Mol Biol Rep (2010) 37:345–354
DOI 10.1007/s11033-009-9749-2

Cloning, structural features, and expression analysis of resistance gene analogs in Tobacco

Yulong Gao · Zhaoli Xu · Fangchan Jiao · Haiqin Yu · Bingguang Xiao · Yongping Li · Xiuping Lu

Received: 22 July 2009 / Accepted: 5 August 2009 / Published online: 30 August 2009

Abstract Using degenerate primers based on the conserved nucleotide binding site (NBS) and protein kinase domain (PKD), 100 resistance gene analogs (RGAs) were isolated from tobacco variety *Nicotiana repanda*. BLASTx search against the GenBank database revealed that 27 belong to the NBS class and 73 belong to the protein kinase (PK) class. Cluster analysis and multiple sequence alignment of the deduced protein sequences indicate that RGAs of the NBS class can be divided into two groups: toll/interleukin receptor (TIR) and non-TIR types. Both types possess 6 conserved motifs (P-loop, RNBS-A, Kinase-2, RNBS-B, RNBS-C, GLPL). Based on their sequence similarity, the tobacco RGAs of the PK class were assigned to 8 subclasses. We examined their expression after infection with either Tobacco mosaic virus (TMV) or the tobacco black shank pathogen (*Phytophthora parasitica* var. *nicotianae*). The expression levels of 4 RGAs of the PK class were significantly elevated by TMV and 1 RGA of the PK class and 3 RGAs of the NBS class were up-regulated by *P. parasitica* var. *nicotianae*. The expression of two RGAs of the PK class was induced by *P. parasitica* var. *nicotianae*. Infection by either TMV or *P. parasitica* var. *nicotianae* enhanced the expression of *NtRGA2*, a RGA of the PK class. The present study shows that RGAs are abundant in the tobacco genome and the identification of tobacco RGAs induced by pathogens should provide valuable information for cloning related resistance genes in tobacco.

Keywords Tobacco · Resistance gene analogs · Nucleotide binding site · Protein kinase

Y. Gao · Z. Xu · F. Jiao · H. Yu · B. Xiao · Y. Li · X. Lu (✉)
Yunnan Academy of Tobacco Agricultural Science,
653100 Yuxi, China
e-mail: xplu1970@163.com

Abbreviations

NBS	Nucleotide binding site
PKD	Protein kinase domain
RGAs	Resistance gene analogs
PK	Protein kinase
TIR	Toll/interleukin receptor
TMV	Tobacco mosaic virus
LRR	Leucine-rich repeat
ORF	Open reading frame
RT	Reverse transcription
MAPK	Mitogen-activated protein kinase

Introduction

Constant exposure to pathogen attack during their long evolutionary history of host plants has resulted in plant-pathogen coevolution. Interactions between plant pathogens and their host plants involve specific recognition and subsequent activation of a cascade of plant defense responses. Plant resistance gene (*R*-gene) plays an important role in plant-pathogen recognition [1]. About 50 *R*-genes have been cloned so far from diverse plant species by transposon-tagging and map-based methods [2]. The protein encoded by the majority of the disease resistance genes present several highly conserved domains: nucleotide binding site (NBS), leucine-rich repeat (LRR), protein kinase (PK) domain, toll/interleukin receptor (TIR) domain etc. [2–5]. The presence of highly conserved domains of plant *R*-genes provides a convenient means for cloning additional *R*-genes or RGAs by a PCR-based approach.

Tobacco is an economically important crop in China and is seriously harmed by Tobacco common mosaic and

Tobacco black shank. Tobacco common mosaic, caused by TMV, is a major tobacco disease in the tobacco production areas worldwide [6]. TMV infection of tobacco causes reductions in leaf size, single leaf biomass and the number of leaves. Additionally, TMV reduces tobacco quality such as decreased sugar contents, increased total amounts of nitrogen and proteins, higher levels of pungency and undesirable volatile compounds, and decreased concentrations of nicotine etc. Tobacco black shank, caused by the soil-borne fungal pathogen *Phytophthora parasitica* var. *nicotianae*, is also one of the major tobacco diseases in China. Black shank severity on susceptible genotypes is increased under several abiotic stresses such as a hot, dry growing season and the presence of root-knot nematodes.

Planting disease-resistant varieties is the most economical and effective approach to tobacco disease management. Classical resistance breeding program is unable to meet farmers' requirement for resistant crop varieties due to both the longer breeding process and either the lack of resistance sources or the linkage of some *R*-genes to certain undesirable quality traits. Application of genetic engineering and molecular maker-assisted selection can considerably shortern the breeding cycle of developing a disease-resistant crop variety. Using PCR amplification with degenerate primers targeting the conserved motifs of respective NBS, LRR and PK domains of known *R*-genes, a great number of RGAs were cloned from diverse crops including soybean [7, 8], corn [9], lettuce [10], chickpea [11], cotton [12]. Some of these RGAs may be *R*-genes, and others could be linked to *R*-genes [13–17]. However, isolation of RGAs from tobacco had not been reported. In the present study we cloned RGAs from tobacco by a PCR-based strategy with degenerate primers designed based on the conserved motifs of known plant *R*-genes. Expression profiles of the cloned tobacco RGAs in response to TMV and *P. parasitica* var. *nicotianae* challenges were examined. Our results provide a basis for developing molecular markers linked to genes resistant to TMV and *P. parasitica* var. *nicotianae*, and eventually facilitate the cloning of these *R*-genes in tobacco.

Materials and methods

Plant materials and DNA extraction

The wild tobacco *N. repanda* resistant to TMV and *P. parasitica* var. *nicotianae* was used for cloning RGAs. Genomic DNA was isolated from tobacco seedlings with three true leaves using the DNeasy Plant mini Kit (Qiagen; Hilden, Germany).

Primer design and PCR amplification

Degenerate primers were designed based on the conserved motifs of the cloned *R*-genes (Table 1). The predicted PCR fragments of NBS-encoding tobacco RGAs are about 500 bp. Tobacco RGAs of the PK class either about 500 or 900 bp in length. PCR reactions were carried out in a volume of 20 µl containing 1× reaction buffer, 0.2 mmol/l dNTPs, 1.5 mmol/l $MgCL_2$, 50 ng DNA template, 1 µmol/l each of forward and reverse primers, 2 U *Taq* DNA polymerase.

Table 1 The primers used for the isolation of RGAs in tobacco (*Nicotiana repanda* L.)

Primer name	Conserved amino acid motif	Primer sequence (5′ to 3′)	References
F1	P-loop	GGNATGGGYGGBRTHGGYAARAC	[18]
R1	Hydrophobic domain (GLPL)	CARMGCYAAWGGYAADCC	
F2	P-loop	GGNGGNRTNGGNAARACCAC	[19]
F3	P-loop	GGNGGNRTNGGNAARACAAC	
R1	Hydrophobic domain (GLPL)	CAHHGCNAAHGGHAAHCC	
R2	Hydrophobic domain (GLPL)	CAANGCCAANGGCAANCC	
R3	Hydrophobic domain (GLPL)	CAGNGCNAGNGGNAGNCC	
F4	P-loop	GGNGTNGGNAARACNAC	[20]
	DMGRDL	AAGATCTCGTCCCATATC	
RLKF1	Kinase domain	ATGGGAAGCAAGTATTCCAA	[21]
RLKR1	Kinase domain (XI)	AGTTTCCACAGCACATCACC	
RLKF2	Kinase domain (I)	GGIGGITTYGGIATHGTITWYAARGG	
RLKR2	Kinase domain (VII)	ARIARYTTIGCIARICCRAARTC	
RLKR3	Kinase domain (VII)	AAIATICKIGCCATICCRAARTC	
RLKF3	Kinase domain	ATCGGKAARGGCGGMGCKGGRATYGTSTAC	[22]
RLKR4	Kinase domain	GGSGCGATGTAKCCRTARGAGCCAGC	

Reactions were performed using the PE9600 (PE, USA). Amplification conditions: 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 50–55°C for 1 min, 72°C for 1 min, and a final step of 72°C for 7 min.

Recovery of PCR products

Amplified products were separated on 1.5% agarose gels and bands of expected sizes were excised and purified using the TaKaRa Agarose Gel DNA Purification Kit. The recovered DNA fragments were ligated to the pGEM-T easy Vector (Promega; Madison, Wis.) overnight and transformed into competent *Escherichia coli* DH5a cells (TaKaRa, Shiga, Japan) according to the heat shock procedure. The treated bacterial cells were mixed with X-gal and IPTG and plated on LB agar plates containing ampicillin (50 μg/ml). After overnight incubation at 37°C, individual white colonies were picked from LB plates and inoculated into liquid LB containing ampicillin (50 μg/ml) and shaken at 37°C for 12 h. The presence of insert was checked by PCR amplification with the universal primers T7 and SP6. Positive clones were sent to Shanghai Sangon Biological Engineering Technology Service Co., Ltd for DNA sequencing.

Sequence analysis

Sequences of the cloned PCR products were used to search the GenBank databases by the BLAST X algorithm. Open reading frame (ORF) search for the cloned putative RGAs was conducted with the ORF finder at the NCBI Website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). RGAs sequences possessing uninterrupted ORF were translated into amino acid sequences with Lasergene software package. Clustal X software [23] with default options was used to conduct multiple sequence alignment of the deduced amino acid sequences, and then the output was edited by the GeneDoc software [24]. Construction of phylogenetic trees was performed with the MEGA4 software [25].

TMV and *P. parasitica* var. *nicotianae* inoculation

TMV broad bean strain was maintained in *Nicotiana tabacum* Samsun nn and used to inoculate TMV-resistant variety Ti245 seedlings at the stage of 4–5 leaves. Virus inoculum was prepared by grounding small amount of TMV-infected tobacco leaves with adequate volumes of inoculation buffer and the resultant crude sap was applied evenly on Carborundum-dusted (200–400 mesh) seedling leaves with a hand dipped in the inoculum, and finally the treated leaves were rinsed with sterile water. The inoculated plants were kept at 22–25°C in a greenhouse.

Phytophthora parasitica var. *nicotianae* race 0, a highly virulent strain, was used to inoculate wild tobacco *N. repanda*. Mycelia of *P. parasitica* var. *nicotianae* race 0 were first grown for 5–7 days on oatmeal agar, and then transferred to sterile unhulled rice media and allowed to grow for 18–28 days to prepare for inoculums consisting of rice and *P. parasitica* var. *nicotianae* mycelia. For virulence assay, 5 g of inoculums was applied to the stem base of tobacco seedlings at the stage of 5 leaves and followed by covering with soil and adding adequate water to keep moisture. The treated plants were kept at 28°C in an incubator.

RNA isolation and RT-PCR

RNA was extracted using Qiagen RNA Mini Kit (Qiagen; Hilden, Germany) from tobacco leaves of Ti245 and roots of *N. repanda* collected at 0, 3, 6, 9, 12 days after inoculation, respectively. The expression levels of RGAs possessing undisrupted ORF was assessed by semiquantitative reverse transcription (RT)-PCR and the constitutively expressed *EF1* gene was served as an internal control.

Results and analysis

Isolation of RGAs from tobacco

NBS-encoding RGAs were PCR-amplified using eight pairs of primers (Table 1) with the following combination: F1/R1; F2, F3/R2, R3, R4; F4/R5. The tobacco RGAs of the PK class were amplified from tobacco with four pairs of primers: RLKF1/RLKR1; RLKF2/RLKR2; RLKF3/RLKR3, RLKR4. PCR products were checked on a 1.5% agarose gel and the band of the bands expected were recovered, cloned and sequenced. A total of 261 DNA fragments were sequenced. BLAST X searches against the GenBank databases revealed that 100 DNA fragments (GenBank accession Nos. FJ787333 to FJ787432) showed significant sequence homology with the known plant *R*-genes or deposited RGAs. Thus these 100 PCR-amplified DNA sequences represent tobacco RGAs. Of which, 27 belong to the NBS class and 73 belong to the PK class.

Sequence analysis of NBS-encoding RGAs

Of the 27 cloned tobacco NBS-encoding RGAs, 21 sequences have uninterrupted ORF. These RGA sequences showed respectively 37% to 77% identity and 55% to 87% similarity to the known *R*-genes *RPS2* (*Arabidopsis*), *I2* (tomato), *PRF* (tomato), *BS2* (pepper), and *RX* (potato). Phylogenetic analyses based on amino acid sequences of NBS-RGAs having uninterrupted ORF along with known *R*-genes: *RX*

(CAB50786, potato), *XA1* (BAA25068, rice), *I2* (AAD27815, tomato), *RPS2* (AAA21874, *Arabidopsis*), *L6* (AAD25965, flax), *RPP1* (AAC72977, *Arabidopsis*), *N* (Q40392, tobacco) revealed the two expected major groups representing the TIR- and the non-TIR NBS sequences (Fig. 1). Of NBS-encoding RGAs having uninterrupted ORF, NtRGA83 and NtRGA84 belonged to TIR-NBS which includes *R*-genes *L6*, *N*, *RPP1*. At the amino acid level, sequence identity between NtRGA83 and NtRGA84 was 39%, and the similarity was 62%. The other cloned tobacco NBS RGAs with read-through ORF were assigned to the non-TIR type, and they could further classified into 3 subfamilies (I–III). The subfamily I contains 3 tobacco RGAs and *RPS2*, and the sequence similarity among 3 RGAs was 93–96% and sequence identity was 88–92% at the amino acid level. The subfamily II contains 7 tobacco RGAs sequences and the *R*-genes *XA1*, *I2*. Among the 7 RGAs the similarity ranged from 82 to 100%, and the identity from 70 to 98%. The subfamily III includes 9 tobacco RGAs and *RX*, and these 9 RGAs exhibit sequence similarity ranging from 53 to 100% and the sequence identity from 32 to 99%.

The tobacco NBS RGAs isolated in this study contain the characteristic six conserved motifs including P-loop, RNBS-A, Kinase-2, RNBS-B, RNBS-C, GLPL of NBS-encoding *R*-genes (Fig. 2). Of which, P-loop, Kinase-2 and GLPL showed a high degree of conservation. The conserved motif of kinase-2 of the TIR-NBS type contains the conserved D residue and that of kinase-2 of the non-TIR type has the conserved W residue [26]. In addition to the 6 conserved motifs mentioned above, tobacco NBS-encoding RGAs also showed a certain degree of conservation in other regions.

Sequence analysis of tobacco RGAs of the PK class

Of 73 isolated tobacco RGAs of the PK class, 49 RGAs had uninterrupted ORF. A phylogenetic tree (Fig. 3) was constructed based on the deduced amino acid sequences of these 49 RGAs and the known *R*-genes of PK *XA21* (rice), *PBS1* (*Arabidopsis*), *PTO* (tomato), *RPG1* (barley). Based on the amino acid sequence similarity, the tobacco PK-RGAs were classified into 8 subfamilies. Subfamily I contained *RPG1* and two tobacco RGAs, and *XA21* and 3 tobacco RGAs belonged to subfamily VII. Subfamily VI included *PBS1* and 10 tobacco RGAs. All the other subfamilies comprised only tobacco RGAs. Of the 8

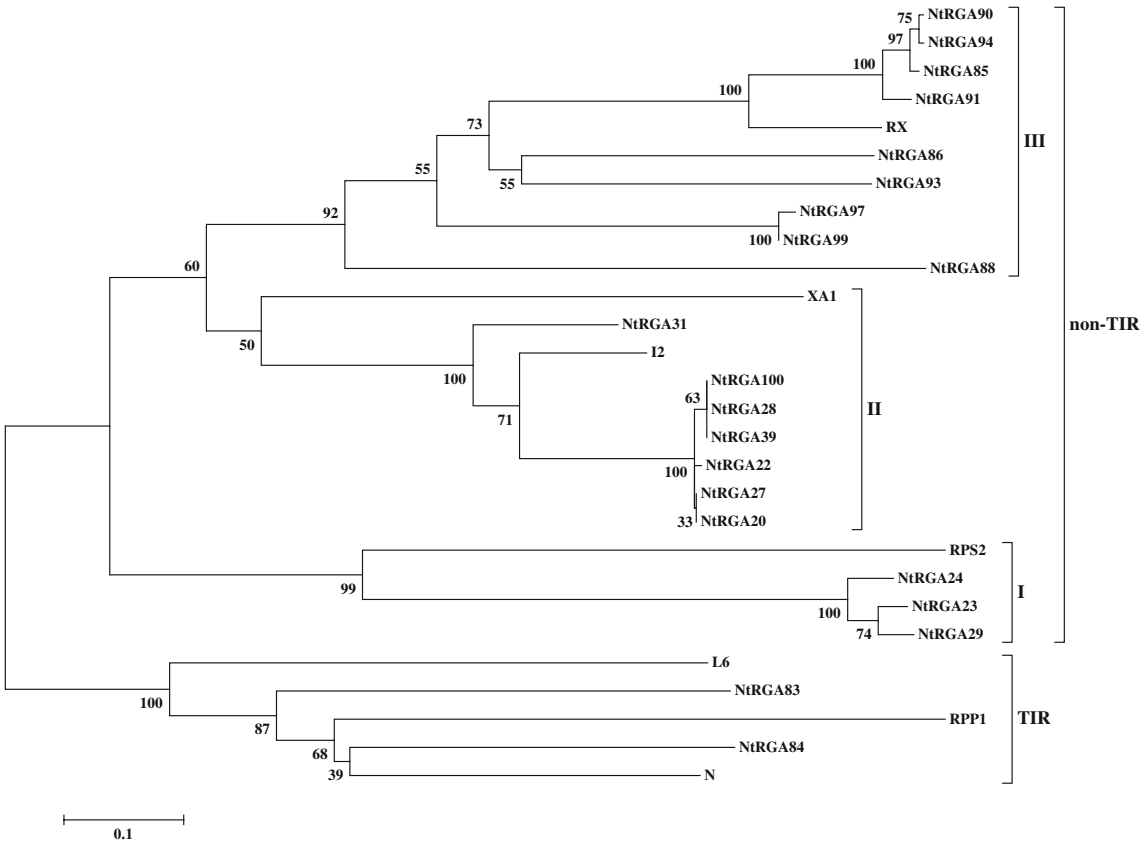

Fig. 1 Neighbor-joining tree based on the Clustal X alignment of the amino acid sequences of NBS-RGAs in *Nicotiana repanda* and the NBS domain of seven known resistance genes. *Numbers* on the branches represent bootstrap values (for 1,000 replicates)

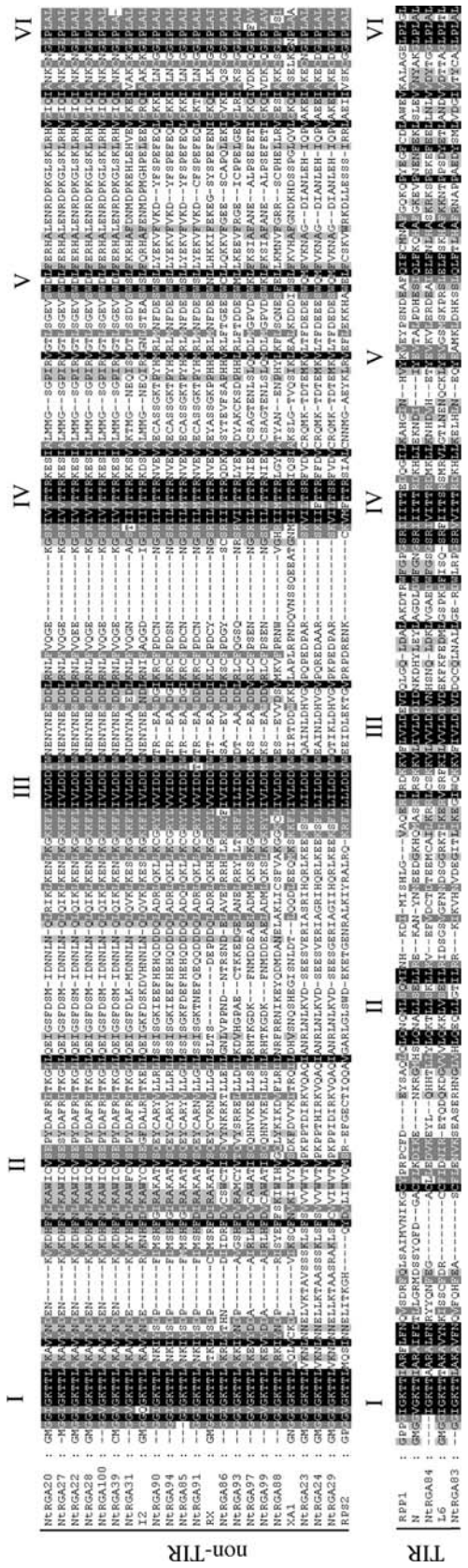

Fig. 2 Alignment of amino acid sequences of in-frame uninterrupted NBS-RGAs found in this study, with other NBS-R-genes. Conserved motifs, P-loop, RNBS-A, Kinase-2, RNBS-B, RNBS-C, GLPL, within the NBS domain were indicated at the *top* of the sequence by I–VI

subfamilies, only subfamily II had one member. The largest group is subfamily VIII containing 25 RGAs. Sequence identity between the tobacco RGAs of PK type and *R*-genes *XA21* etc. ranged from 24.1 to 74%, and the sequence identity among tobacco PK type RGAs from 34.4 to 100% at the amino acid level.

Multiple alignment of deduced amino acid sequences of tobacco PK-RGAs with uninterrupted ORF and *R*-genes *PTO*, *XA21* revealed that all tobacco RGAs of the PK class contained 8 conserved motifs (I–VII). Furthermore, NtRGA2, NtRGA17, NtRGA9, NtRGA15, NtRGA18, NtRGA1, NtRGA16 and NtRGA12 had 2 additional conserved motifs: VIII and IX (Fig. 4).

Expression analysis of tobacco RGAs

To determine whether transcription of tobacco RGAs were induced by pathogen challenge, the expression profiles of cloned tobacco RGAs containing uninterrupted ORFs were determined by RT-PCR in leaves or roots after inoculations with either TMV or *P. parasitica* var. *nicotianae* for 0, 3, 6, 9, and 12 days. The primer pairs used for RT-PCR were listed on Table 2. The results showed that the levels of transcription of 4 tobacco PK RGAs were up-regulated by TMV inoculation, and 1 tobacco RGA of the PK class and 3 tobacco NBS-encoding RGAs were up-regulated by inoculation with *P. parasitica* var. *nicotianae*. The expression of 2 tobacco RGAs of the PK class were induced by *P. parasitica* var. *nicotianae* challenge. In addition the expression of NtRGA2, a tobacco PK RGA, was up-regulated by challenge inoculations with either TMV or *P. parasitica* var. *nicotianae* (Fig. 5).

Discussion

RGAs have been successfully isolated from various plants using PCR amplification with degenerate primers based on the conserved motifs of known plant *R*-genes. The relation of RGAs to plant *R*-genes may fall into three categories: (1) RGAs are actual *R*-genes. For example, the full-length cDNA of the soybean *R*-gene *KR1* was cloned using an RGA fragment as a hybridization probe to screen a cDNA library [27]; (2) RGAs are linked to plant *R*-genes, for instance, NBS-encoding RGAs of sunflower are linked to down mildew-resistant locus *P15/P18* [28], and three RGA sequences were shown to be co-segregated with wheat rust resistance gene [29]; (3) RGAs are not functionally related to *R*-genes.

NBS-encoding genes are widely distributed in diverse plant genomes. For instance, about 150 NBS-encoding genes were predicted to exist in the *Arabidopsis* genome,

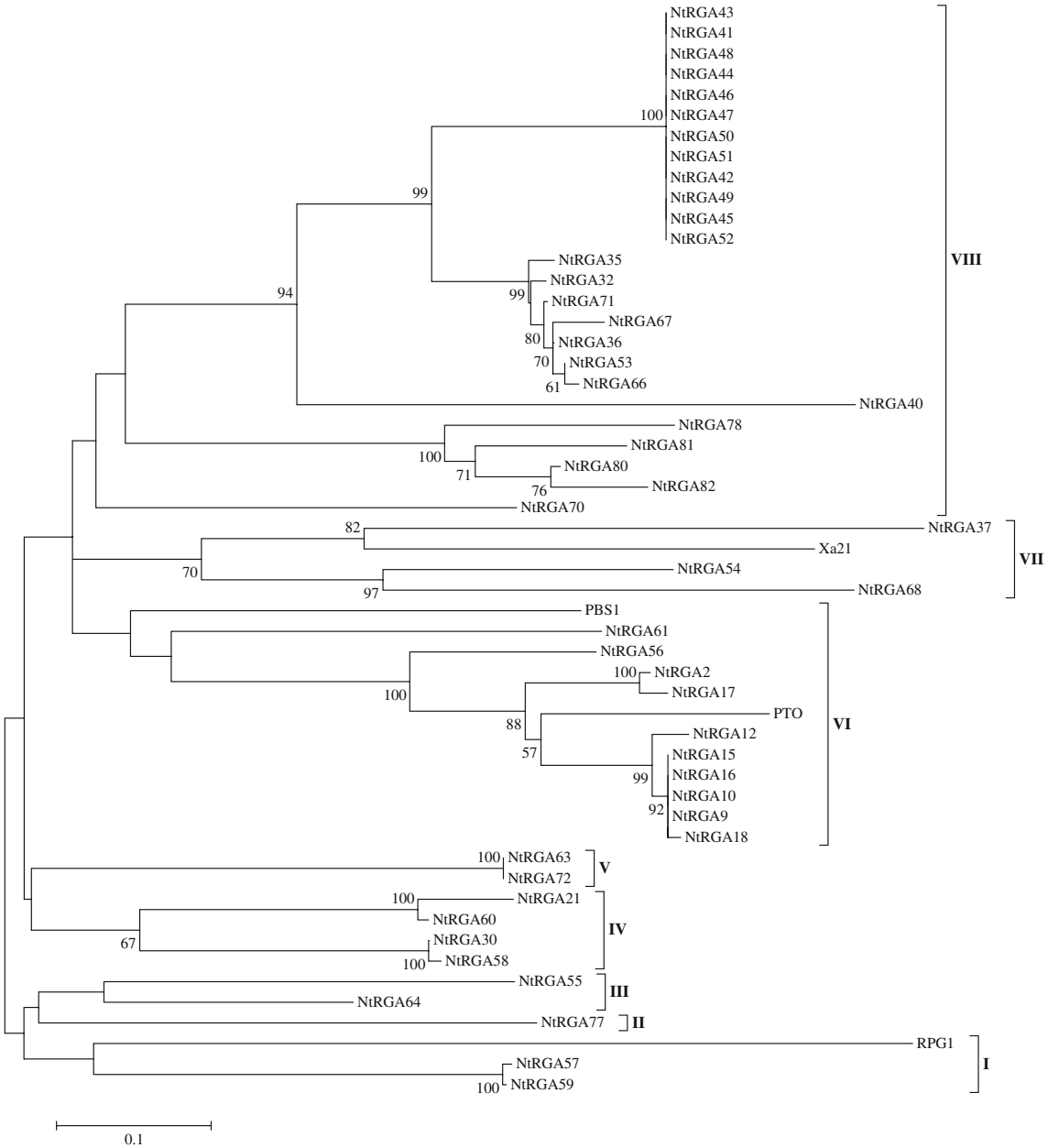

Fig. 3 Phylogenetic tree based on the amino acid sequences of PK-RGAs in this study and PK-R-genes cloned from other plants

accounting for 0.5% of the predicted ORFs of the entire genome [30]. About 600 NBS-containing genes were predicted in the rice genome, which represents 1.5% of the predicted genes of the whole genome [26, 31]. Of the plant *R*-genes cloned, 71% possess the conserved NBS domain [12]. Of the 27 tobacco NBS-encoding RGAs identified in this study, 21 RGA sequences had uninterrupted ORF and contained the characteristic 6 conserved motifs of NBS-encoding *R*-genes: P-loop, RNBS-A, Kinase-2, RNBS-B, RNBS-C, GLPL. The cloned tobacco NBS-encoding RGAs can be further divided into two types: TIR-NBS- and non-TIR-NBS types. Furthermore, for the identified tobacco TIR-NBS RGAs, the last residue of the kinase 2 motif was the conserved residue D. For the cloned tobacco non-TIR-NBS RGAs, the residue in this position of the kinase 2 motif was the conserved residue W. These findings are consistent with the previous results [26]. Of the tobacco NBS-encoding RGAs cloned, only 2 RGA sequences were members of the TIR-NBS type, and all the other belonged to the non-TIR-NBS type.

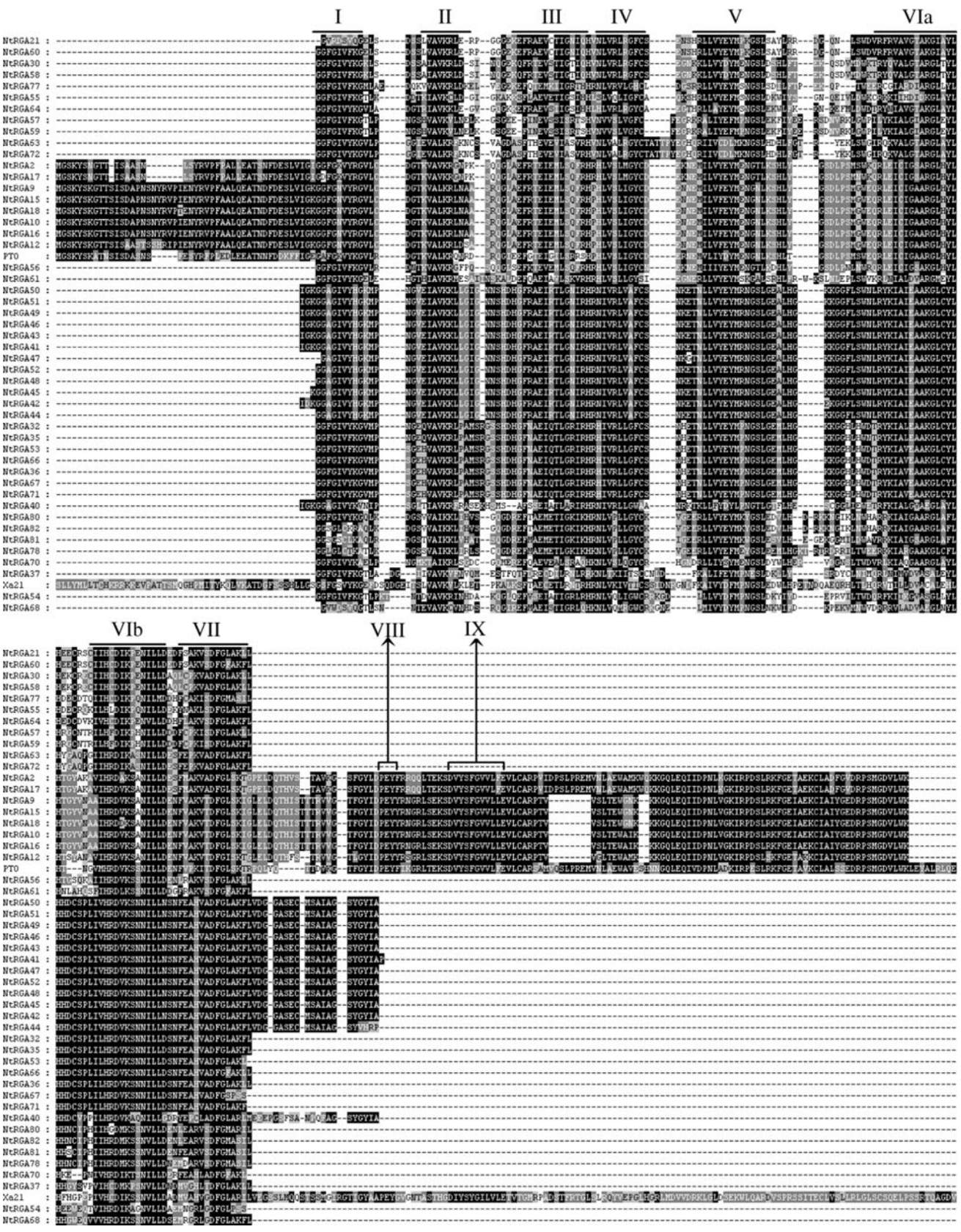

Fig. 4 Alignment of the predicted amino acid sequences of PK-RGAs from tobacco. Tomato *PTO* and rice *Xa21* were included in this analysis

Table 2 RGAs-specific primer sequences used for RT-PCR analysis

RGA	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Class
NtRGA2	GTAATCCCAAGTCCCAACA	CTTCATAGCCCATTCAGC	PK
NtRGA1	CGTCATCCGCATCTGGTT	GAAAGCCGTCCGTTTCTA	PK
NtRGA12	TCTCAGTTCCGCCATCCA	TCCACAGCACATCACCCA	PK
NtRGA63	GCACGGCTACAACTCCTT	CCGAAATCTGCCACCTTA	PK
NtRGA53	AAGGGGTAATGCCGAGTG	AATGACCGCCTTTCTTGC	PK
NtRGA18	ATGGGAAGCAAGTATTCCAA	AGTTTCCACAGCACATCACC	PK
NtRGA29	GGTAAGACGACTTTGGTGAA	ATGTCAACACCTTCATTTCG	NBS
NtRGA86	ATCGCTTTGATGTTTGTTCGTG	TCCGCAGCTTTGTGCTATTTTC	NBS
NtRGA91	TGACATTCGGGCAAAAGCAACT	CATTCAGCCACCTCCACATTCC	NBS

This result may be related to our choice of the primers or the limited in number of RGAs sequenced as well. The cloned tobacco non-TIR-NBS RGAs can be further classified into 3 subfamilies.

Genes encoding protein kinases constitute a large family and play important roles in various physiological and biochemical reactions of plants in response to hormones and environment such as self-incompatibility, pollen and endosperm development, flower abscission, environmental stresses and plant defense responses. Known plant *R*-genes encoding kinase include *PTO* of tomato, *XA21* of rice, *FLS2* and *PBS1* of *Arabidopsis*, and *RPG1* of barley etc. Products of kinase genes with diverse functions contain 12 conserved motifs (I–XI) [32]. In the present study, 73 tobacco RGA sequences of the PK class were cloned by PCR amplification with degenerate primers based on some of the above-mentioned conserved motifs of protein kinases. The deduced amino acid sequences of 49 tobacco RGAs with read-through ORF of the PK class contained 10 conserved motifs (I–IX). The cloned tobacco RGAs of the PK class can be further grouped into distinct 8 subfamilies, suggesting a high level of diversity of the PK class RGAs in tobacco. However, the predicted amino acid sequence identity between NtRGA10 and NtRGA16, between NtRGA9 and NtRGA15 of the VI subfamily; and among NtRGA41, NtRGA46 and NtRGA49, between NtRGA50 and NtRGA51, between NtRGA48 and NtRGA52 of the VIII subfamily respectively was shown to be 100%, suggesting the conserved nature of RGAs of the PK class in tobacco. The very high degree of conservation of PK class RGAs in tobacco provides a compensatory mechanism for certain function: the functional loss of one gene due to its mutation can be compensated for by another highly homologous gene, which might be the consequence of long-term evolution.

Most cloned plant *R*-genes encode proteins with the NBS region, which is required for ATP or GTP binding. However, genes involved in development process and other signal transduction pathways not relevant to plant disease resistance also encode proteins containing a conserved NBS region [33]. Likewise, apart from their role in plant disease resistance, genes encoding kinases also function in other aspects of plant physiology. Thus, localization study and expression analysis will help determine whether cloned RGAs are actually involved in disease resistance. The present study examined the expression profiles of cloned tobacco RGAs with uninterrupted ORF after pathogen challenge. We found that expression levels of 4 tobacco PK RGAs were elevated by TMV inoculation, and 1 tobacco PK RGA and 3 NBS-encoding RGAs were up-regulated by *P. parasitica* var. *nicotianae* inoculation. Transcription of two tobacco PK RGAs were induced by *P. parasitica* var. *nicotianae*. It is likely that these genes are involved in resistance to TMV and *P. parasitica* var. *nicotianae* since their expression was responsive to the challenge with the two pathogens. It is interesting that the expression levels of tobacco PK NtRGA2 were up-regulated by challenge inoculation with either TMV or *P. parasitica* var. *nicotianae*, suggesting that the products of NtRGA2 possibly involved in defense pathways against both TMV and *P. parasitica* var. *nicotianae*. Despite a decade of research on the structure and function of the tobacco TMV *R*-gene *N* [34–37], it remains unclear in terms of the molecular and physiological aspects of *N*-mediated defense signal transduction in pre-hypersensitive after TMV infection. It is currently believed that mitogen-activated protein kinase (MAPK) plays a role in the recognition between *N* gene and the pathogen. Inoculation of the tobacco *NN* cultivar with TMV resulted in the transcriptional induction of 2 MAPK genes [38]. In the current investigation, we showed that the expression levels of four cloned tobacco RGAs of the PK class were elevated after challenge inoculation with TMV, indicating that these 4 tobacco RGA sequences may play a functional role in defense response against TMV. The isolation of tobacco RGA sequences will provide valuable resources for further elucidating the molecular mechanism of TMV resistance in tobacco.

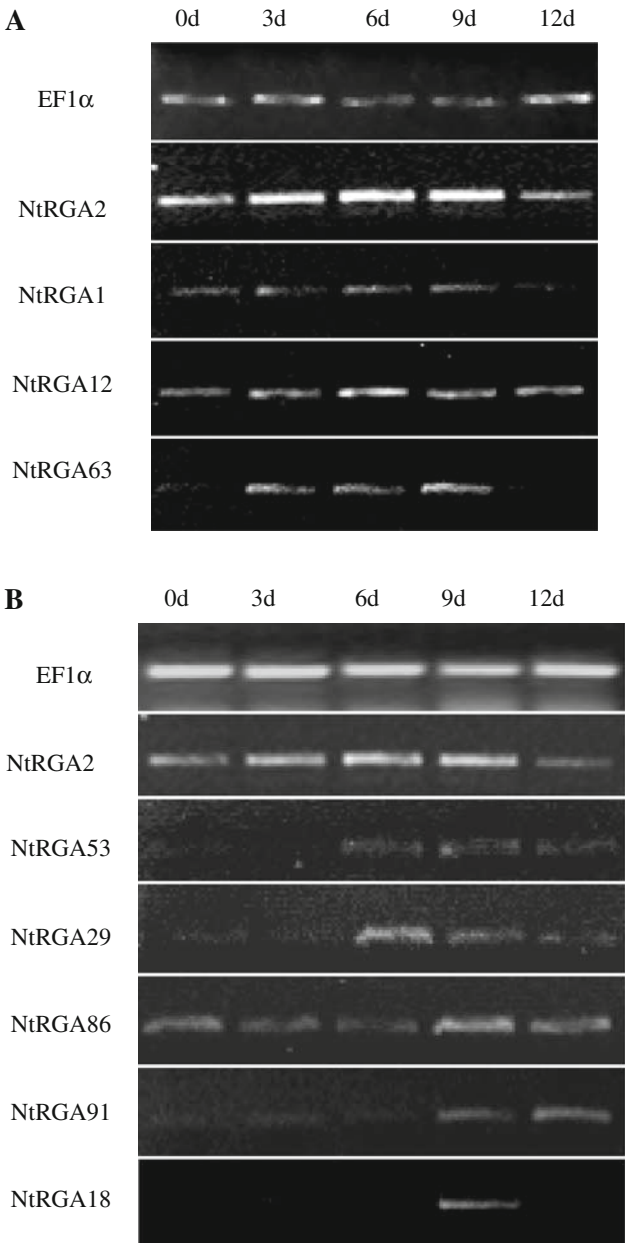

Fig. 5 RT-PCR analysis to determine the expression profiles of tobacco RGAs. **a**, **b** Show expression profiles of RGAs after inoculated by TMV B and *Phytophthora parasitica* var. *nicotianae*; EF1α, RT-PCR control; 0–12 days indicate 0–12 days post inoculation

Acknowledgments This study was financially supported by Yunnan Science and Technology Project (2008CD194) and Science and Technology Project of Yunnan Provincial Tobacco Monopoly Bureau (07A03 and 05-03).

References

1. Bendahmane A, Farnham G, Moffett P, Baulcombe DC (2002) Constitutive gain-of-function mutants in a nucleotide binding site-leucine rich repeat protein encoded at the Rx locus of potato. Plant J 32:195–204
2. Xiao WK, Xu ML, Zhao JR, Wang FG, Li JS, Dai JR (2006) Genome-wide isolation of resistance gene analogs in maize (*Zea mays* L.). Theor Appl Genet 113:63–72
3. McDowell JM, Woffenden BJ (2003) Plant disease resistance genes: recent insights and potential applications. Trends Biotechnol 21:178–183
4. Dangl JL, Jones JDG (2001) Plant pathogens and integrated defense responses to infection. Nature 411:826–833
5. Jones JDG (2001) Putting knowledge of plant disease resistance genes to work. Curr Opin Plant Biol 4:281–287
6. Maule AJ, Daowen W (1996) Seed transmission of plant viruses: a lesson in biological complexity. Trends Microbiol 4:153–158
7. Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc Natl Acad Sci USA 93:11746–11750
8. Yu Y, Buss G, Maroof M (1996) Isolation of a superfamily of candidate resistance genes in soybean based on a conserved nucleotide binding site. Proc Natl Acad Sci USA 93:11751–11756
9. Collins NC, Webb CA, Seah S, Ellis JG, Hulbert SH, Pryor A (1998) The isolation and mapping of disease resistance gene analogs in maize. Mol Plant Microbe Interact 11:968–978
10. Meyers BC, Shen KA, Rohani P, Gaut BS, Michelmore RW (1998) Receptor-like genes in the major resistance locus of lettuce are subject to divergent selection. Plant Cell 10:1833–1846
11. Huettel B, Santra D, Muehlbauer J, Kahl G (2002) Resistance gene analogues of chickpea (*Cicer arietinum* L.): isolation, genetic mapping and association with a Fusarium resistance gene cluster. Theor Appl Genet 105:479–490
12. Gao YL, Guo WZ, Wang L, Zhang TZ (2006) Isolation and characterization of resistance and defense gene analogs in cotton (Gossypium barbadense L.). Sci China C Life Sci 49:530–542
13. Pflieger S, Palloix A, Caranta C, Blattes A, Lefebvre V (2001) Defense response genes co-localize with quantitative disease resistance loci in pepper. Theor Appl Genet 103:920–929
14. Chen X, Soria MA, Yan G, Sun J, Dubcovsky J (2003) Development of sequence tagged site and cleaved amplified polymorphic sequence markers for wheat stripe rust resistance gene Yr5. Crop Sci 43:2058–2064
15. Shi ZX, Chen XM, Line RF, Leung H, Wellings CR (2001) Development of resistance gene analog polymorphism markers for the Yr9 gene resistance to wheat stripe rust. Genome 44:509–516
16. Radwan O, Bouzidi MF, Nicolas P, Mouzeyar S (2004) Development of PCR markers for the Pl5/Pl8 locus for resistance to *Plasmopara halstedii* in sunflower, *Helianthus annuus* L. from complete CC-NBS-LRR sequences. Theor Appl Genet 109:176–185
17. Zhang ZZ, Xu JS, Xu QJ, Larkin P, Xin ZY (2004) Development of novel PCR markers linked to the BYDV resistance gene Bdv2 useful in wheat for marker-assisted selection. Theor Appl Genet 109:433–439
18. Lanaud C, Risterucci AM, Pieretti I, N'Goran AK, Fargeas D (2004) Characterization and genetic mapping of resistance and defence gene analogs in cocoa (*Theobroma cacao* L.). Mol Breed 13:211–227
19. Noir S, Combes MC, Anthony F, Lashermes P (2001) Origin, diversity and evolution of NBS-type disease-resistance gene homologues in coffee trees (Coffea L.). Mol Gen Genomics 265: 654–662
20. Liu JJ, Ekramoddoullah AKM (2003) Isolation, genetic variation and expression of TIR-NBS-LRR resistance gene analogs from western white pine (*Pinus monticola* Dougl. ex. D. Don.). Mol Gen Genomics 270:432–441
21. Pflieger S, Lefebvre V, Caranta C, Blattes A, Goffinet B, Palloix A (1999) Disease resistance gene analogs as candidates for QTLs involved in pepper-pathogen interactions. Genome 42:1100–1110
22. Yamamoto E, Knap HT (2001) Soybean receptor-like protein kinase genes: paralogous divergence of a gene family. Mol Biol Evol 18:1522–1531

23. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
24. Nicholas KB, Nicholas HB Jr, Deerfield II DW (1997) GeneDoc: analysis and visualization of genetic variation. EMBnet News, 4: 14–17. Available from http://www.no. embnet.org/embnet.news/vol4_2/contents.html
25. Kumar S, Dudley J, Nei M, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299–306
26. Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral BW, Young ND (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide binding superfamily. Plant J 20:317–332
27. He CY, Tian AG, Zhang JS, Zhang ZY, Gai JY, Chen SY (2003) Isolation and characterization of a full-length resistance gene homolog from soybean. Theor Appl Genet 106:786–793
28. Radwan O, Bouzidi MF, Vear F, Philippon J, De Labrouhe DT, Nicolas P et al (2003) Identification of non-TIR-NBS-LRR markers linked to the Pl5/Pl8 locus for resistance to downy mildew in sunflower. Theor Appl Genet 106:1438–1446
29. Yan GP, Chen XM, Line RE (2003) Resistance gene-analog polymorphism markers co-segregating with the Yr5 gene for resistance to wheat stripe rust. Theor Appl Genet 106:636–643
30. Meyers BC, Kozik A, Griego A, Kuang HH, Michelmore RW (2003) Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. Plant Cell 15(4):809–834
31. Goff SA, Ricke D, Lan TH, Presting G, Wang RL, Dunn M et al (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. japonica). Science 296:79–92
32. Hanks SK, Hunter T (1995) The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J 9:576–596
33. Shirasu K, Schulze-Lefert P (2003) Regulators of cell death in disease resistance. Plant Mol Biol 44:371–385
34. Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. Cell 78:1101–1115
35. Dinesh-Kumar SP, Tham WH, Baker BJ (2000) Structure-function analysis of the tobacco mosaic virus resistance gene *N*. Proc Natl Acad Sci USA 97:14789–14794
36. Dinesh-Kumar SP, Baker BJ (2000) Alternatively spliced *N* resistance gene transcripts: their possible role in tobacco mosaic virus resistance. Proc Natl Acad Sci USA 97:1908–1913
37. Mestre P, Baulcombe DC (2006) Elicitor-mediated oligomerization of the tobacco N disease resistance protein. Plant Cell 18: 491–501
38. Zhang S, Klessig DF (2001) MAPK cascade in plant defense signaling. Trends Plant Sci 6:520–527