\#C115-01). For truncated domain fragments of \textit{GmRUN1} gene, three different domains TIR (1–165 aa), NBS (166–474 aa), LRR (475–1088 aa) were identified by SMART website (http://smart.embl-heidelberg.de/ (Accessed: 1 October 2021)), and the PCR product was cloned into the pCB301-2µ-HDV vector linearized by PCR.

To generate truncated promoter clones of \textit{GmRUN1} gene, genomic DNA was isolated from soybean cv. Hefeng25 leaves using the CTAB method according to the manufacturer’s instructions, and the \textit{GmRUN1} truncated promoter regions were amplified and cloned into the pBI121 vector by replacing the CaMV 35S promoter before the \textit{β}-glucuronidase (\textit{GUS}) gene.

To generate exogenous intron insert clones of \textit{GmRUN1} gene, the Luciferase gene was cloned from pGWB435-LUC (GenBank No. AB294455.1) and then recombined into the entry vector pHSG299. Four introns of \textit{GmRUN1} gene were amplified from the genomic DNA and cloned into recombined vector pHSG299-LUC digested by \textit{SacI} site. The recombined entry clones were then recombined with the binary vector pCambia1300 for luciferase assay.

2.3. Transient Expression and Virus Inoculation

\textit{Agrobacterium tumefaciens} GV3101 strains carrying recombinant binary vectors were used to infiltrate \textit{N. benthamiana} leaves. Liquid cultures of all \	extit{Agrobacterium} strains were initially grown at 28 °C with agitation in Luria–Bertani (LB) media supplemented with the appropriate antibiotics. The bacterial cells were pelleted by centrifugation at 8000 rpm for 1 min, resuspended in infiltration buffer (10 mM \textit{MgCl}_2, 10 mM MES, and 200 \textit{µM} AS) and adjusted to an appropriate OD600 for infiltration. PJL24 which carried GFP (green fluorescent protein) in TMV genome was used as infectious clones for verification of \textit{GmRUN1} resistance, and GFP fluorescence was detected by a handheld long-wave (365 nm) UV lamp. SMV-N1 strain was used to infect \textit{N. benthamiana} leaves with mechanical inoculation, the SMV-infected leaves under quartz sand grinding in 1× phosphate buffer was daubed to infiltrated site using a writing brush.

2.4. Promoter Analysis and In Situ GUS Activity Assay

Promoter elements were predicted for the 3000 bp genomic sequence upstream of \textit{GmRUN1} gene by PlantCare website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (Accessed: 1 October 2021)). The promoter-GUS recombinant vectors, alone or together with the recombinant vectors expressing \textit{GmDREB3} proteins, were infiltrated into \textit{N. benthamiana}. SMV and SA were also applied to the leaves for induction analysis by rub and spray, respectively, and the leaf discs (1 cm in diameter) were cut at 2 dpi (day post inoculation) for GUS staining with X-Gluc as the substrate according to the literature [38].

2.5. Luciferase Reporter Assay and Fluorescence Quantitative Analysis

\textit{Agrobacterium} strain GV3101 carrying \textit{LUC} gene inserted different \textit{GmRUN1} introns that recombined in pCambia1300 expression vector were infiltrated into \textit{N. benthamiana} leaves. After 24–48 h, 20 \textit{µL} 0.5 mM D-luciferin was applied to infiltrated site in the dark. After dark treatment for 3–5 min, the \textit{LUC} expression was detected by CCD imaging system (Berthold Technologies, Bad Wildbad, Germany, LB 985) with IndiGO software at 560 nm for exposure time of 1–3 min, and the fluorescence intensity was visualized to assay the effect of different \textit{GmRUN1} introns. The infiltrated sites of leaves without imaging in the same batch were cut and quick freezing in liquid nitrogen for qRT-PCR analysis. To quantitatively analyze the level of \textit{GmRUN1} resistance, Gel-Pro analyzer software was used.
for the detection of TMV-GFP intensity in the green channel, and the data were imported to GraphPad Prism7 software to analyze statistically significant and draw graph.

2.6. Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA extraction and reverse transcription were performed as described above, and the quantitative RT-PCR using gene-specific primers was carried out subsequently in an Analytikjena qPCR instrument using TransStrat® Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China, Cat#AQ141). The data were normalized to ACTIN expression by the cycle threshold (CT) 2-ΔΔCT method according to the literature [39] and analyzed by Graphpad Prism7 software. All experiments were repeated at least three times. Primers used in this study are listed in Table S1.

3. Results

3.1. GmRUN1 cDNA Shows Partial Resistance to TMV and SMV in N. Benthamiana Transient Expression Assay

Previously, we identified NBS-LRR family genes that were involved in SMV–soybean interactions [36]. In the present study, we characterized one such gene with sequence ID of XM_006592417.3 (gene locus: G12g132200) and designated it as GmRUN1 as it was homologous with Vitis rotundifolia RUN1 (resistance to Uncinula Necator 1) gene (Figure 1A,B) [40,41]. ORF (open-reading frame) sequence or genomic DNA sequence of GmRUN1 was amplified from soybean cDNA or genomic DNA and were ligated into binary vectors pCB301 and pBI121 to obtain recombinant overexpression vectors. We used N. benthamiana transient expression system to investigate the role of GmRUN1 for SMV resistance. In the parallel experiment, TMV-GFP infectious clone (pJL24) was also used to investigate the antiviral role of GmRUN1 toward TMV [42]. The above recombinant vectors were transformed into Agrobacterium strain GV3101 and transiently expressed in tobacco leaves to detect their resistance to TMV and SMV. In this study, a TMV-resistant tobacco gene N [43] and a SMV/TMV resistant soybean gene SRC7 (unpublished data from the same lab) were used as positive control, while Agrobacterium carrying empty vector pBI121 was used as negative control. We defined almost no GFP fluorescence (for TMV) or severe hypersensitive response (HR) (for SMV) as full resistance, weak GFP fluorescence or mild HR as partial resistance, and strong GFP fluorescence or no HR as no resistance. As a result, GmRUN1 genomic DNA showed full resistance to both TMV and SMV, while its cDNA displayed only partial resistance when it was expressed from either of the binary vectors (Figure 1C–E; Tables 1 and 2).

Table 1. Phenotypic statistics of TMV appearance upon transient expression of different genes.

| Gene Name          | Full Resistance | No Resistance | Partial Resistance |
|--------------------|-----------------|---------------|--------------------|
| pBI121-N           | 30/30 (100%)    | 0/30 (0%)     | 0/30 (0%)          |
| pBI121-SRC7        | 30/30 (100%)    | 0/30 (0%)     | 0/30 (0%)          |
| pBI121-GmRUN1 gDNA | 24/30 (80%)     | 0/30 (0%)     | 6/30 (20%)         |
| pCB301-GmRUN1 cDNA | 0/30 (0%)       | 13/30 (43%)   | 17/30 (57%)        |
| pBI121-GmRUN1 cDNA | 0/30 (0%)       | 24/30 (80%)   | 6/30 (20%)         |
| pBI121             | 0/30 (0%)       | 30/30 (100%)  | 0/30 (0%)          |

Table 2. Phenotypic statistics of SMV upon transient expression of different genes.

| Gene Name          | HR/Total Leaves |
|--------------------|-----------------|
| pBI121-GmRUN1 gDNA | 14/30 (47%)     |
| pCB301-GmRUN1 cDNA | 9/30 (30%)      |
| pBI121-GmRUN1 cDNA | 7/30 (23%)      |
| pCB301             | 0/30 (0%)       |
Figure 1. Antiviral activity of GmRUN1. (A) Gene architecture of GmRUN1. Block and line indicate exon and intron, respectively. (B) Evolutionary analyses of GmRUN1. The evolutionary tree was built using the neighbor-joining method conducted in MEGA7. All positions with less than 50% site coverage were eliminated. (C) Transient expression assay for GmRUN1 antiviral activity for TMV. N. benthamiana leaves were infiltrated with Agrobacterium tumefaciens GV3101 inocula (OD600 = 1.0) carrying different recombinant vectors and co-infected with TMV-GFP. GFP was visualized under hand-held UV lamp (Wavelength = 365 nm) at 5 dpi (days post infiltration). N: tobacco N protein. SRC7: SMV resistance cluster 7. EV: empty vector. GmRUN1 cDNA-1: GmRUN1 cDNA expressed from pCB301 vector. GmRUN1 cDNA-2: GmRUN1 cDNA expressed from pBI211 vector. (D) Fluorescence quantification of GmRUN1 transient expression assay. TMV-GFP intensity was analyzed by Gel-Pro analyzer software and normalized against positive control (N). (E) Transient expression assay for GmRUN1 antiviral activity for SMV.

3.2. Three Canonical Domains of GmRUN1 Are Indispensable for Its Antiviral Activity

GmRUN1 is a typical TNL (TIR-NBS-LRR) protein, containing three domains, including a typical N-terminal TIR domain, central NBS domain, and C-terminal LRR domain (Figure 2A). To investigate the importance of these domains, we made truncation analysis. We expressed five truncations for GmRUN1, including GmRUN1TIR, GmRUN1NBS, GmRUN1LRR, GmRUN1TN (abbreviation for TIR-NBS of GmRUN1), and GmRUN1NL (abbreviation for NBS-LRR of GmRUN1) (Figure 2A). Truncation analysis showed that all of the TIR, NBS, and LRR domains were indispensable for GmRUN1 antiviral activity, as the deletion of any of them abolished its resistance to both TMV and SMV (Figure 2B–D; Tables 3 and 4).

3.3. GmRUN1 Has IME Phenomena

As GmRUN1 gDNA is resistant to TMV and SMV and its cDNA without the introns has been proved to be partially resistant, we speculate that the introns of GmRUN1 might be involved in its antiviral activity. GmRUN1 gDNA contains four introns with the sizes...
of 6738, 243, 134, and 102 nt, and all of them follow the “GT-AG” rule (Figure 1A). Some introns were reported to enhance gene expression, and this phenomenon was named intron-mediated enhancement (IME) [44,45]. To determine the possible IME of four 
GmRUN1
introns, we inserted them at the +166 site of luciferase (LUC) reporter gene (Figure 3A). Interestingly, the insertion of any of four introns from 
GmRUN1
abolished LUC signal (Figure 3B). We speculate that the insertion of these introns may disturb the LUC ORF and subsequent protein expression. The PCR amplicons still contained the introns when total RNA was extracted from the inoculated leaves, and LUC cDNA was amplified, indicating that the introns were not correctly spliced from LUC mRNA (Figure 3C). Therefore, these introns led to insertion mutation in LUC gene, showing that the introns of one gene may not be spliced normally when was inserted into other genes. However, we observed that LUC mRNA level was significantly upregulated by 3.3 times upon insertion of intron2, showing that intron2 has IME effects at transcriptional level (Figure 3D). We then replaced the intron of soybean antiviral gene SRC7 with 
GmRUN1
intron2 (Figure 4A), and the mRNA level of SRC7 was increased by 2.6 folds, further demonstrating the IME effect of 
GmRUN1
intron2 at the transcriptional level (Figure 4B). Furthermore, the insertion of 
GmRUN1
intron2 did not abolish SRC7 antiviral activity, indicating that 
GmRUN1
intron2 was correctly spliced from SRC7 mRNA (Figure 4C; Table 5). It also indicates that the splicing of intron depends on inserted genes. SRC7 was homologous to 
GmRUN1
; therefore, it is reasonable that 
GmRUN1
intron2 can be spliced in SRC7 but not in nonhomologous LUC gene. To further demonstrate the importance of these introns, we made 
GmRUN1
expression constructs with truncations in different introns (Figure 5A). The depletion of intron1 increased while further deletion of intron2 decreased antiviral activity, indicating the enhancement of intron2 for antiviral activity (Figure 5B, Table 6). Taken together, 
GmRUN1
intron2 has an IME effect.

Figure 2. Truncation analysis of 
GmRUN1
. (A) Domain architecture of 
GmRUN1
. Yellow, green, and blue boxes indicate TIR, NBS, and LRR domains, respectively. TN: TIR-NBS, NL: NBS-LRR, TNL: TIR-NBS-LRR. (B, D) Transient expression assay of different domains for antiviral activity. 
N. benthamiana
leaves were infiltrated with 
Agrobacterium GV3101 inocula (OD600 = 1.0) carrying different recombinant vectors and co-infected with TMV-GFP (B) or SMV (D). GFP was visualized under hand-held UV lamp (Wavelength = 365 nm) at 5 dpi (days post infiltration). (C) Fluorescence quantification of truncated 
GmRUN1
transient expression assay. TMV-GFP intensity was analyzed by Gel-Pro analyzer software and normalized against positive control (GmRUN1 gDNA).
Table 3. Phenotypic statistics of TMV appearance upon transient expression of different genes.

| Gene Name          | Full Resistance | No Resistance | Partial Resistance |
|--------------------|-----------------|---------------|--------------------|
| pBI121-GmRUN1 gDNA| 17/25 (68%)     | 0/25 (0%)     | 8/25 (32%)         |
| pCB301-GmRUN1TIR  | 0/25 (0%)       | 25/25 (100%)  | 0/25 (0%)          |
| pCB301-GmRUN1NBS  | 0/25 (0%)       | 25/25 (100%)  | 0/25 (0%)          |
| pCB301-GmRUN1LRR  | 0/25 (0%)       | 25/25 (100%)  | 0/25 (0%)          |
| pCB301-GmRUN1TN   | 0/25 (0%)       | 25/25 (100%)  | 0/25 (0%)          |
| pCB301-GmRUN1NL   | 0/25 (0%)       | 6/25 (24%)    | 19/25 (76%)        |
| pCB301             | 0/25 (0%)       | 25/25 (100%)  | 0/25 (0%)          |

Table 4. Phenotypic statistics of SMV upon transient expression of different genes.

| Gene Name          | HR/Total Leaves |
|--------------------|-----------------|
| pBI121-GmRUN1 gDNA| 10/25 (40%)     |
| pCB301-GmRUN1TIR  | 1/25 (4%)       |
| pCB301-GmRUN1NBS  | 0/25 (0%)       |
| pCB301-GmRUN1LRR  | 0/25 (0%)       |
| pCB301-GmRUN1TN   | 1/25 (4%)       |
| pCB301-GmRUN1NL   | 0/25 (0%)       |
| pCB301-GmRUN1TNL  | 6/25 (24%)      |
| pCB301             | 0/25 (0%)       |

Figure 3. Influence of GmRUN1 introns insertions on LUC reporter gene expression. (A) Schematic diagram of LUC recombinant vector construction (top panel) and transient expression (bottom panel). (B) Luciferase reporter assay of GmRUN1 introns. LUC recombinant vectors with or without different GmRUN1 introns were transiently expressed in N. benthamiana leaves. Images were taken using a Berthold camera 24 and 30 h after infiltration. (C) Semiquantitative PCR for transcription assay of LUC inserted different GmRUN1 introns. Lines: (1), LUC cDNA, 1653 bp; (2), LUC-intron2 cDNA, 1896 bp; (3), LUC-intron3 cDNA, 1787 bp; (4), LUC-intron4 cDNA, 1755 bp; (5–8), N. benthamiana ACTIN of line 1~4; and (9) Negative control. (D) qRT-PCR assays of LUC mRNA level after inserted with GmRUN1 introns. Error bars show the SD between biological replicates performed (n = 3), and Tukey’s multiple comparisons test was performed between samples in different groups.
Figure 4. GmRUN1 intron2 enhances SRC7 expression. (A) Gene architecture of SRC7. Block and line indicate exon and intron, respectively. (B) qRT-PCR assays of SRC7 mRNA level after replaced by GmRUN1 intron2. Error bars show the SD between biological replicates performed (n = 3), and Tukey’s multiple comparisons test was performed between samples in different groups. (C) Transient expression assay for recombinant SRC7-intron2 antiviral activity. N. benthamiana leaves were infiltrated with Agrobacterium tumefaciens GV3101 inocula carrying different recombinant vectors (OD$_{600}$ = 0.01) and co-infected with TMV-GFP. N: tobacco N protein. SRC7: SMV resistance cluster 7. SRC7-intron2: SRC7 intron replaced by GmRUN1 intron2. EV: empty vector.

Table 5. Phenotypic statistics of TMV appearance upon transient expression of different genes.

| Gene Name                       | Full Resistance | No Resistance | Partial Resistance |
|---------------------------------|-----------------|---------------|--------------------|
| pCambia1300-SRC7-intron2         | 15/20 (75%)     | 0/20 (0%)     | 5/20 (25%)         |
| pCambia1300-SRC7                | 7/20 (35%)      | 0/20 (0%)     | 13/20 (65%)        |
| pCambia1300-N                   | 8/20 (40%)      | 0/20 (0%)     | 12/20 (60%)        |
| pCambia1300                     | 0/20 (0%)       | 20/20 (100%)  | 0/20 (0%)          |

Figure 5. Antiviral activity of GmRUN1 truncations in different introns. (A) Schematic diagram of truncated vector construction with different GmRUN1 introns. Block and line indicate exon and intron, respectively. (B) Transient expression assay of truncated GmRUN1 introns for antiviral activity. N. benthamiana leaves were infiltrated with Agrobacterium tumefaciens GV3101 inocula (OD$_{600}$ = 0.5) carrying different recombinant vectors and co-infected with TMV-GFP. GFP was visualized under hand-held UV lamp (Wavelength = 365 nm) at 5 dpi (days post infiltration).
Table 6. Phenotypic statistics of TMV appearance upon transient expression of different genes.

| Gene Name      | Full Resistance | No Resistance | Partial Resistance |
|----------------|-----------------|---------------|--------------------|
| pCB301-gDNA    | 9/35 (26%)      | 0/35 (0%)     | 26/35 (74%)        |
| pCB301-GmRUN1-ExIn-1 | 15/35 (43%)  | 0/35 (0%)     | 20/35 (57%)        |
| pCB301-GmRUN1-ExIn-5 | 2/35 (6%)     | 0/35 (0%)     | 33/35 (94%)        |
| pCB301-GmRUN1-ExIn-11 | 2/35 (6%)    | 5/35 (14%)    | 28/35 (80%)        |
| pCB301-cDNA    | 0/35 (0%)       | 17/35 (49%)   | 18/35 (51%)        |
| pCB301        | 0/35 (0%)       | 35/35 (100%)  | 0/35 (0%)          |

3.4. GmRUN1 Expression Is Transcriptionally Regulated by SA

Having confirmed the IME effect of intron2 on GmRUN1 expression, we next intended to clone the GmRUN1 promoter and further examine the transcriptional regulation of GmRUN1. We used GUS reporter to assess promoter activity. The promoter region of GmRUN1 was amplified from soybean genomic DNA and was cloned into binary vector pBI121 to obtain Pro:GUS reporter vectors. We made four different Pro:GUS constructs, harboring different lengths of the promoter region, namely Pro2415:GUS, Pro2592:GUS, and Pro2237:GUS (Figure 6A). When these Pro:GUS constructs are transiently expressed in N. benthamiana, they did not show any GUS signal, demonstrating that they possessed very low basal transcriptional activity (Figure 6B). The infection of SMV did not induce the GUS expression in any of the Pro:GUS constructs, indicating that GmRUN1 expression is not inducible by SMV infection (Figure 6B). Salicylic acid (SA) is a well-known defense hormone which is generally implicated in plant immunity against plant viruses including SMV [36]. Treatment with MeSA, an analog of SA, elevated GUS expression for the Pro2592:GUS and Pro2414:GUS reporters but not in Pro2415:GUS and Pro2237:GUS reporters (Figure 6B).

Figure 6. Transcriptional regulation of GmRUN1 promoter. (A) Schematic diagram of truncated vector construction with different region of GmRUN1 promoter. Colored dashed lines and boxes indicate cis-acting regulatory element predicted by PlantCare database. (B) GUS activity assay of different region of GmRUN1 promoter. GUS activity was detected at 3 days post SA or SMV induction. EV: pBI121 empty vector. Pro1–4: Pro2415:GUS, Pro2592:GUS, Pro2237:GUS, and Pro2414:GUS. EVΔGUS: pBI121 empty vector removed GUS gene.

3.5. GmRUN1 Expression Is Transcriptionally Induced by Transcription Factor GmDREB3

Based on the above results, we further analyzed cis-regulatory element present in GmRUN1 promoter using PlantCare website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Accessed: 1 October 2021)). Interestingly, we found two cis-elements immediately upstream of GmRUN1 ORF which were present in SA-inducible constructs Pro2592:GUS and Pro2414:GUS, while lacking in SA noninducible Pro2415:GUS and Pro2237:GUS constructs (Figure 6A). One cis element is the SA-responsive as-1, which might contribute to SA induction of GmRUN1 promoter. Another cis element is the ERF binding element ERE, which is close to as-1 element on GmRUN1 promoter (Table 7). Previously, we showed that GmRUN1 expression is repressed upon SMV infection in soybean [36]. From our RNA-seq data, we also found that GmDREB3 is significantly repressed by SMV infection, and GmDREB3 expression pattern is positively correlated with that of GmRUN1 (Figure 7A). The data lead us to the assumption...
that GmDREB3 might be a positive regulator of GmRUN1. Coexpression of GmDREB3 elevated GUS expression for the Pro2592:GUS and Pro2414:GUS reporters but not in Pro2415:GUS and Pro2237:GUS reporters, further demonstrating positive regulation of GmRUN1 expression by GmDREB3 via the ERE cis element at GmRUN1 promoter (Figure 7B).

### Table 7. Promoter elements and their functions of GmRUN1.

| Promoter Element | Sequence | Function |
|------------------|----------|----------|
| TATA-box         | TATAA    | Core promoter element around -30 of transcription start |
| CAAT-box         | CCAAT    | Common cis-acting element in promoter and enhancer regions |
| CGTCA-motif      | CGTCA    | Cis-acting regulatory element involved in the MeJA-responsiveness |
| TGACG-motif      | TGACG    | Cis-acting regulatory element involved in the MeJA-responsiveness |
| as-1             | TGACG    | Cis-acting element related to salicylic acid induction |
| TCA-element      | TCAGAAGAGG | Cis-acting element involved in salicylic acid responsiveness |
| TCA              | TCATCTTCAT | Unknown functional element |
| ABRE             | ACGTG    | Cis-acting element involved in the abscisic acid responsiveness |
| ERE              | ATTTAAA  | Cis-acting element involved in ethylene response |
| AARE             | AAACCA   | Cis-acting regulatory element essential for the anaerobic induction |
| LTR              | CCGAAA   | Cis-acting element involved in low-temperature responsiveness |
| MBS              | CAACGTG  | MYB binding site involved in drought inducibility |
| W-box            | TGACCC   | Cis-acting element involved in disease resistance |
| CAT-box          | GCCACT   | Cis-acting regulatory element related to meristem expression |
| AT1-motif        | AATTTTTTTAT | Part of a light-responsive module |
| GT1-motif        | GGTTAAT  | Light-responsive element |
| TCT-motif        | TCTTACC  | Part of a light-responsive element |
| G-Box            | CACGTG   | Cis-acting regulatory element involved in light responsiveness |
| MRE              | AACCTAA  | MYB binding site involved in light responsiveness |
| Box 4            | ATTTAT   | Part of a conserved DNA module involved in light responsiveness |

**Figure 7.** GmDREB3 positively regulates GmRUN1. (A) Venn diagrams of predicted TFs transcription level consistent with GmRUN1 at 1/5/10 dpi (left panel). Red number indicates GmDREB3 which shows consistent expression pattern with GmRUN1 in graph at 1/5/10 dpi (right panel). (B) GUS activity assay of different region of GmRUN1 promoter coexpressed with GmDREB3. GUS activity was detected at 3 dpi. EV: pBI121 empty vector. Pro1–4: Pro2415:GUS, Pro2592:GUS, Pro2237:GUS, and Pro2414:GUS. EVΔGUS: pBI121 empty vector removed GUS gene.

### 4. Discussion

In this study, we identified an antiviral gene GmRUN1 from soybean. GmRUN1 encodes a typical TIR-NBS-LRR protein and gives resistance to both a Potyvirus SMV and a Tobamovirus TMV. Truncation analysis showed that the all of the canonical domains TIR,
NBS, and LRR were necessary for GmRUN1 antiviral activity. In some reports, TIR-NBS domains but not LRR domain were sufficient to trigger immune responses, especially in transient expression assay. For example, *Arabidopsis* powdery mildew resistance gene TN2 and autoimmune-related gene CHS1 encode functional TIR-NBS proteins [46,47]. Furthermore, TIR-NBS genes were broadly reported in variety of plant species, including the leguminous plants such as soybean and common bean [48,49]. Therefore, the antiviral mechanism of GmRUN1 might be different from those of TIR-NBS genes and deserves further investigation.

Overexpression of GmRUN1 showed HR upon infection with SMV. Cell-death-triggering activity of R proteins should be under strict control so as to trigger timely immune response only upon pathogen infection and to also avoid fitness costs at pathogen-free conditions [30]. Therefore, multiple layers of regulation at transcriptional, post-transcriptional, and protein activity levels exist for expression control of R genes [22]. The expression of GmRUN1 is inducible by the treatment with major defense hormone SA. Promoter truncation analysis narrowed down the SA-responsive element to a −326~−336 nt (10 nt) region, where we identified a SA-responsive cis element as-1, which is most likely contributed to SA induction on GmRUN1 expression [51,52]. SA is an important defense hormone which contributes to the immunity against various pathogens, especially plant viruses, and leads to systemic acquired resistance (SAR) [53]. For example, SA was reported to be involved in defense response mediated by R genes such as tobacco N and *Arabidopsis* AtTN10 [48,54]. Besides this as-1 element, we also found a ERE element in the SA-responsive promoter region. A putative transcription factor GmDREB3 upregulates GmRUN1 promoter activity via ERE-dependent manner, and the expression of GmRUN1 is positively correlated with SA induction on GmRUN1 expression [51,52]. GmDREB3 is an important defense hormone which contributes to the immunity against various pathogens, especially plant viruses, and leads to systemic acquired resistance (SAR) [53]. For example, GmDREB3 was found to be involved in defense response mediated by R genes such as tobacco N and *Arabidopsis* AtTN10 [48,54]. Besides this as-1 element, we also found a ERE element in the SA-responsive promoter region. A putative transcription factor GmDREB3 upregulates GmRUN1 promoter activity via ERE-dependent manner, and the expression of GmRUN1 is positively correlated with SA induction on GmRUN1 expression [51,52]. SA is an important defense hormone which contributes to the immunity against various pathogens, especially plant viruses, and leads to systemic acquired resistance (SAR) [53]. For example, SA was reported to be involved in defense response mediated by R genes such as tobacco N and *Arabidopsis* AtTN10 [48,54]. Besides this as-1 element, we also found a ERE element in the SA-responsive promoter region. A putative transcription factor GmDREB3 upregulates GmRUN1 promoter activity via ERE-dependent manner, and the expression of GmRUN1 is positively correlated with SA induction on GmRUN1 expression [51,52]. GmDREB3 is an important defense hormone which contributes to the immunity against various pathogens, especially plant viruses, and leads to systemic acquired resistance (SAR) [53]. For example, SA was reported to be involved in defense response mediated by R genes such as tobacco N and *Arabidopsis* AtTN10 [48,54].

It has been shown that some introns have positive regulatory roles on gene expression, but some others possess inhibitory effects [58]. In 1987, Callis et al. first discovered that introns can mediate the enhancement of gene expression in maize cells [44]. Subsequently, the phenomenon of IME was observed in mammals, nematodes, and yeasts [59–61]. Some endogenous introns can compensate for the low-level expression driven by the weak promoter [62]. IME is also associated with specific sequence motifs, such as TTNGATYTG and CGATT [63]. Several introns were also shown to have both promoter activity and enhancer function [64]. In most studies, IME was attributable to increased mRNA accumulation at the transcriptional level; however, there are also some data that provide evidence of enhanced translation [65,66]. Here, we provided evidence that intron2 of GmRUN1 increased mRNA level of native gene GmRUN1, a homologous gene SRC7 and also a reporter gene Luciferase, indicating the general transcriptional enhancement of this intron2 in different genes. However, we also showed that correct splicing of intron2 might depend on the inserted genes. The in-depth study of IME phenomenon provides a basis for the wide application of functional introns.

5. Conclusions

Plant viruses pose threats to agriculturally important crops. SMV is a major pathogen of soybean and causes heavy yield losses worldwide. Although several R genes have been cloned from multiple host varieties, soybean–SMV interactions are still elusive. In this study, we identified a TNL (TIR-NBS-LRR)-type antiviral gene GmRUN1 from soybean. GmRUN1 genomic DNA showed full resistance to both TMV and SMV, while its cDNA displayed only partial resistance, and three canonical domains of GmRUN1 are indispensable for its antiviral activity. GmRUN1 is spectacular as its expression is regulated at multiple layers, such as SA induction, GmDREB3 transcriptional activation, and IME from intron2. GmRUN1 represents a novel SMV-resistance gene and deserves further functional study.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v13102032/s1, Table S1: Primers used in this study.
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