



Complementary DNA (cDNA) cloning and functional verification of resistance to head smut disease (*Sphacelotheca reiliana*) of an NBS–LRR gene *ZmNL* in maize (*Zea mays*)

Hong Di  · Tao Yu · Yanxue Deng · Xiaojie Dong · Rou Li · Yu Zhou · Zhenhua Wang 

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Abstract The nucleotide-binding site (NBS)-leucine-rich repeat (LRR) gene family comprises the largest number of known disease resistance (*R*) genes and is one of the largest gene families in plants. In the present study, the full-length cDNA of *ZmNL* (GenBank Accession Number KF765443) was isolated using Rapid Amplification of cDNA Ends. The nucleotide sequence of *ZmNL* contains an open reading frame of 3156 bp that encodes the *ZmNL* protein, which is comprised of 1051 amino acid residues. This putative protein has high homology to other known resistance proteins (84% to *Triticum aestivum* *LR10*) and belongs to the CC–NBS–LRR type *R* gene family. The *ZmNL* gene was introduced into

the maize inbred line of Huangzao4 which was highly susceptible to head smut under the control of the maize ubiquitin promoter by *Agrobacterium*-mediated transformation. The head smut disease incidence of 3 T₂ transgenic lines was significantly reduced (by 18.38–29.40%) compared with the wild type, which indicated that the overexpression of *ZmNL* gene in maize enhanced the resistance to the fungus *Sporisorium reilianum* (Kühn) Clint of these plants.

Keywords *ZmNL* gene · Transgenic maize · Resistance to head smut disease · (NBS)-leucine-rich repeat (LRR) gene family

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Hong Di and Tao Yu contributed equally to this research.

H. Di · Y. Deng · X. Dong · R. Li · Y. Zhou · Z. Wang (✉)
Northeast Agricultural University,
Harbin 150030, Heilongjiang Province, China
e-mail: zhenhuawang_2006@163.com

T. Yu
The Postdoctoral Workstation of Heilongjiang
Academy of Agricultural Sciences, Maize Institute of
Heilongjiang Academy of Agricultural Sciences,
Harbin 150086, Heilongjiang Province, China

Introduction

Fewer disease resistance (*R*) genes have been reported in maize (*Zea mays*) than in other grains, such as rice (*Oryza sativa*) and common wheat (*Triticum aestivum*). Maize *R* genes that have been studied include *Hm1*, which confers *Cochliobolus carbonum* race 1 resistance; *Rp1-D*, which confers common rust (*Puccinia sorghi*) resistance; and *Rxo1*, which controls resistance to *Burkholderia andropogonis* (Johal and Briggs 1992; Collins et al. 1999; Zhao et al. 2005). Therefore, the isolation and study of *R* genes from maize is important in terms of understanding the resistance mechanisms against these diseases and

facilitate methods for development of disease-resistant maize varieties.

R genes in plant encode receptors that specifically recognize pathogen avirulence (*avr*) gene products (Lamb et al. 1989), in a manner consistent with Flor's gene-for-gene hypothesis (Flor 1942). The interaction of proteins encoded by *R* and *avr* genes has been suggested to trigger a series of signaling cascades including influxes of ions, increases in oxidative activity that generate reactive oxygen intermediates, production of nitric oxide, activation of the hypersensitive response (HR; programmed cell death at infection sites that inhibits growth of pathogens), and production of toxic antimicrobial substances, all of which result in disease resistance (Dangl and Jones 2001). Most *R* genes encode proteins containing a nucleotide-binding site (NBS) domain and C-terminal leucine-rich repeats (LRR) (Dangl and Jones 2001) that mediate plant defenses against fungal, bacterial, viral, and nematode pathogens (Hammond-Kosack and Jones 1997; Ellis and Jones 1998; Meyers et al. 1999; Williamson 1999). Binding of ATP and GTP by the NBS domain helps to transduce signals during disease resistance responses (Walker et al. 1982; Saraste et al. 1990; Tameling et al. 2006). The C-terminal LRR domains, which are often present in proteins that perceive signals, mediate interactions between proteins or between peptides and their ligands (Dangl and Jones 2001; Holt et al. 2002). The LRR domain specifically recognizes pathogens that express a corresponding *avr* gene, binds the NBS domain, and keeps the *R* protein in an inactive state (Ellis et al. 2000; Tameling et al. 2002).

Since the *R* gene *Hm1* was cloned (Johal and Briggs 1992), more than 70 other *R* genes have been cloned from numerous monocot and dicot crops and model plant species such as *Arabidopsis*, *Zea mays*, *Sorghum bicolor*, *Solanum tuberosum*, *Oryza sativa*, etc. (Wei et al. 2013). Most of plant *R* genes have been cloned using positional information from linkage studies or by tagging with transposons (Collins et al. 1999; Zhou et al. 2006; Lee et al. 2009). In the first approach, a high-resolution linkage map of the area around the gene is used to isolate the *R* genes for which there is only phenotypic and map location information. In the second method, the target of *R* gene is inactivated by insertion of a transposon into its coding or regulatory sequences. However, few transposons are suitable for use in tagging, and the likelihood that

they will insert within a target *R* gene is very low (Brutnell 2002). Thus, for the above reasons and due to the complexity of the maize genome, progress in map- and transposon-based cloning of *R* genes has been relatively difficult and slow. One solution is to use degenerate primers to target and amplify the sequences encoding conserved NBS domains of NBS-containing maize *R* genes. This method has been used to clone the NBS-containing *R* genes from soybean (*Glycine max*) (Kanazin et al. 1996; Yu et al. 1996; Penuela et al. 2002), potato (*Solanum tuberosum*) (Leister et al. 1996), lettuce (*Lactuca* sp.) (Shen et al. 1998), bean (*Vicia* sp.) (Creusot et al. 1999; Rivkin et al. 1999), the *Arabidopsis* (Aarts et al. 1998), maize (Collins et al. 1998), rice (*Oryza sativa*) (Yuan et al. 2011), barley (*Hordeum vulgare*) (Leister et al. 1998), and other angiosperms. The present study builds on earlier work in which we identified a putative NBS-LRR gene that was expressed differentially in different maize lines under artificial inoculation of the fungus *Sporisorium reilianum* (Kühn) Clint.

In the present study, we cloned the full-length cDNA of this NBS-LRR gene, the *ZmNL*, from maize using 5'-RACE and 3'-RACE, analyzed its expression profile by qRT-PCR in maize after infection with the *Sphacelotheca reiliana*. We used established the *Agrobacterium*-mediated methods to introduce the *ZmNL* gene into the maize inbred line and subsequently evaluated the *Sphacelotheca reiliana* incidence of the transgenic plants. Here, we aimed to understand the function of the *ZmNL* gene in the resistance to the head smut.

Materials and methods

Plant materials

The elite Chinese inbred maize (*Zea mays*) line (Huangzao4) is highly susceptible to head smut with about 75% of plants showing susceptibility in the field (Chen et al. 2008; Weng et al. 2012). In addition to this, the line of L282 was developed from a cross between the (resistant) line Qi319 and the Huangzao4 and subsequent backcrosses with the Huangzao4. Evaluations of resistance for the head smut disease under artificial inoculation in the field were revealed that the L282 was highly resistant to head smut with disease incidence of 0% (Di et al. 2015a).

Extraction of genomic DNA and amplification of genomic NBS fragments

Genomic DNA was extracted from leaves of the L282 using the DNeasy Kit (Qiagen, CA, USA) according to the manufacturer's instructions. DNA quality was assessed by electrophoresis in 1.0% agarose gel (Biowest, Madrid, Spain) in $1 \times$ TAE buffer at 120 V for 30 min, and concentrations were determined by measuring absorbance at 260 nm using an ultraviolet (UV) spectrophotometer.

A pair of degenerate primers, mZmNL-F and mZmNL-RP1, targeting the conserved P-loop and GLPL domains, respectively, was designed to amplify an internal conserved fragment of the NBS domain from the line L282 (See Table 1). PCR reactions were performed in 25 μ L containing 2.5 μ L of $10 \times$ PCR Buffer (Mg²⁺ Plus), 2 μ L of dNTPs (2.5 mM each), 1 μ L of each primer (10 μ M), 17.5 μ L of sterile ddH₂O, 0.5 μ L (5 U/ μ L) of LA Taq DNA polymerase (TaKaRa, Dalian, China), and 0.2 μ g of DNA template. Temperature cycling conditions were: 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 60 s; followed by 72 °C for 10 min. PCR products were then separated by size on a 1.0% agarose gel in $1 \times$ TAE buffer and the appropriately sized band was recovered using a Quick Gel Extraction Kit (TransGen, Beijing, China) and cloned into the pEasy-T1Simple vector (TransGen). Recombinant clones were isolated, and then sequenced at Sheng-gong Biotechnology Company (Shanghai, China).

Cloning of the full-length of *ZmNL* cDNA

Total RNA was isolated from leaves of the L282 with a Plant RNA Kit (TransGen) following the manufacturer's procedure and treated with DNase I (Omega, GA, USA) to remove residual DNA. The first-strand of cDNA was synthesized from 1 μ g of total RNA using 1 μ L (200 U) PrimeScriptTM RT Enzyme Mix I (TaKaRa) according to the manufacturer's protocol. To clone the full-length of cDNA of *ZmNL* from maize, 5' RACE and 3' RACE were carried out using the SMARTTM RACE Amplification Kit (Clontech, Palo Alto, USA) with 5' RACE and 3' RACE gene-specific primers (GSP) (See Table 1). RACE reactions were set up in volumes of 50 μ L, as recommended by the manufacturer. The temperature cycling conditions were: 5 cycles of 94 °C for 30 s and 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; followed by 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. All PCR products were cloned into the pEasy-T1Simple vector (TransGen) and sequenced by Sheng-gong Biotechnology Company (Shanghai, China). The sequence of this *ZmNL* cDNA clone has been submitted to GenBank (GenBank Accession No. KF765443).

Sequence analysis

The sequence analysis as below was conducted in 2012. Analysis of the *ZmNL* cDNA ORF was performed using ORFfinder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The amino acid sequence of the putative ZmNL protein was deduced using the

Table 1 Primer sequences of *ZmNL* for gene cloning

Primers	Sequences (5'→3')	Amplified region	Amplification length (bp)
m <i>ZmNL</i> -F	AAGTGGACTGGGATGTAAG	Partial region	678
m <i>ZmNL</i> -R	TTCGCAGACTAAGGTATTT	Partial region	
5' RACE GSP-1	GGAAGCCCGTGAAGTCATCATCAATAGC	5'RACE	2009
5' RACE GSP-2	TTCACCCTGCCGTTGCTGCTGTTGT	5'RACE	
3' RACE GSP-1	CCCACGTTTCTCAGAAGCCTTATCA	3'RACE	2020
3' RACE GSP-2	CCTGCCTACTCTACCTGAGCATGTT	3'RACE	
p <i>ZmNL</i> -F	CGGTTTCGAAATGGACCTCGTGGTTCGGCG	<i>ZmNL</i> clone	3156
p <i>ZmNL</i> -R	ATAGCGCCGCTTATAGGTCGTTGTACT	<i>ZmNL</i> clone	
q <i>Actin</i> -F	GTTGGGCGTCCTCGTCA	<i>Actin1</i> gene	250
q <i>Actin</i> -R	TGGGTCATCTTCTCCCTGTT	<i>Actin1</i> gene	

Expert Protein Analysis System (<http://www.expasy.org/>). Determinations of the amino acid composition and the secondary and tertiary structure of the ZmNL protein were carried out using DNA tool 6.0, SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html), and Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre/>). Conserved motifs and signal sequences of the ZmNL protein were predicted at the Conserved Domain Search Service (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/>), and SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). A phylogenetic tree of the aligned protein sequences was constructed using the neighbor-joining (NJ) method in MEGA 5.0 (<http://www.megasoftware.net/>) with the bootstrap value set to 1000.

Artificial inoculation of plants with the *S. reilianum*

The compatible of *S. reilianum* mating-type strains SRZ1 and SRZ2 (Schirawski et al. 2005) were inoculated into 2 mL potato dextrose broth (2.4%) (PDB, Difco, Detroit, USA) and incubated with shaking at 200 rpm at 28 °C for 8–12 h. Each culture was then used to inoculate 50 mL of PDB (Difco, Detroit, USA) and allowed to grow overnight to an optical density (OD) of 0.5–1.0 at 600 nm. The cultures were then centrifuged at 3500 rpm for 5 min. Cell pellets from each culture were then re-suspended in water to an OD of 2.0 at 600 nm. The SRZ1 and SRZ2 suspensions were then mixed and used as inoculum for 7-days-old maize seedlings (Gillissen et al. 1992). Similar inoculation was performed with deionized water as a control. At 7 days after inoculation (DAI), infection of maize leaves by the *S. reilianum* was observed by light microscopy, scanning electron microscopy, and transmission electron microscopy, as described by Martinez et al. (1999).

Inducible expression analysis by real-time quantitative PCR

The expression of the *ZmNL* gene was assayed by quantitative real-time PCR (qRT-PCR) on a Chromo4™ Real-Time Detector (Bio-Rad, CA, USA) using the TransStart Top Green qPCR SuperMix (TransGen). Three biological replicate leaf samples were collected for qPCR. Total RNA was extracted

from infected leaves collected from the L282 and Huangzao4 at 0, 1, 2, 3, 4, 5, or 6 DAI using the Plant RNA Kit (TransGen) according to the manufacturer's instructions. RNA quality was assessed by electrophoresis on 1.0% agarose (Biowest, Madrid, Spain) gels in 1 × TAE buffer at 120 V for 15 min, and concentrations were determined by measuring absorbance at 260 nm in a UV spectrophotometer. First-strand cDNA was synthesized using the Prime-Script™ RT Enzyme Mix I (TaKaRa) for RT-PCR. The primers used in these PCR reactions are shown in Table 1. The qRT-PCR template consisted of 1 µL (100 ng) of cDNA in reactions containing 10 µL 2 × SYBR Green PCR Master Mix, 0.5 µL (10 µM) each of forward and reverse primer, and 8 µL water. The thermal cycling conditions were 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 12 s, and 72 °C for 18 s. The maize *Actin1* gene (Gene ID: 100282267) was amplified with two primers (See Table 1) as an internal control.

Binary plant expression vector construction and maize transformation

The whole length cDNA of *ZmNL* was subcloned into the plasmid pTF101-Ubi-Nos which contained a maize constitutive ubiquitin promoter, the *Bar* gene driven by the CaMV 35S promoter and a *Nos* terminator according to the standard protocol using the In-Fusion Cloning Kit [Takara Biomedical Technology (Beijing) Co., Ltd]. The freeze–thaw technique method was used to introduce the recombinated plasmid pTU-ZmNL into the *Agrobacterium tumefaciens* strain EHA101 (Höfgen and Willmitzer 1988).

Seeds of maize inbred line Huangzao4, which was used as the genetic materials in this study, were surface sterilized and were incubated on autoclaved solid MS medium at 28 °C in darkness until the etiolated seedlings had grown to 3.0–4.0 cm in length. After immersed in the suspension of transformed *Agrobacterium tumefaciens* (OD₆₀₀ = 0.8) for 10 min under 0.05 MPa pressure, maize shoot tips with exposed meristems were co-cultivated on solid MS medium in the dark at 24 °C for 3 days. Then the plantlets were transplanted into pots and cultivated in the greenhouse. The herbicide Basta with concentration of 0.1% was sprayed on the seedlings (Li et al. 2008). Then the seeds of survived seedlings and PCR-positive plants, which were assayed for the presence of the transgene by PCR,

were harvested. The T₂ generation seedlings were confirmed the integration and stability of the *ZmNL* gene with RT-PCR, Southern blotting, and RT-PCR (Di et al. 2015b). These experiments were conducted in the Molecular Breeding Experimental Base of Northeast Agricultural University located in Harbin City, Heilongjiang Province of China. A special room was used to store the plants. The related law of China were abided to burn the dedicated spot when the experiments were over.

PCR, RT-PCR and southern blotting analysis

The CTAB procedure was used to extract Genomic DNA of maize plants (Murray and Thompson 1980). PCR assays were performed with specific primers NF2 (5'- CGCGAATTTGGGAATGAC -3') and NR2 (5'- CGACGGAAGAGTTTGTAGGAG -3'). Due to the *ZmNL* gene was an endogenous gene of maize, the primer designed by the sequences of *ZmNL* gene and *Nos* terminator. The *Bar* gene was assayed by the

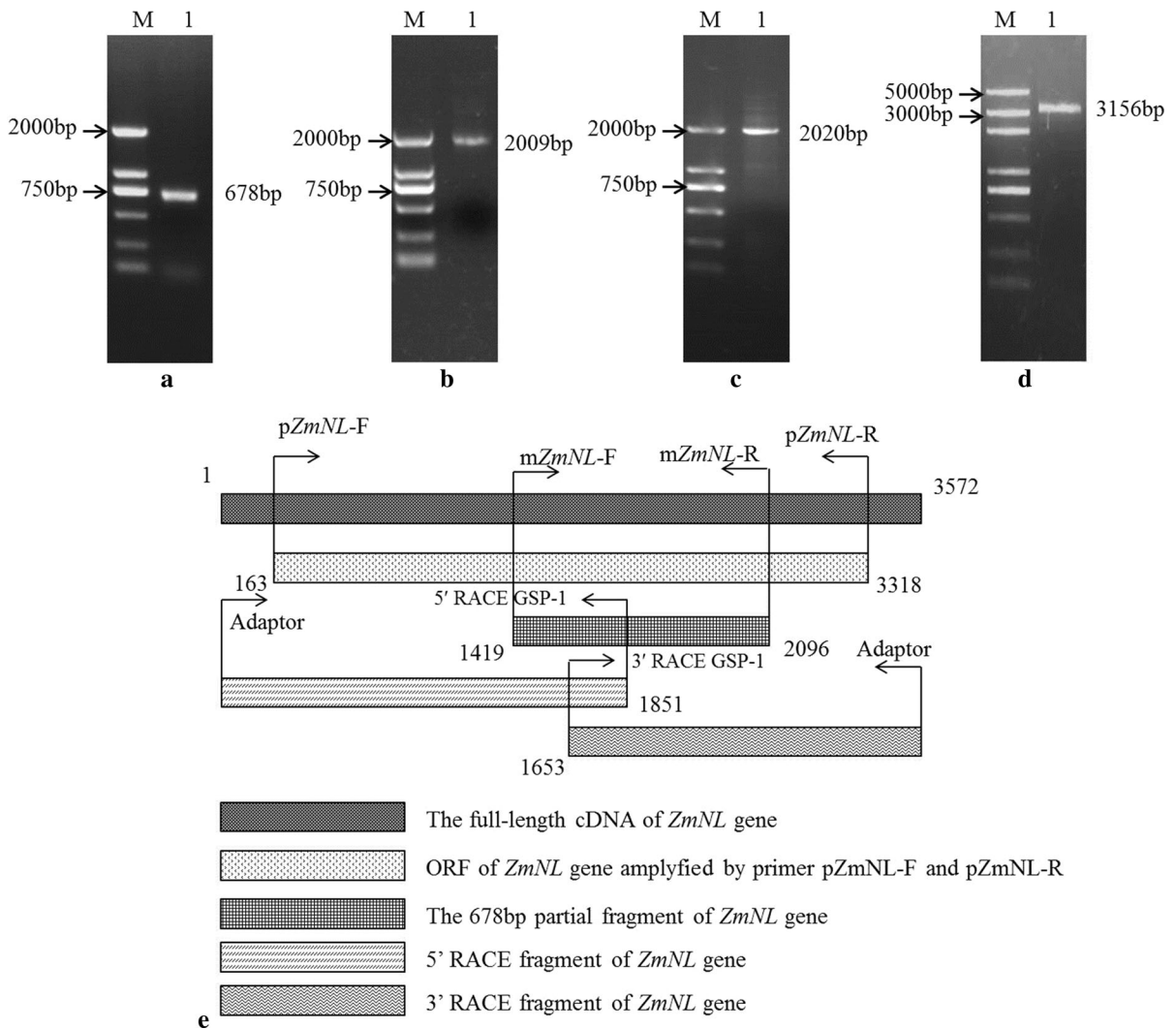


Fig. 1 Cloning of the full length cDNA of *ZmNL* gene from maize line L282. M, DL2000 DNA marker; Lane 1, *ZmNL* cDNA fragment; **a** The *ZmNL* cDNA was amplified from maize line L282 by a pair of primers mZmNL-F and mZmNL-R designed according to coding sequence of *ZmNL*. **b** 5'-end of *ZmNL* cDNA obtained by 5'RACE GSP-1 and 5' RACE GSP-2.

c 3'-end of *ZmNL* cDNA obtained by 3'RACE GSP-1 and 3' RACE GSP-2. **d** The *ZmNL* clone obtained by RT-PCR with a pair of gene specific primers pZmNL-F and pZmNL-R designed according to ORF sequence. **e** Diagram of overlapping relationship of subclone fragments and positions of corresponding primers

specific primers BF1 (5'-CCATCGTCAACCACTA-CATCG -3') and BR1 (5'- AGCTGCCAGAAACC-CACGT -3'). Total RNAs were extracted by the Trizol reagent (Tiangen, Beijing, China), and the RNA were reverse transcribed using the RT Reagent Kit (Transgene, Beijing, China). The gene-specific primers same as PCR assay from sequences of the conserved region

of the *Bar* gene were used to amplify a 439 bp transcript. The adigoxigenin (DIG)-labelled probe consisting of the *Bar* gene fragment was used as template of Southern blotting, as described in the DIG System Manual (Roche, Inc., Basel, Switzerland). The *EcoR* V and *Hind* III were taken to digest the Genomic DNA from each of the PCR-positive maize plants, the

Table 2 Amino acid compositions of *ZmNL*

Types	Percentage (%)	Name	Quantity (%)		
Hydrophobic amino acids	44.90	Ala	7.20		
		Ile	4.70		
		Leu	12.70		
		Met	1.90		
		Phe	2.80		
		Pro	3.50		
		Asn	3.80		
		Val	7.30		
		Trp	1.00		
		Hydrophilic amino acids	39.50	Asp	5.90
Cys	1.90				
Glu	6.00				
Gly	6.10				
Ser	8.40				
Thr	5.30				
Tyr	1.70				
Gln	4.20				
Basic amino acids	15.60			Arg	5.80
				His	2.90
		Lys	6.90		
Acidic amino acids	11.80	Asp	5.70		
		Glu	5.90		

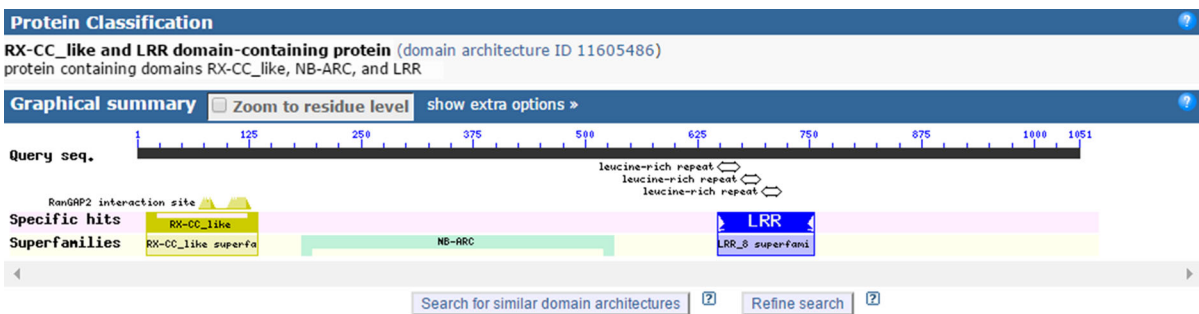


Fig. 2 Conserved domains of the *ZmNL* protein predicted by CD-Search. The light and dark gray rectangles indicate the major domains predicted by CD-Search, including an RX-CC-

like domain (from Ala11 to Glu134), a P-loop_{NTPase} superfamily (Asp207-305 Ile), an NB-ARC domain (from Ile183 to Gly531), and an LRR domain (from Leu647 to Gly755)

restriction enzymes that cut a single site in the T-DNA and cut no sites within the *Bar* sequence. Digested genomic DNA was electrophoresis on 0.8% agarose gels and transfer onto a nylon membrane with positively charged (Roche, USA). After drying at 80 °C for 2 h, as described in the DIG System Manual, the membrane was hybridized to digoxigenin (DIG)-labelled probe.

Evaluation of resistance against head smut disease in the field

The randomized complete blocks with three replicates was used to evaluate the resistance to the *S. reiliana* of both plant materials in disease ‘hot spot’, Harbin, Heilongjiang Province, China (45.8°N, 126.5°E) during April to September in 2016. Each plot consisted of two 5 m rows with 0.67 m between rows, which were over-planted and later thinned to 25 plants per row. The inoculum was collected from the previous crop season and stored in cloth bags in a dry and well-ventilated environment. Artificial inoculation was applied by sowing seed together with the 0.1% *S. reiliana* inoculum and soil, with normal agronomic practices in field management. When disease symptoms were fully expressed, individual plants were scored at crop maturity, and the mean of three replicates per plot was determined.

Results

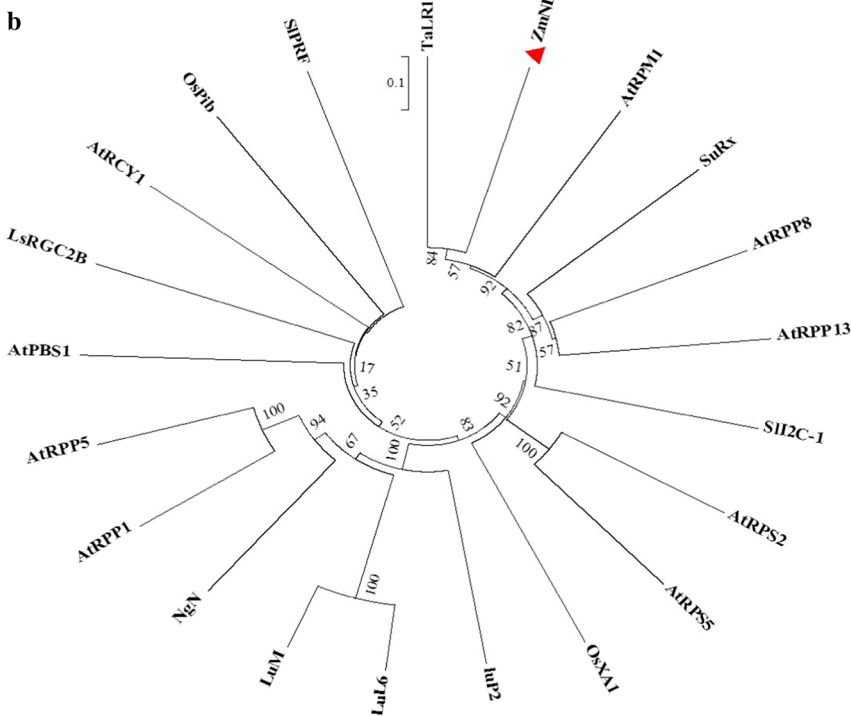
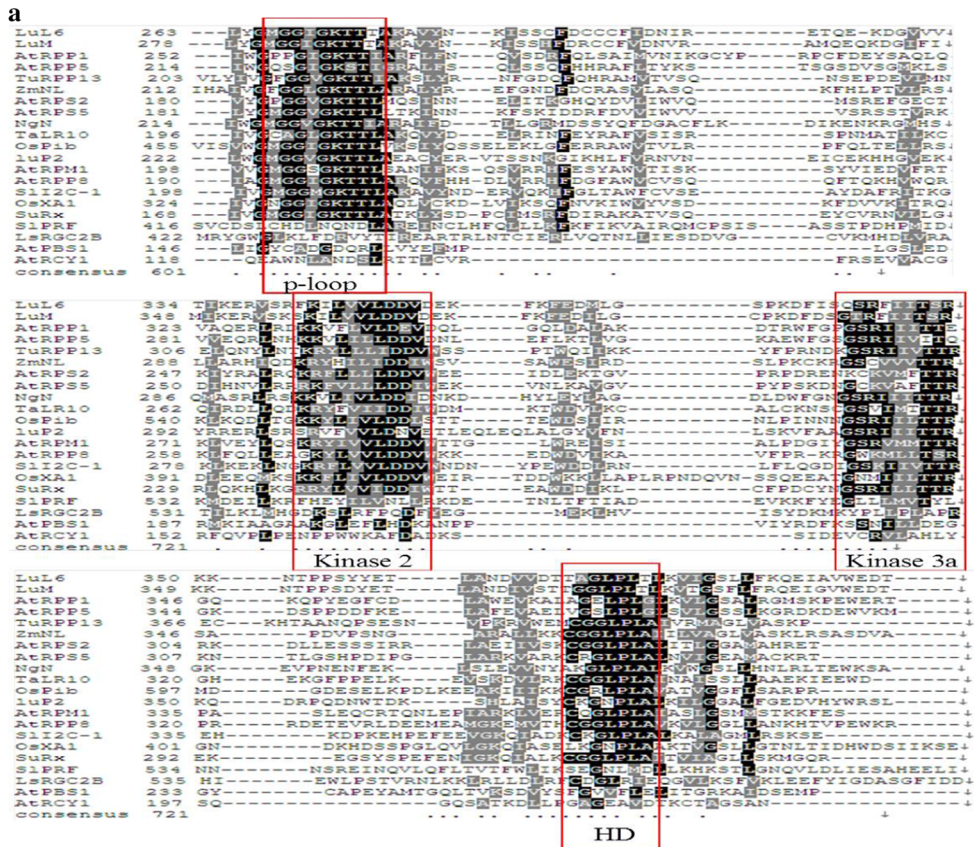
Isolation and characterization of the *ZmNL* cDNA

In our preliminary study, the *ZmNL* DNA sequences of B73 RefGen_v2 in bin 2.09 were confirmed by the presences of conserved nucleotide binding site (*NBS*) and leucine rich repeat (*LRR*) *R* gene domains using Fgenesh software (www.softberry.com/berry.phtml), ORF Finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) and InterProScan search (<https://www.ebi.ac.uk/Tools/pfa/iprscan/>). The specific primer m*ZmNLF* and m*ZmNLR* was designed according to the coding sequence of *ZmNL*. The RT-PCR reaction was carried out using RNA isolated from leaf of the line L282 as template for the synthesis of cDNA. A cDNA fragment showed a 678-bp band in a 1% agarose gel (Fig. 1a) was cloned into pEasy-T1Simple vector. Sequencing results of various clones showed the same

nucleic acid sequence which was fully consistent with the candidate sequence in MaizeGDB (<http://www.maizegdb.org/>). Based on the obtained fragment, RACE strategies were conducted at cloning the full-length cDNA sequence of the *ZmNL*. The 5'-end (Fig. 1b) and the 3'-end (Fig. 1c) of cDNA with the specific primers and universal primers were amplified using first strand cDNA derived from total RNA as template. Then the *ZmNL* gene was assembled according to overlapping sequences from the three fragments above (Fig. 1e). The reconstructed full length of the cDNA sequence, 3572-bp long (Supplementary Fig. 1), was identified from the sequence of the *ZmNL* clone, including 3156 bp CDS (Fig. 1d), 163 bp of 5'-UTR and 253 bp of 3'-UTR. The *ZmNL* sequence was deposited in GenBank with Accession Number of KF765443.

The *ZmNL* gene encodes a putative protein of 1051 amino acids with a predicted molecular mass of 116.6 kD and a predicted PI of 8.47. Table 2 showed the comparison of the amino acid sequence of *ZmNL* protein via ExPASy ProtParam. Its putative secondary structure includes 587 α -helices (55.85%), 341 random coils (32.35%), 100 extended strands (9.90%), and 23 β -helices (1.90%). CD-Search results indicated that the *ZmNL* protein contains conserved NBS and LRR domains (Fig. 2).

Figure 3 indicated the comparison of the amino acid sequence of *NBS*-containing *R* protein in some higher plant. The typical conserved motif were presented in *ZmNL* protein and other plant *R* proteins, including P-loop(GMGGVVGKTT), Kinase-2(LIVLDDVW), Kinase-3a(GSR/KILVTTR), and hydrophobic domain HD(GLPLAL) (Fig. 3a). Based on nucleotide and amino acid primary structure homology, the *ZmNL* showed higher homology to the sequence of the plant *NBS*-containing *R* gene (nucleotide comparison not shown). Evolutionary relationships between the *ZmNL* and *R* proteins from other plant species demonstrated that *ZmNL* is 84% similar to the *Triticum aestivum* resistance protein *LR10* and showed extensive amino acid sequence similarity to other coiled-coil-*NBS*-*LRR* (*CC*-*NBS*-*LRR*) proteins (Fig. 3b). The protein sequences used for comparison and phylogenetic trees were from *Linum usitatissimum* (AAA91021.1, AAB47618.1, AAK28806.1, AAC83165.1), *Arabidopsis thaliana* (AAC72977.1, AAB58295.1, Q42484.1, NP_172686.1, NP_187360.1, NP_196820.1, NP_001077962.1), *Triticum aestivum*



◀ **Fig. 3** Comparison of encoding amino acid sequence of ZmNL protein with other plants. **a** ClustalW was conducted at Comparison of encoding amino acid sequence of ZmNL protein with other plants. **b** Phylogenetic tree based on alignment of the deduced amino-acid sequences of *ZmNL* and other known R proteins from different plant species. The scale bar represents conversion of branch length to genetic distance between clades (0.1 = 10% genetic distance). The protein sequences used for phylogenetic analysis include: LuL6 (AAA91021.1) from *Linum usitatissimum*, LuM (AAB47618.1) from *Linum usitatissimum*, AtRPP1 (AAC72977.1) from *Arabidopsis thaliana*, AtRPP5 (AAB58295.1) from *Arabidopsis thaliana*, TuRPP13 (EMS68464.1) from *Triticum urartu*, AtRPS2 (Q42484.1) from *Arabidopsis thaliana*, AtRPS5 (NP_172686.1) from *Arabidopsis thaliana*, NgN (Q40392.1) from *Nicotiana glutinosa*, TaLR10 (AAQ01784.1) from *Triticum aestivum*, OsPib (BAA93618.1) from *Oryza sativa*, LuP2 (AAK28806.1) from *Linum usitatissimum*, AtRPM1 (NP_187360.1) from *Arabidopsis thaliana*, AtRPP8 (AAC83165.1) from *Arabidopsis thaliana*, SI2C-1 (AAB63274.1) from *Solanum lycopersicum*, OsXA1 (O48647) from *Oryza sativa*, SuRx (CAB50786.1) from *Solanum tuberosum*, SIPRF (AAC49408.1) from *Solanum lycopersicum*, LsRGC2B (AAD03156.1) from *Lactuca sativa*, AtPBS1 (NP_196820.1) from *Arabidopsis thaliana*, and AtRCY1 (NP_001077962.1) from *Arabidopsis thaliana*

(EMS68464.1, AAQ01784.1), *Nicotiana glutinosa* (Q40392.1), *Oryza sativa* (BAA93618.1, O48647), *Solanum lycopersicum* (AAB63274.1, AAC49408.1), *Solanum tuberosum* (CAB50786.1), and *Lactuca sativa* (AAD03156.1).

Inducible expression of *ZmNL* in response to the *S. reilianum*

To confirm that artificial inoculation was successful, hyphae of the *S. reilianum* were observed in the leaves at 7 DAI. Comparing the mock- and the *S. reilianum*-infected L282 leaves, chlorotic flecks were only observed on the newly emerged leaves of infected Huangzao4 (Fig. 4a). Hyphae in the infected leaves were stained with cotton blue and observed under light microscopy (Fig. 4b). Scanning electron micrographs showed numerous hyphae growing around the stomata (Fig. 4c). Cross-sections of the leaves showed infection hyphae growing in the intercellular spaces inside the leaves (Fig. 4d, e). Thus, the hyphae of *S. reilianum* could penetrate into the host tissues and cause disease symptoms. To determine its contribution to resistance to *S. reilianum* infection, the expression of *ZmNL* was analyzed using qRT-PCR. As shown in Fig. 5, *ZmNL* expression was up-regulated in the L282

and Huangzao4 after inoculation with the *S. reilianum* and reached a peak on the second DAI, but peak expression was significantly higher in the L282 (4.29) than in the Huangzao4 (2.99). The expression of *ZmNL* in the both L282 and Huangzao4 then decreased continually from 3 to 6 DAI. These results indicate that the expression of *ZmNL* is likely induced by the *S. reilianum* infection.

Molecular characterization of transgenic maize plants

Transgenic maize plants overexpressing the *ZmNL* gene were successfully generated by the *Agrobacterium*-mediated transformation of binary vector pTF101-*ZmNL* into the inbred line Huangzao4. More than 20 T0 independent transgenic plant lines were established and propagated in the greenhouse. A 398-bp PCR product amplified with *ZmNL* gene-specific primers is shown in Fig. 6. Three transgenic maize lines (the *ZmNL*-1, *ZmNL*-4 and *ZmNL*-6) with single or double transgenes integration events were verified via Southern blotting analysis, and no integration of *Bar* was detected in the WT plants (Fig. 7).

The expression of the *ZmNL* gene in T2 plants leaves was monitored by reverse transcriptase-polymerase chain reaction (RT-PCR), and the results are shown in Fig. 8. The RT-PCR did not produce a product in wild type plant samples.

Over-expressing the *ZmNL* gene increased the resistance of maize to head smut

The mean disease incidence of 3 transgenic maize lines and acceptor line Huangzao4 was observed (Table 3). The highest level was 81.52% in Huangzao4 line, and the lowest level was 0 in control line L282. The disease incidence of transgenic lines *ZmNL*-1, *ZmNL*-4, and *ZmNL*-6 decreased from wild line by 29.40, 29.51 and 18.38% respectively. The introduction of *ZmNL* gene declined the head smut disease incidence by an average of 25.76 percent.

Discussion

The ORF of the 3156-bp *ZmNL* cDNA that we isolated from the *S. reilianum*-resistant maize line L282

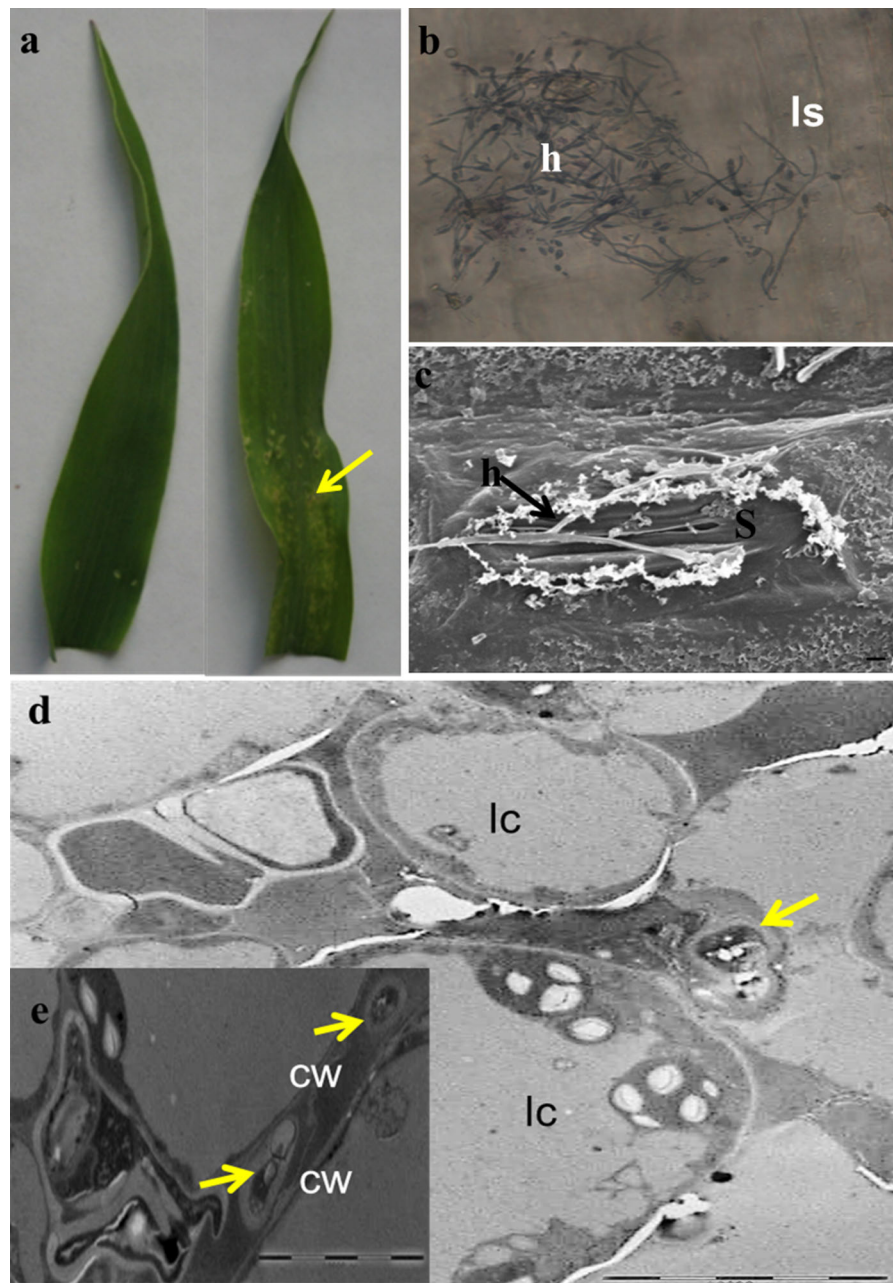


Fig. 4 Symptoms of infection with *S. reilianum* and hyphae of *S. reilianum*-infected Huangzao4 leaves observed under light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). **a** *S. reilianum*-infected plants (right) show chlorotic flecks (arrow) on leaves; no symptoms were observed on leaves of mock-infected plants

(left). **b** Hyphae (h) on the leaf surfaces (ls) of the *S. reilianum*-infected plants. Magnification $\times 400$. **c** Stomata surrounded by numerous long hyphae, bar = 2 μm . **d** Hyphae (h) in the intercellular spaces within the leaves (lc), bar = 10 μm . **e** Hyphae (h) in the intercellular space between two leaf cells, bar = 10 μm

encodes a deduced protein of 1051 amino acids with high sequence similarity to other *CC-NBS-LRR R* proteins. The *NBS-LRR* proteins can be further

distinguished by the particular motif located at the N-terminus into two subclasses: Toll and human interleukin-1 receptor (*TIR*)-*NBS-LRR* proteins and

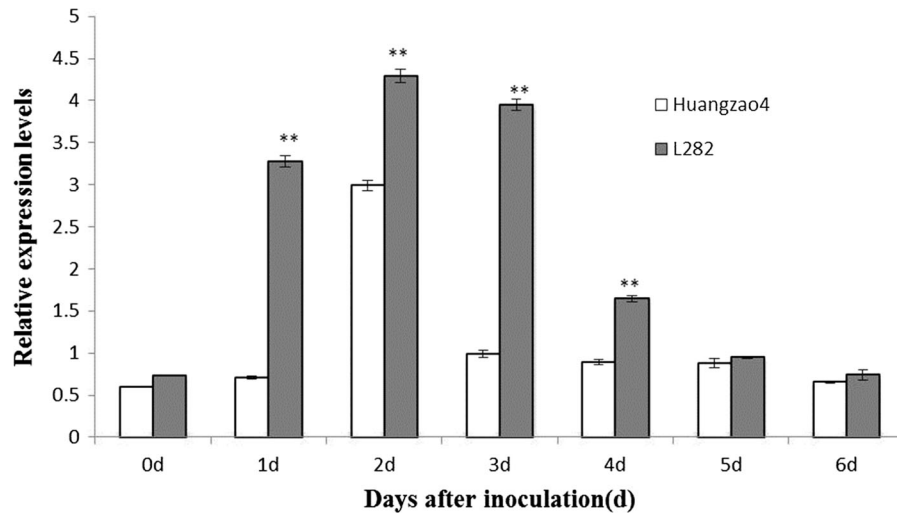


Fig. 5 qRT-PCR to monitor *ZmNL* transcript expression in L282 and Huangzao4 leaves inoculated with *S. reilianum* at 0, 1, 2, 3, 4, 5 and 6 days. The relative expression of *ZmNL* transcripts was normalized to the expression of maize *Actin1* mRNA. Leaves were injected with water as the mock-

inoculation control. The error bars denote standard deviations of the qRT-PCR signals ($n = 3$). The expression levels of *ZmNL* transcript in L282 and Huangzao4 were significantly different (** $p < 0.01$, Student's *t* test)

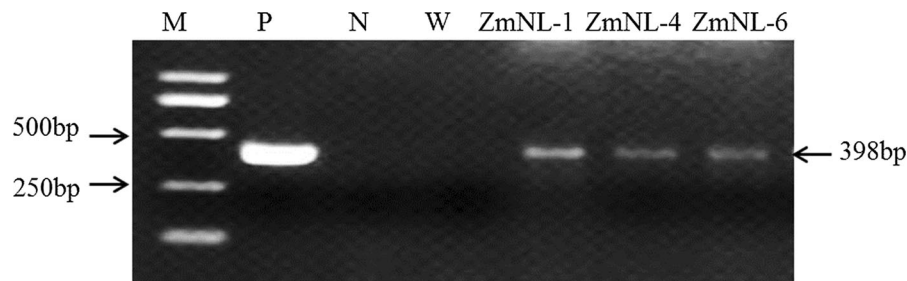


Fig. 6 PCR analysis of transformed samples using primers specific for the *ZmNL* gene and *Nos* terminator. A 398-bp PCR product was amplified from three transgenic maize lines (ZmNL-1, ZmNL-4, and ZmNL-6) with primers specific for

the *ZmNL* gene and *Nos* terminator. M, DNA Marker DL2000 (TaKaRa); P, pTU-ZmNL plasmid; N, wild-type plants of Huangzao4; W, water (no template DNA) control; ZmNL-1, ZmNL-4, and ZmNL-6, transgenic maize lines

CC-NBS-LRR proteins. It is noteworthy that the *CC* domains of the *NRG1* from *Nicotiana benthamiana*, and *ADR1* (Collier et al. 2011), *RPS2*, *RPS5*, and *RPM1* from the *Arabidopsis* (Tao et al. 2000; Ade et al. 2007; Gao et al. 2011) can trigger the PCD. Their *NBS* domains help transduce signals for plant defense responses. The *LRR* domain can determine the specificity of pathogen recognition via protein–protein and protein–ligand interactions with effector molecules derived from pathogens (Jia et al. 2013). The protein encoded by the *ZmNL* gene might recognize the corresponding avirulence protein expressed the head smut pathogen, bind with ATP or GTP, and activate a kinase or G protein involved in protein

phosphorylation to amplify the disease resistance signal. Its *LRR* domain might then interact with intracellular disease-related proteins to transmit the disease resistance signal and trigger the expression of downstream defense genes.

The 2000 bp upstream sequence of *ZmNL* of B73 RefGen_v2 was analyzed via PlantCARE to explore the cis-acting elements in the promoter region (Supplementary Fig. 2). Except *TATA*-box and *CAAT*-box, the multiple cis-acting elements related to pathogens responses, such as MeJA response elements TGACG-motif (– 882 to – 886 bp) and CGTCA-motif (– 1292 to – 1296 bp). Defense response element TC-rich repeat (– 1025 to – 1033) was also found in

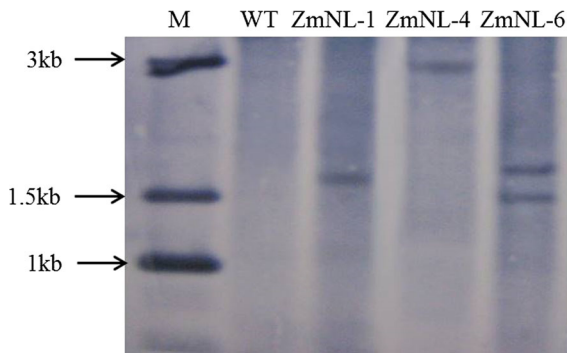


Fig. 7 Southern blot analysis of transgenic plants and the wild type. Total genomic DNA of three transgenic maize lines (ZmNL-1, ZmNL-4, and ZmNL-6) and WT was digested with *EcoR* V and *Hind*III. A single-copy insertion of the *Bar* gene was detected in transgenic maize lines, and no integration of *Bar* was detected in the corresponding WT plants. M, DNA Marker DL15000 (TaKaRa); WT, wild-type plants of KF513; AKF58, AKF61, and AKF65, transgenic lines

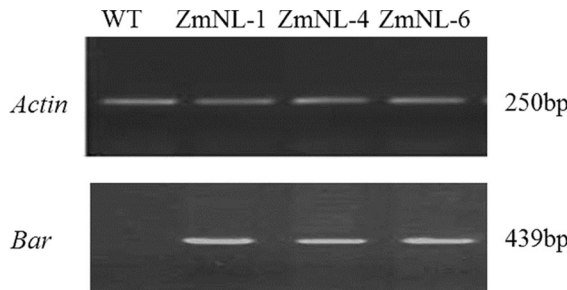


Fig. 8 RT-PCR analysis of transformed samples using *Bar* gene-specific primers. A 439-bp PCR product was amplified with *Bar* gene-specific primers from three transgenic maize lines (ZmNL-1, ZmNL-4, and ZmNL-6) by reverse transcriptase-polymerase chain reaction (RT-PCR). In WT plants, no expression of the *Bar* gene was detected. WT, wild-type plants of Huangzao4; ZmNL-1, ZmNL-4, and ZmNL-6, transgenic lines

Table 3 Incidence investigation of head smut disease for transgenic lines

Lines	Disease incidence (%)	Increased range (%)
ZmNL-1	52.12 ± 0.07c	29.40
ZmNL-4	52.01 ± 0.12c	29.51
ZmNL-6	63.14 ± 0.06b	18.38
Huangzao4	81.52 ± 0.10a	–

Values followed by different letters in each column are significantly different as compared with wild type at $P < 0.05$

the promoter region of *ZmNL*. In our study, the *ZmNL* gene was introduced into maize line of Huangzao4,

which was highly susceptible to head smut. The transgenic lines showed the increased disease resistance under artificial inoculation in the field. These results proved further the strong correlation between the expression of *ZmNL* gene and disease responses of maize.

As for most plant *R* genes, the *ZmNL* gene was expressed at a low constitutive level in uninfected plants. When plants were treated with the *S. reilianum*, the expression of *ZmNL* increased in the L282 more than the Huangzao4. During earlier stages of the infection, *ZmNL* expression increased. At later stages of infection with the *S. reilianum*, the expression *ZmNL* decreased. The higher expression of *ZmNL* in the smut-resistant line L282 than in the Huangzao4 suggests a positive role for the expression of *ZmNL* in response to the *S. reilianum* infection in the L282 and Huangzao4. Further, the delayed response and lower level of *ZmNL* expression in the Huangzao4 upon *S. reilianum* infection correlates with the weaker resistance of the Huangzao4 to the *S. reilianum* infection.

A wall-associated kinase gene *ZmWAK* was reported in the same region of *ZmNL* (Zuo et al. 2015). The expression of the two genes was analyzed after inoculation with *S. reilianum* using qRT-PCR (Fig. 5, Supplementary Fig. 3). The similar up-regulated changed trend was observed in the L282 and the Huangzao4 and the expression of two genes both reached a peak on the second DAI. These results indicate that the *ZmNL* protein might cooperate with other proteins associated with resistance to the *S. reilianum*, such as the *ZmWAK*, to stimulate HR around attempted pathogen infection sites in maize and limit the further expansion of the pathogen via PCD. Future studies will be more precisely address the *ZmNL* function.

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