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RESEARCH ARTICLE

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Cloning and structure analysis of an NBS-LRR disease-resistant gene from *Setaria italica* Beauv

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Abstract Degenerate PCR primers targeting conserved motifs of most NBS-LRR disease-resistant genes in plants were tested in *Setaria italica* Beauv. cultivar Shilixiang, which is resistant to *Uromyces setariae-italicae*. A sequence with a length of 2673 bp has been obtained by using Genomic Walking technology. The nucleotide sequence contained an open reading frame that encoded 891 amino acid residues with a calculated molecular mass of 101.44 kDa. It was named *RUS1* (Resistance against *Uromyces setariae-italicae*, GenBank No. FJ467296). It contained an NB-ARC domain and three conserved motifs P-loop, kinase 2, and kinase 3, which had the characteristics of NBS-LRR type resistant gene of plant. Phylogenetic analysis indicated that it was similar to *RPM1* and might belong to LZ-NBS-LRR type disease resistance gene. Southern blotting result displayed that there were at least three copies of *RUS1* in the foxtail millet genome.

Keywords *Setaria italica*, *RUS1*, NB-ARC domain

1 Introduction

To ward off infection, plants have evolved sophisticated mechanisms to recognize and guard against pathogens. In the infected leaf, these defenses include strengthening of cell walls, activation and/or synthesis of antimicrobial compounds, and expression of many defense-associated proteins, including the pathogenesis-related (PR) proteins. Disease resistance is frequently governed by specific recognition between pathogen AVIRULENCE genes and corresponding plant disease RESISTANCE (*R*) genes. This type of gene for gene interaction usually is accompanied by a hypersensitive response leading to the restriction of pathogen growth (Carine et al., 2008). Forty-eight *R*-genes have been cloned from numerous plant species (*Arabidopsis thaliana*, rice, wheat, etc.) using map-based cloning and transposon tagging (Li and Zhang, 2004). These *R*-genes conferred resistant to a wide range of plant pathogens including bacteria, fungi, oomycetes, and viruses (Dangl and Jones, 2001; Meyers et al., 2003; Deyoung and Innes, 2006; Tan et al., 2007).

Despite the wide range of recognized pathogen taxa, *R*-genes encode proteins that share significant sequence similarity and structural motifs, suggesting common protein-protein interactions as components of receptor systems and common roles in signaling events in plant defense responses. Degenerate primers targeting conserved motifs have been used to amplify resistance gene analogs (RGAs) from diverse plant taxa such as soybean, *Arabidopsis thaliana*, rice, wheat, tomato, etc. (Leister and Katagiri, 2000; Robert et al., 2008). Many RGAs are phylogenetically related to the known *R*-genes, and a number of studies have shown homologues mapping to *R*-gene loci, providing evidence that such genomic regions are likely coded for resistance.

Foxtail millet (*Setaria italica*) is a member of the grass family, Poaceae (Gramineae), which is grown for its edible starchy seeds (Kothari et al., 2005). It is widely taken as people's food in China, Japan, India, and African countries. Foxtail millet has a number of advantages that have made it the traditional staple cereal crop in subsistence or low-resource agriculture in semiarid regions. It is also an excellent forage crop, and because of its low hydrocyanic acid content, it is the best annual grazing crop in China. Some of the most threatening diseases of foxtail millet include leaf rust, downy mildew, ergot, and leaf spot (Budak et al., 2003). Foxtail millet leaf rust (*Uromyces setariae-italicae*) is one of the most serious constraints affecting its production in China. At present,

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the control of it primarily depends on the frequent applications of fungicides and host resistance. Frequent applications of fungicides not only pollute the environment but also harm human beings and other living things. The use of resistant varieties is an effective way to control the diseases. However, the development of disease-resistant varieties is costly and time-consuming when using the traditional breeding method. Molecular cloning of disease-resistant gene technology will speed up the process of plant breeding. In this paper, taking *Setaria italica* cultivar with high resistance to leaf rust as material, the resistance gene related to millet leaf rust was cloned by the method of resistance gene analogs. Our results provided a shortcut for the cloning of foxtail millet leaf rust resistance genes and the process of plant breeding.

2 Materials and methods

2.1 Plant materials and strain

Seeds of *Setaria italica* cultivar Shilixiang and Yugu No. 1 were germinated, sown in composite soil (peat compost to vermiculite, 3∶1), and then grown for 30 days after germination in greenhouse in plastic pots. All the millet seeds and the millet leaf rust isolates were kindly provided by the Institute of Millet Crops, Hebei Academy of Agriculture and Forestry Sciences, China.

2.2 Disease assay

Infection with *Uromyces setariae-italicae* isolate 93–5 was performed by spraying it onto 4-week-old plants. For mock 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 with a temperature range of 28–30°C and a photoperiodic regime of 12-hour light and 12-hour darkness.

2.3 DNA isolation

DNA extraction method of CTAB was appropriately modified based on the description by An et al. (2001). One gram of leaf tissue was ground under liquid nitrogen and suspended in a buffer containing 100 mmol·L^{-1} Tris-HCl (pH 8.0), 1.4 mmol·L^{-1} NaCl, 20 mmol·L^{-1} EDTA, 2% CTAB and 2% β-mercaptoethanol. After being incubated at 65°C for 30 min, the suspension was centrifuged at 12000 r·min^{-1} for 10 min at 4°C. The DNA was extracted with chloroform-isoamyl alcohol (24∶1) and precipitated with 0.45 mL ice-cold isopropanol. The pellet containing DNA was dissolved in TE buffer.

2.4 Degenerate primer design

Based upon conserved motifs in NBS-LRR domain-containing disease resistant genes, three degenerated primers were designed in this study. Sequences of primers were the following: NBS-1-L/R 5'-GGYATGGGNG-GYMTHGGNAARAC-3' and 5'-CCANACATCATCMA-GSACAA-3', NBS-2-L/R: 5'-GGNATGGGNGGNNTN-GGNAARACNAC-3' and 5'-NACYTTNAGNGC-NAGNGGNAGNCC-3', and NBS-3-L/R: 5'-GGTGGG-GTTGGGAAGACAACG-3' and 5'-CAACGCTAGTGG-CAATCC-3' (Code base: R = A/G, Y = C/T, M = A/C, S = G/C, H = A/T/C, N = A/T/G/C). Primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.

2.5 Cloning and sequencing of resistance gene analogs

Taking genomic DNA of foxtail millet as template, resistance gene analogs were cloned. PCR reactions were carried out using 20 ng·μL^{-1} genomic DNA, 10×PCR buffer, 2.5 mmol·L^{-1} of dNTPs, 10 μmol·L^{-1} of each degenerate primer, and 0.5 U of *Taq* DNA polymerase in a total volume of 25 μL. The PCR conditions for the amplifications included advance denaturation at 94°C for 7 min, followed by denaturation at 94°C for 30 s, annealing at 45–55°C for 30 s, elongation at 72°C for 40 s, and 35 cycles elongation at 72°C for 10 min in the end. The production of PCR was analyzed by 6% denatured polyacrylamide gel electrophoresis (PAGE). Process of silver-staining detection was identified to the description of Creste et al. (2001).

The differential fragments were cut using sterile scalpel and placed in a centrifugal tube. Thirty μL sterile ddH_2O was added to the centrifugal tube. Thawed and frozen for some times, the tube was then centrifuged for 10 min at the speed of 10000 r·min^{-1}. About 2 μL of the aliquot was used as template for reamplification in a total volume of 25 μL PCR reaction mix containing 10×PCR buffer, 2.5 mmol·L^{-1} dNTPs, primer NBS-L/R, and Taq Polymerase (Takara). PCR conditions were at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min for 30 cycles and with final extension for 10 min at 72°C. The PCR products were resolved on 1.2% TAE-agarose gel and purified from an agarose gel slice using the UNIQ-10 Gel Extraction Kit (Sangon). The purified PCR products were cloned into the plasmid pMD-19 simple vector (TaKaRa) following the manufacturer's instructions. M13 reverse primers were used to generate single pass partial sequences of the plasmid with the differential band inserts. The positive clones were sequenced using an automated sequencer (ABI PRISM 3730XL, Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.).

2.6 Obtained full length of resistance gene analogs by Genomic Walking

Approximately, 2.5 μg of genomic DNA was digested with 10 U each of the *Dra*I, *Pvu*II, *Eco*RV, and *Swa*I 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. The total reaction volume was 100 μL. Digestion was carried out in a water bath at 37°C for 16–18 h. From each reaction tube, 5 μL was removed and run on a 0.8% agarose/EtBr gel to determine whether digestion was complete. Genomic DNA was purified by phenol/chloroform method. Subsequently, purified genomic DNA was mixed with ligation mixture containing adapter, 2.5 U of T4 DNA ligase (TaKaRa), 10×T4 DNA ligase buffer (supplied with the enzyme). Ligation was carried out overnight at 16°C. PCR reactions were carried out using 1.4 μL of adaptor DNA, 10×PCR buffer, 10 μmol·L^{-1} of dNTPs, 10 μmol·L^{-1} of each gene-specific primer and universal primer, and 0.5 U of *Taq* DNA polymerase in a total volume of 25 μL. PCR was carried out in TGRADIENT hermocycles (Biometra). The PCR conditions for the amplifications included 7 cycles at 94°C for 25 s and then at 72°C for 4 min, which is followed by 32 cycles at 94°C for 25 s and 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% TAE-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.

2.7 Sequence and phylogenetic analysis

Sequences were processed to remove vector and identified on the basis of sequence similarity using the program BLAST. Phylogenetic analysis was done using DNAMAN software.

2.8 Electrophoresis and Southern blotting

Genomic DNA was digested with restriction enzymes cut at a single site within the probe-specific sequence (*Hin*dIII) and with restriction enzymes that were not cut within the probe-specific sequence (*Pst*I, *Xba*I, *Eco*RV). The digested DNA was electrophoresed on a 0.8% TAE agarose gel and transferred to a nylon membrane (Total BLOT + TM NYLON MEMBERANES, and AMRESCO). Hybridization, washes, and hybrid detection were done according to the instructions provided with DIG DNA labeling and detection kit (Roche).

3 Results

3.1 Disease assay

After being inoculated with *Uromyces setariae-italicae* virulent isolate 93–5, *Setaria italica* cultivars Shilixiang, and Yugu No.1 displayed different symptoms. Shilixiang displayed a resistance symptom and no uredospores were obtained on the inoculated leaf. Infected with *Uromyces setariae-italicae* virulent isolate, uredospores were obtained on the inoculated leaf of Yugu No. 1. It was susceptible to *Uromyces setariae-italicae*. The reproduction of uredospores was increased gradually as time prolonged. Until 10 days after inoculation, the leaves were covered with a lot of uredospores (Fig. 1).

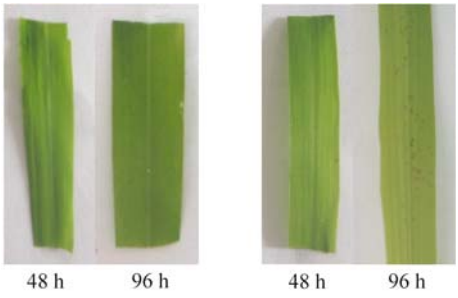

Fig. 1 Symptom of *Setaria italica* cultivar inoculated with *Uromyces setariae-italicae* virulent isolate 93–5
Note: Left and right represent Shilixiang and Yugu No. 1, respectively.

3.2 Obtaining of resistance gene analogs

Taking genomic DNA of *Setaria italica* cultivars Shilixiang and Yugu No. 1 as template, the amplification was done using degenerate primers combination, respectively. The product was analyzed by 6% denatured polyacrylamide gel electrophoresis (PAGE). A special fragment was generated from *Setaria italica* cultivar Shilixiang (Fig. 2). After thawing and freezing for some times, the differential fragment was obtained. The product of reamplification was cloned and sequenced. It showed a significant similarity to the known NB-ARC domain contained in related disease resistance proteins of *Oryza sativa* and of *Triticum aestivum*, based upon the search result using the BLASTX program. This sequence was obtained using primer combination NBS-1-L/R. Its length was 309 bp. The

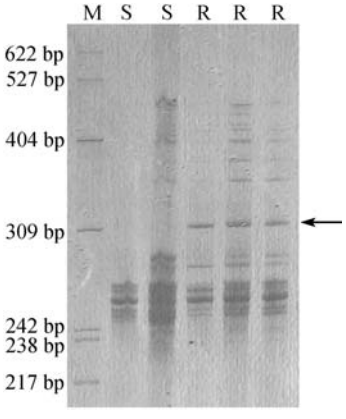

Fig. 2 PCR amplification result of disease resistant gene of *Setaria italica*
Note: Arrowhead indicates the specific band presented in Shilixiang; M represents pBR322.

PCR product obtained from primers combination NBS-2-L/R and NBS-3-L/R were also analyzed by 6% PAGE. No special fragment was generated from *Setaria italica* cultivar Shilixiang.

3.3 Obtaining of full length of resistance gene analogs by Genome Walking

According to the known sequence, 5' end and 3' end of gene special primers were designed using Primer 5.0 software. The amplification product was obtained using a gene-specific primer and universal primer combination. PCR products were analyzed on 1.2% TAE-agarose gel (Fig. 3). Thereafter, the PCR products were purified and sequenced. Through spliced between the known sequence and 5' end, 3' end sequence, a sequence with a length of 2673 bp was obtained. The nucleotide sequence contained an open reading frame that encoded 891 amino acid residues with a calculated molecular mass of 101.44 kDa. It was named *RUS1* (Resistance against *Uromyces setariae-italicae*) gene of *Setaria italica* (GenBank No. FJ467296).

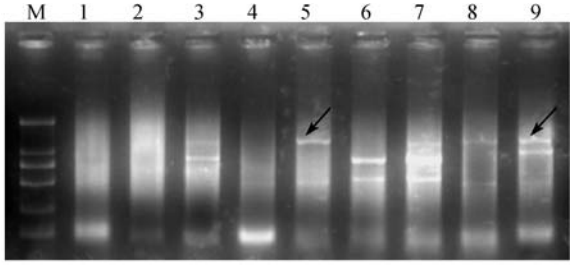

Fig. 3 Amplification result of Genome Walking
Note: M represents DL2000, Lanes 1–5 represent PCR product of 3' end, Lanes 6–9 represent PCR product of 5' end, and arrowhead indicates the specific band.

3.4 Sequence and phylogenetic analysis

Using DNAMAN software, alignment between *RUS1* and the known plant resistance disease gene that contained NB-ARC domains such as *I2*, *L6*, *RPS2*, *RPP13*, *RPP8*, etc. was done. Align results displayed that, like for a known plant disease-resistant gene, *RUS1* contained three conserved motifs including P-loop, kinase 2, and kinase 3 (Fig. 4). These motifs had the characteristics of NBS-LRR type resistance gene of plants.

BLASTP results indicated that *RUS1* contained a conserved NB-ARC domain, which was a novel signaling motif shared by plant resistance gene products (Fig. 5). It had 56%–64% homology to proteins containing the NB-ARC domain of *Oryza sativa*, NBS-LRR disease resistance protein homologue of *Hordeum vulgare*, and disease resistance protein of *Triticum aestivum*.

Using DNAMAN software, phylogenetic analysis was done between *RUS1* and the known NBS–LRR type resistance disease of plant such as *Prf*, *RPS2*, *RPS4*, *RPP1*, *RPP5*, and *RPP8*, etc. Results indicated that *RUS1* might belong to LZ-NBS-LRR type disease resistant gene and had the most similarity to *RPM1* (Fig. 6).

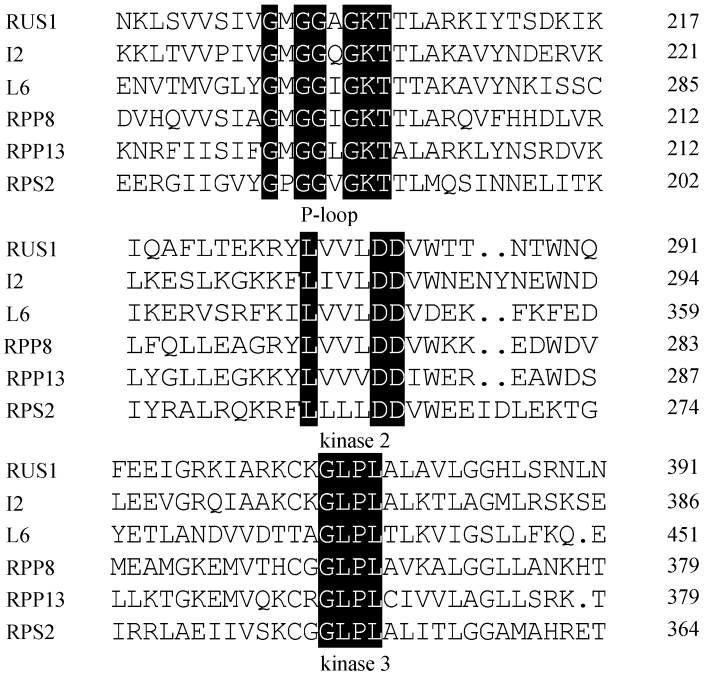

Fig. 4 Alignment of deduced amino acid sequences of *RUS1* with NB-ARC domains of five known *R*-gene

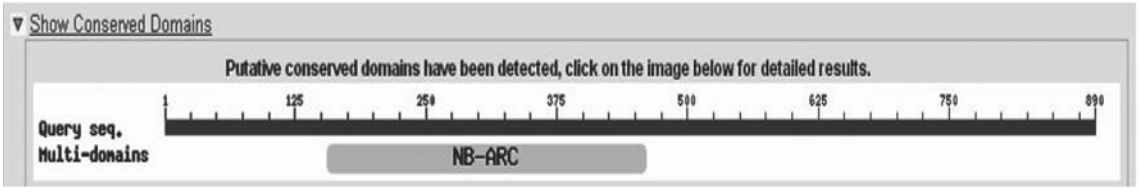

Fig. 5 Conserved domain of *RUS1* gene of *Setaria italica*

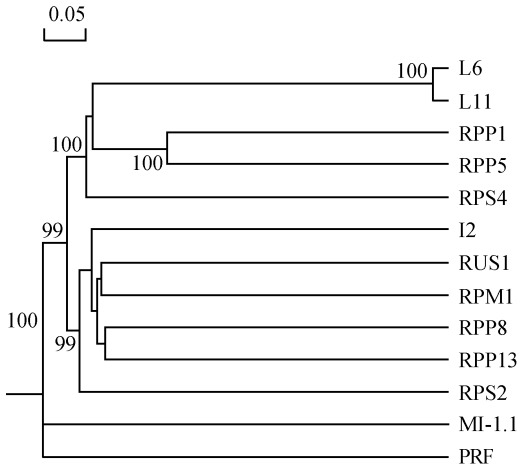

Fig. 6 Phylogenetic tree based on alignment of the deduced amino-acid sequences of *RUS1* and the NBS domains of known *R*-genes

3.5 Electrophoresis and Southern blotting

Southern blotting analysis was performed to determine the copy number of *RUS1* in the genome of Shilixiang. Treatment with enzymes that were not cut within the sequence consistent with the probe produced two bands (Fig. 7, Lanes 1, 2, and 4), and three bands were observed after the treatment with enzymes cut at a single position within the probe sequence (Fig. 7, Lane 3). These results indicated that there were at least three copies of *RUS1* genes in the genome.

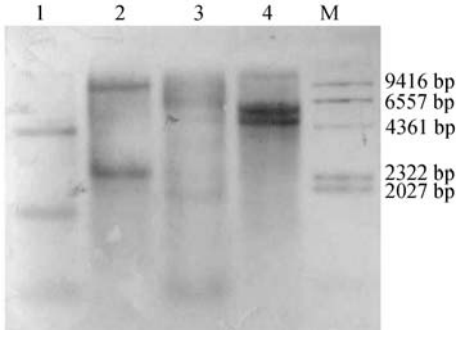

Fig. 7 Copy number analysis of *RUS1* in genome of *Setaria italica*

4 Discussion

Plants defend themselves against pathogen invasion through the action of specific *R*-genes and various nonspecific host responses. Genetic studies have established that dominant *R*-genes generally function as a gene-for-gene manner, whereby resistance is afforded based on an interaction between the *R*-gene encoded protein and the cognate pathogen elicitor, commonly referred to as an avirulence (Avr) factor or effector protein (Seo et al., 2006). To date, five principal classes of *R*-genes have been identified, based upon conserved protein domains. The most abundant class is the cytoplasmic nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins. The NBS-LRR R proteins contain distinct domains, several of which are composed of characteristic motifs. Conserved amino acid motifs existed in NBS-LRR R proteins included the phosphate binding loop or 'P-loop' (also called kinase 1), kinase 2, GLPL (also called kinase 3), and hydrophobic structure (Robert et al., 2008). In our research, *RUS1* gene of *Setaria italica* was obtained using degenerate primers based conserved motifs in NBS-LRR domain-containing *R*-genes of plant. Characteristic motifs of NBS-LRR R proteins such as phosphate binding, kinase 2 and GLPL existed in *RUS1*. The final amino acid within the kinase 2 motif could commonly reveal differences between TIR and non-TIR types, with an aspartic acid (D) residue in TIRs and a tryptophan (W) in non-TIRs. Non-TIR NBS subfamily R proteins could contain an N-terminal coiled-coil (CC) motif, a subset of which coded for a leucine zipper sequence (LZ). *RUS1* with a tryptophan (W) existing in the kinase 2 motif belonged to the non-TIR NBS subfamily R proteins. Phylogenetic analysis result indicated that *RUS1* had the most similarity to *RPM1*, which encodes an LZ-NBS-LRR protein and confers the resistance to the bacterial pathogen *Pseudomonas syringae* expressing either of two sequence-unrelated *avr* genes, *avrRpm1*, or *avrB* (Douglas et al., 1998).

In plant R proteins, the NBS region is a conserved domain that is responsible for the binding and the hydrolysis of ATP and GTP (Tameling et al., 2002). LRRs are typically involved in protein-protein interactions, and various studies indicate that the LRR motif is at least partly responsible for recognition specificity (Leister and Katagiri, 2000). Between the NBS and LRR domains, the ARC domain has recently been identified to play a role

in the recruitment of the LRR domain to the N-terminal region and in the molecule inactive/active statement (Rairdan and Moffett, 2006). Disease resistance is the predominant function so far demonstrated for plant NBS-LRR encoding genes. *Arabidopsis* genome is estimated to have 200 genes coding for NBS-LRR proteins and confer resistance against a wide variety of pathogens and pests, including viruses, bacteria, fungi, oomycetes, nematodes, and insects (Yi et al., 2000). *Lr10* (resistant to leaf rust) encodes a CC-NBS-LRR type of protein with an N-terminal domain, which is under diversifying selection. When overexpressed in transgenic wheat plants, *Lr10* confers the enhanced resistance to leaf rust (Catherine et al., 2003). *Lr21* is a potentially durable and highly effective leaf rust resistance gene in wheat and belongs to the NBS-LRR group (Li et al., 2003). In flax (*Linum usitatissimum*), at least 31 rust resistance specificities to different flax rust (*Melampsora lini*) strains have been identified, and they are distributed among five polymorphic loci, K, L, M, N, and PR proteins encoded by the genes at the L, M, N, and P loci are members of the intracellular NBS-LRR class (Ching-I et al., 2007). The *rp1* (resistant against *Puccinia sorghi*) complex is the best characterized resistance gene family from maize. The genes in the *rp1* complex belong to the most common class of resistance genes that code for nucleotide binding site-leucine-rich repeat (NBS-LRR) proteins (Craig et al., 2003). *RUS1* gene of *Setaria italica* can be obtained from cultivar Shilixiang, with some resistance symptoms after being inoculated with *Uromyces setariae-italicae* virulent isolate 93–5. Analysis results indicated that *RUS1* gene may encode an LZ-NBS-LRR protein and confer its resistance to the pathogen *Uromyces setariae-italicae*. While, the function of *RUS1* gene in the anti–infection process of *Setaria italica* against *Uromyces setariae-italicae* needs to be further identified by the methods of real-time PCR, Northern blotting, gene knockout, etc.

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