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RESEARCH ARTICLE

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Expression analysis of *RUS1* and construction of *RUS1* plant expressing vector

Abstract *RUS1* was one of the disease resistance gene analogs obtained from *Setaria italica* Beauv. Semi-quantitative RT-PCR analysis result showed that *RUS1* gene could be induced by *Uromyces setariae-italicae* and had relation to the resistance response of *Setaria italica* Beauv. against *Uromyces setariae-italicae* infection. Promoter sequence of *RUS1* was obtained by the method of Genome Walking, and its length was 675 bp. *RUS1* promoter and pCAMBIA1300 vector were fused to construct *RUS1*::GUS vector. GUS histochemical staining result showed that promoter could activate gene expression. *RUS1* gene (including the promoter sequence) was obtained through PCR amplification and then fused with pCAMBIA1300 vector to construct pCAMBIA1300:*RUS1* plant expressing vector. The research laid a foundation for gene functional identification of *RUS1*.

Keywords *Setaria italica*, *RUS1*, semi-quantitative RT-PCR, GUS histochemical staining, plant expressing vector

1 Introduction

Foxtail millet (*Setaria italica* Beauv.) has a lot of advantages that have made it to become one of the traditional staple cereal crops in semi-arid regions of China and other countries. It is an excellent forage and best annual grazing crop in China. Leaf rust is one of the most threatening diseases of millet. The epidemic of millet leaf rust (*Uromyces setariae-italicae*) can cause serious loss of production, and how to control the disease effectively has become a serious problem. With the rapid development of molecular biology technology, cloning of disease-resistant gene is easy to be done, and the process of plant breeding can be speeded up. Reports about disease-resistant gene clone and function analysis of Foxtail millet are very limited. *RUS1* (Resistance against *Uromyces setariae-italicae*) obtained from Foxtail millet cultivar "Shilixiang" is highly resistant to the infection of *Uromyces setariae-italicae* by the method of RGA (Resistance gene analogs). Using Genome Walking technology, the full-length DNA sequence of *RUS1* was obtained (GenBank No. FJ467296), and it was identified as a putative NBS-type disease-resistant gene, containing an NB-ARC domain and conserving motifs P-loop, kinase 2, kinase 3 and hydrophobic domain which are the characteristics of NBS-LRR-type resistant gene of plant. Phylogenetic analysis indicated that it was similar to *RPM1* and belonged to the NBS-LRR-type disease resistance gene. *RUS1* gene function and relationship to millet resistance response are not clear. In this paper, gene expressing of *RUS1* was analysed, and plant expressing vectors of *RUS1* were constructed. Our result will lay a foundation for the functional analysis of *RUS1*.

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2 Materials and methods

2.1 Plant materials and agrobacteria strains

Foxtail millet leaf rust-resistant cultivar "Shilixiang" and *Uromyces setariae-italica* were kindly provided by the Institute of Millet Crops, Hebei Academy of Agricultural and Forestry Sciences. Plant expressing vectors pCAMBIA1300 and *Agrobacterium* GV3101 were kindly provided by Prof. Yanyun Pan from the College of Life Sciences, Agricultural University of Hebei. *Agrobacterium* LBA4404 was kindly provided by Dr. Lei Wang from Biotechnology Research Institute, Chinese Academy of Agricultural Sciences (CAAS).

2.2 Material treatment

Infection with *Uromyces setariae-italicae* was performed on 4-week-old plants. To infect plants, the fungal spore was sprayed onto the leaves of plants. For placebo treatment, sterile water was sprayed onto the leaves. After inoculation, plants were kept under a transparent cover to maintain 100% relative humidity (RH) and transferred to a growth chamber at 28–30°C with a 12-h light/12-h dark photoperiodic regime. Samples were taken at 0 h, 24 h, 48 h and 72 h after inoculation.

2.3 Semi-quantitative RT-PCR

Total RNA was isolated from 100-mg fresh plant material finely ground in liquid nitrogen and extracted using Trizol reagent (TIANGEN BIOTECH CO.). RT was performed using an oligodT_{18} for a primer and M-MLV Reverse transcriptase (TAKARA). To determine the expression pattern of *RUS1*, semi-quantitative RT-PCR was performed with specific primers (F: 5′-GGAAAAACTACACTTGC-TAG-3′, R: 5′-CTACCATCATCTCTGCCC-3′) corresponding to the coding region of *RUS1*. First-strand cDNA was quantified with 18S rRNA primers (F: 5′-GTTGCAGTTAAAAAGCTCGT-3′, R: 5′-TTGATTTCT-CATAAGGTGCC-3′) in PCR reactions. The volume of PCR reaction was 25 μL with 0.5-μL first-strand cDNA as template. Reactions were performed with Taq polymerase (TaKaRa), at 94°C for 7 min, followed by 24 cycles for 30 s at 94°C, 30 s at 52°C, and 50 s at 72°C. The PCR products were separated on 1.2% (w/v) agarose gel.

2.4 Obtained promoter of *RUS1* gene by Genome Walking

Approximately 2.5 μg of genomic DNA was digested with *Sac*II, *Dra*I, *Pvu*II, *Sma*I, *Eco*RV and *Swa*I restriction enzymes (Fermentas) in a restriction buffer (supplied with the enzyme). Digestion was carried out in a water bath at 37°C for 16 h–18 h. From each reaction tube, 5 μL was removed and run on a 1.0% (w/v) agarose gel to determine whether the digestion was complete or not. Genomic DNA was purified by phenol/chloroform method. Subsequently, purified genomic DNA was mixed with ligation mixture containing an adaptor, 2.5 U of T_4 DNA ligase (TaKaRa) and 10 × T_4 DNA ligase buffer (supplied with the enzyme). Ligation was carried out overnight at 16°C. PCR reactions were carried out using 1.4 μL adaptor DNA, 10 × PCR buffer, 10 μmol·L^{-1} dNTPs, 10 μmol·L^{-1} of each gene-specific primer and universal primer, and 0.5 U Taq DNA polymerase in a total volume of 25 μL. The PCR conditions for the amplifications included: 7 cycles at 94°C for 25 s, then at 72°C for 4 min, followed by 32 cycles at 94°C for 25 s, then 4 min at 67°C with final incubation at 67°C for 7 min before cooling at 4°C. PCR products were resolved on 1.2% agarose gel. Each single band was isolated and extracted using the UNIQ-10 Gel Extraction Kit (Sangon). The extracted PCR products were cloned and sequenced.

2.5 Activity analysis of *RUS1* promoter

GUS histochemical staining assay was used for analysis of *RUS1* promoter expression. The staining solution included 100 mmol·L^{-1} sodium phosphate buffer solution (pH 7.0), 0.5 mmol·L^{-1} $K_3[Fe(CN)_6]$, 0.5 mmol·L^{-1} $K_4[Fe(CN)_6]$, 10 mmol·L^{-1} EDTA, 0.1% Triton X-100, 20% Methanol, and 1.0 mg·mL^{-1} X-Gluc.

2.6 Construction of plant expressing vector

2.6.1 Gene cloning

Primer with enzyme restriction sites was designed according to known sequence. Taking genome DNA of Shilixiang as template, the PCR reactions were carried out using 10 × PCR buffer, 2.5 mmol·L^{-1} dNTPs, 10 μmol·L^{-1} each gene-specific primer and 0.5 U Taq DNA polymerase in a total volume of 25 μL. The PCR conditions for the amplifications included: pre-denaturation at 94°C for 7 min, then denaturation at 94°C for 30 s, followed by 35 cycles at 60°C for 30 s, 72°C for 4 min, and with final incubation at 72°C for 10 min before cooling at 4°C. The PCR products were resolved on 1.0% agarose gel. The target band was isolated and extracted using the UNIQ-10 Gel Extraction Kit (Sangon).

2.6.2 Enzyme digest and ligation reaction

PCR products and vectors were digested with two different restriction enzymes (Fermentas) in a restriction buffer containing 33 mmol·L^{-1} Tris-acetate (pH 7.9), 10 mmol·L^{-1} Mg-acetate, 66 mmol·L^{-1} K-acetate and 0.1 mg·mL^{-1} bovine serum albumin. Digestion was carried out in a water bath at 37°C for 2.5 h–3.0 h. Digested product was analyzed on a 0.8% agarose gel. The target band was purified using the UNIQ-10 Gel Extraction Kit. Purified PCR product and vector were mixed with ligation mixture containing PCR products and vectors, 2.5 U T_4 DNA ligase (TaKaRa) and 10 × T_4 DNA ligase buffer (supplied with the enzyme). Ligation was carried out overnight at 16°C.

2.6.3 Transform and identified of positive clone

The ligation product was transformed to *Agrobacterium* LBA4404. Digestion reaction with two different restriction enzymes was used to identify positive clone.

3 Results

3.1 Expression of *RUS1* using semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to determine the

expression pattern of *RUS1*. Infected with *Uromyces setariae-italicae*, total RNA of 0 h, 24 h, 48 h and 72 h samples was obtained, and the first strand cDNA was synthesized. First-strand cDNA was quantified using PCR amplification with 18S rRNA primers. Intensity of amplified bands was adjusted to the same, indicating that the equal amount of total RNA was used for semi-quantitative RT-PCR. Analysis result showed that expression of *RUS1* gene decreased at 24 h and 48 h after inoculation. While at 72 h, the expression was increased highly than un-inoculated plant (Fig. 1). According to the result, it was suggested that there was a close relation between *RUS1* and resistance response of *Setaria italica* Beauv. against *Uromyces setariae-italicae* infection.

3.2 Obtained promoter of *RUS1* gene by Genome Walking

According to the known sequence of *RUS1* gene, the 5' end of gene special primers was designed using Primer 5.0 software. The PCR amplification was performed using a gene-specific primer and universal primer combination. The PCR products were analyzed on 1.2% agarose gel. The length of about 750-bp band was obtained (Fig. 2). Thereafter, the PCR product was purified and sequenced. Spliced between the known sequence of *RUS1* gene, 5' end sequence and the length of 675-bp promoter sequence were obtained.

The sequence was as follows:

CTCTCTGCTCTCGATCGGAGTCTGAATG-GATGGTTGGAAATGGGGTGACAGAA-TGCGGTTGTGGTTGTGGCAGGAGTGGTTT-TATCTTGGAATGCAAACGTTTGCTTTGGGGTTT-GATCTTCGCTCAAACACCATGGACATCACGGCAA-GAATATGCCAAAGATAGCAAAGCAAAGCAAAG-CACGTCCGCTAGAAAGATAGCCCATTGCAACAC-CATGGACATCACGGAAAGAATATGCCAAAG-CAAAATATCACGACTGAAAGGAGTTGTGTAAGT-CACAGTATCCGGCTATCCGCCGCAACC-GGTCGGTGGCGCAGTCTCCAGGCATCCGG-GAGTCCCTTCAGCGTGCGCCGCCACCTCCTG-GAGTATGAAAATTGGACAATGAGCCAGGGATCA-CAGCAAGCTGAAGCATGTAATGGCAGCTTGCGGT-CAGGGAATTGGATCATCTGGTTGTCACG-TATTGTGGAGGAGACACACAGATGCAACGC-TATTGGAACTAGTTCGTCTTGGACA-GAACTCTGGTGTCGGAGTATTGTCTCGA-TAGCTGGTAGTTCATCCATGCAGGCATAATT-CATGCGGGATATTGCTCACGCAAATGCAACTAGC-TATGCTTTGTCACCTTCGGCTTGGCTACAAATAC-CAATCAACGACCATGGATTTGGCTCATCA-CATCGT.

3.3 Activity analysis of *RUS1* promoter

According to the known sequence of *RUS1* promoter, the primers with enzyme-restricted site of *Pst*I and *Xba*I were designed. Taking Shilixiang genome DNA as template, PCR was performed. Single band was obtained, and the length was the same as *RUS1* promoter (Fig. 3). The target band was purified and cloned. The sequence result showed that it had 100% similarity to *RUS1* promoter. Digesting the result of pMD:*RUS1* promoter vector with *Xba*I and *Pst*I showed that two bands were obtained (Fig. 4). The length of 2.7-kb band was vector, while the length of about 0.7 kb band was *RUS1* promoter. The band of *RUS1* promoter was isolated and purified.

Modified plant expressing vector pCAMBIA1300 had 35S promoter with the length of 875 bp and β-glucuronidase (GUS) reporter gene. The digested product of modified pCAMBIA1300 with *Xba*I and *Pst*I enzyme and vector (containing β-glucuronidase reporter gene) was purified. The purified *RUS1* promoter and vector pCAMBIA1300 were mixed with ligation mixture. Ligation product was transformed to *E. coli* DH 5α. Digestion result of positive clone by *Xba* I and *Pst* I indicated that the *RUS1* promoter and pCAMBIA1300 vector were fused to construct the *RUS1*::GUS vector.

The *RUS1*::GUS vector was transformed to *Agrobacter-*

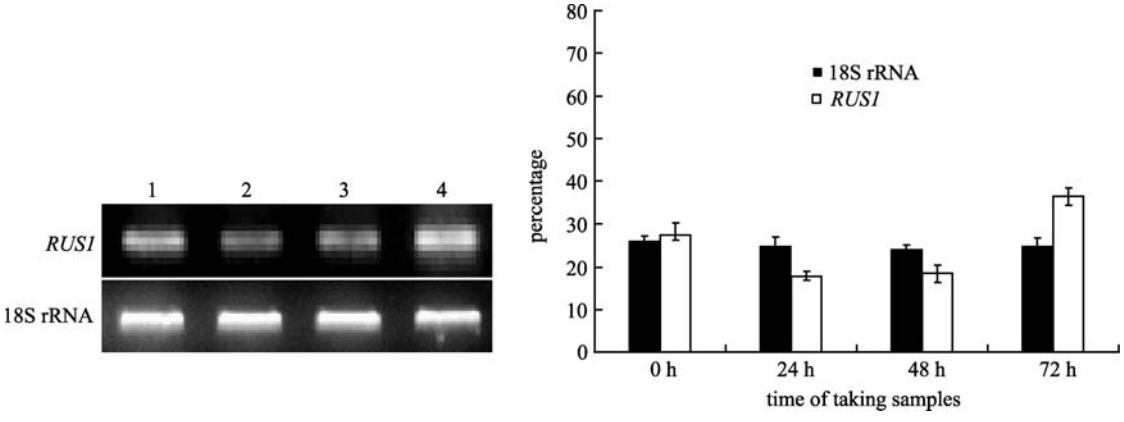

Fig. 1 Expression pattern of the *RUS1* gene after inoculation with *Uromyces setariae-italicae*
Note: 1–4 represent 0 h, 24 h, 48 h and 72 h after inoculating.

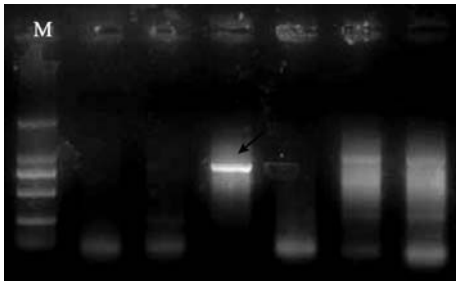

Fig. 2 5′ end Genome Walking amplification result of *RUS1*
Note: Arrowhead represents the target band and M represents DL2000.

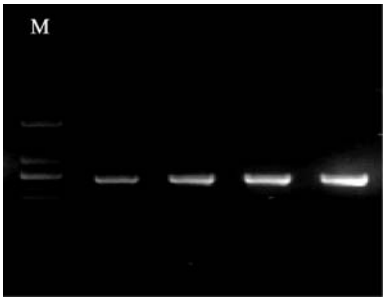

Fig. 3 Amplification results of *RUS1* promoter.
Note: M represents DL2000.

ium GV3101. The activity of *RUS1* promoter was analysed by the method of GUS histochemical staining. Results showed that the color of *Agrobacterium* GV3101 containing the *RUS1*::GUS vector was blue and was the same as that of agrobacterium GV3101 containing 35S promoter (positive control). The color of *Agrobacterium* GV3101 (negative control) was yellow (Fig. 5). The staining result indicated that the *RUS1* promoter could activate gene expression.

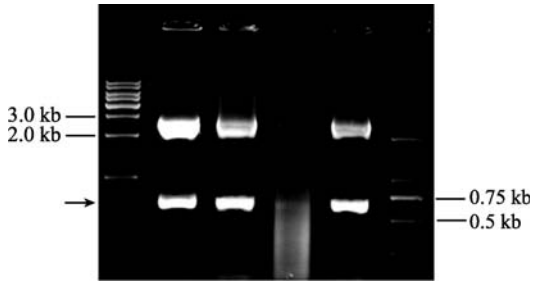

Fig. 4 Enzyme digestion result of pMD:*RUS1* positive clone (*Xba* I, *Pst* I).
Note: Arrowhead represents the target band.

3.4 Construction of plant expressing vector pCAMBIA1300:*RUS1*

Gene special primer with enzyme-restricted site of *Kpn*I and *Bam*HI was designed, and the product contained a promoter. Taking Shilixiang genome DNA as template, the length of 3.3-kb band was obtained through PCR amplification. The target band was purified and cloned. The sequence of 3.3-kb band was obtained with 100% similarity to *RUS1* gene. The PCR product and pCAMBIA1300 vector were digested by *Kpn*I and *Bam*HI. The purified PCR product and pCAMBIA1300 vector were mixed with ligation mixture and transformed. The digestion result of positive clone by *Kpn*I and *Bam*HI showed that *RUS1* and pCAMBIA1300 vector were fused to construct pCAMBIA1300:*RUS1* vector. pCAMBIA1300:*RUS1* vector was transformed to *Agrobacterium* LBA4404. Fig. 6 showed the enzyme digestion result of positive transformant.

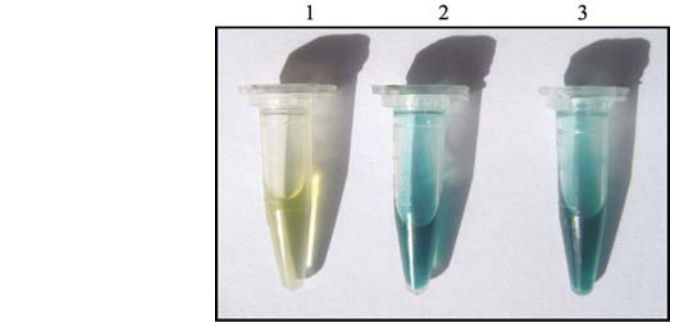

Fig. 5 GUS histochemical staining result
Note: 1 is negative control (*Agrobacterium* GV3101); 2 is positive control (*Agrobacterium* GV3101 containing 35S enhanced promoter); 3 is *Agrobacterium* GV3101 containing the *RUS1*::GUS vector.

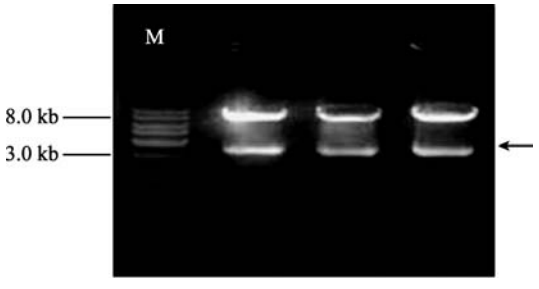

Fig. 6 Enzyme digests result of positive transformant (*Kpn*I, *Bam*HI)
Note: M is 1 kb DNA ladder; arrowhead represents the target band.

4 Discussion

Nucleotide binding site (NBS) is a conserved domain of some plant resistance genes. It has been demonstrated that it is possible to clone resistance gene analogs based on the conserved domain (He et al., 2001; Wang et al., 2003). We isolated *RUS1* gene of *Setaria italica* based on the conserved motifs in NBS-LRR domain-containing R-genes of plant. Sequence and phylogenetic analysis indicated that it had characteristic motifs of NBS-LRR and might belong to LZ-NBS-LRR-type disease resistance

gene. Disease resistance is the predominant function for plant NBS-LRR encoding genes, which has been so far demonstrated (Catherine et al., 2003; Craig et al., 2003; Li et al., 2003; Ching-I et al., 2007). To verify the resistance gene of *RUS1* related to millet leaf rust or disease-related gene, semi-quantitative RT-PCR was used in our experiment. Using housekeeping gene 18S rRNA as control, first-strand cDNA of samples was quantified, and then semi-quantitative RT-PCR was employed. Semi-quantitative RT-PCR analysis results showed that expression of *RUS1* was decreased at first and increased later. At 72 h, the expression was increased and was higher than that of control. According to the results, it was suggested that *RUS1* could be induced and had a relation to resistance response of Foxtail millet against *Uromyces setariae-italicae* infection.

The 35S promoter, derived from the common plant virus, cauliflower mosaic virus (CaMV), was a constitutive promoter and could activate gene expression in every tissue and every growth development stage. Over-expression of 35S enhance promoter infected the development of plant (Hull et al., 2002; Corderode et al., 2004). Future transgenic researches will require suitable promoters, which confer a tissue-specific gene expression, in which the time of activation and the degree of expression can be controlled (Song et al., 2000; Guan et al., 2002; Stangeland and Salehian, 2002; Filho et al., 2006). Therefore, obtaining special promoters of *RUS1* gene becomes more important for the analysis of gene function. In this paper, the length of 675-bp promoter sequence of *RUS1* was obtained, and the Plant-care software analysis result showed that it contained a TATA box and a CAAT box which were common characteristics of a promoter. To further identify the activity of *RUS1* promoter, the *RUS1* gene promoter and pCAMBIA1300 vector were fused to construct *RUS1*::GUS vector. Using the GUS histochemical assay, the activity of *RUS1* promoter was determined. Staining result showed that the *RUS1* promoter had an activity and could activate gene expression. The promoter sequence obtained in our experiment was a special promoter of *RUS1* gene.

Genetic transformation system based on the Ti plasmid of *Agrobacterium* has been reported in many plant species, such as freezing resistance lettuce, aphid resistance transgenic tobacco expressing *pta* gene, aphid resistance transgenic cotton with *aca* gene and brown planthopper and green leafhopper resistance *indica* rice (Pileggi et al., 2001; Nagadhara et al., 2003; Solís et al., 2003; Yao et al., 2003; Wu et al., 2006). Genetic transformation through *Agrobacterium* in foxtail millet has been established (Liu et al., 2007), providing a shortcut for gene function analysis of *RUS1*. On this basis, the *RUS1* gene (included promoter) and pCAMBIA1300 vector were fused to construct pCAMBIA1300:*RUS1* vector. The construction of plant expressing vector of *RUS1* gene was the foundation step for *RUS1* gene function analysis. To obtain more information about the function of *RUS1* gene, we will perform the genetic transformation through *Agrobacterium* with *RUS1* clone in resistance and susceptible materials to observe the changes of the responses to *Uromyces setariae-italicae* infection and the expression of *RUS1*, regulating the resistance path and so on.

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