Theor Appl Genet (2005) 111: 819–830
DOI 10.1007/s00122-005-0002-7

ORIGINAL PAPER

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Isolation of TIR and nonTIR NBS–LRR resistance gene analogues and identification of molecular markers linked to a powdery mildew resistance locus in chestnut rose (*Rosa roxburghii* Tratt)

Received: 11 December 2004 / Accepted: 17 May 2005 / Published online: 2 August 2005

Abstract Toll and interleukin-1 receptor (TIR) and nonTIR nucleotide binding site–leucine rich repeat (NBS–LRR) resistance gene analogues (RGAs) were obtained from chestnut rose (*Rosa roxburghii* Tratt) by two PCR-based amplification strategies (direct amplification and overlap extension amplification) with degenerate primers designed to the conserved P-loop, kinase-2, and Gly-Leu-Pro-Leu (GLPL) motifs within the NBS domain of plant resistance gene (*R* gene) products. Thirty-four of 65 cloned PCR fragments contained a continuous open reading frame (ORF) and their predicted protein products showed homology to the NBS–LRR class *R* proteins in the GenBank database. These 34 predicted protein sequences exhibited a wide range (19.5–99.4%) of sequence identity among them and were classified into two distinct groups by phylogenetic analysis. The first group consisted of 23 sequences and seemed to belong to the nonTIR NBS–LRR RGAs, since they contained group specific motifs (RNBS-A-nonTIR motif) that are often present in the coiled-coil domain of the nonTIR NBS–LRR class *R* genes. The second group comprised 11 sequences that contained motifs found in the TIR domain of TIR NBS–LRR class *R* genes. Restriction fragment length polymorphic (RFLP) markers were developed from some of the RGAs and used for mapping powdery mildew resistance genes in chestnut rose. Three markers, RGA22C, RGA4A, and RGA7B, were identified to be linked to a resistance gene locus, designated *CRPM1* for *c*hestnut *r*ose *p*owdery *m*ildew resistance *1*, which accounted for 72% of the variation in powdery mildew resistance phenotype in an F1 segregating population. To our knowledge, this is the first report on isolation, phylogenetic analysis and potential utilization as genetic markers of RGAs in chestnut rose.

Communicated by H. Nybom

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Introduction

Powdery mildew fungi, belonging to the order Erysiphales of the phylum Ascomycota, are obligate biotrophic pathogens found on numerous economically important cultivated plants like cereals, vegetables, fruits, trees, roses, and many indoor plants (Linde et al. 2004). Powdery mildew disease in roses is mostly caused by *Sphaerotheca pannosa*. The pathogen can infect shoots and flowers but it primarily grows on leaves, causing leaf curling, deformation, and premature defoliation. As a result, both plant quality and vigor may be severely compromised. To date, there have been extensive studies on powdery mildew disease of various plant species such as cereals, vegetables, ornamentals, and the model plant *Arabidopsis* (Kunoh 1995; Huang et al. 2000; Linde et al. 2004; Xiao et al. 2001). However, powdery mildew disease is not well characterized in chestnut rose.

It is believed that chestnut rose (*Rosa roxburghii* Tratt) originated in China. Fruits of chestnut rose have a high content of vitamin C (2,000∼3,000 mg/100 g FW), and display high levels of superoxide dismutase (SOD) activity, and therefore are believed to have senescence-retarding and cancer-preventing effects (Ma et al. 1997; Wen et al. 2004). Chestnut rose is a rare fruit crop in Southwest China, and has recently been labeled as one of the three promising new fruit crops in China (Wen and Deng 2005a). Identification and ge-

netic mapping of powdery mildew resistance resources will facilitate cultivar improvement through marker-assisted selection (MAS) breeding programs. *R. roxburghii* cv. Guinong no. 6, an indigenous diploid ($2n = 14$) chestnut rose cultivar in Guizhou province of China, is highly resistant to powdery mildew. Our previous analysis with an F1 progeny derived from an intraspecific cross between *R. roxburghii* cv. Guinong no. 6 and *R. roxburghii* cv. Guinong no. 5 suggested that the resistance might be controlled by multiple genes (Wen et al. 2005b).

Over 40 *R* genes have been isolated from various plant species in the past decade. These *R* genes can be categorized into several distinct classes based on their predicted protein structures (Dangl and Jones 2001). The largest class (approximately 75%) falls into the NBS–LRR class, which encodes a nucleotide binding site (NBS) domain and a leucine rich repeat (LRR) domain. NBS–LRR *R* genes belong to a highly conserved gene family and are abundant in plant genomes. For example, there are approximately 150 in the *Arabidopsis* genome (Meyers et al. 2003) and nearly 600 in the rice genome (Monosi et al. 2004). NBS–LRR *R* genes are thought to function in pathogen-recognition and subsequent initiation of defense responses (Jones and Jones 1997; Van der Biezen et al. 2000). The molecular mechanisms that govern NBS–LRR function might follow a 'soft-wired signaling complex' model (Teruel and Meyer 2000). The NBS domain, which consists of P-loop, kinase-2, kinase-3a, and GLPL motifs, is highly conserved for ATP or GTP binding and hydrolysis activity; while the C-terminal LRR domain is presumed to ensure recognition-dependent activation of resistance (Tameling et al. 2002). NBS–LRR *R* genes can be further subdivided into toll and interleukin-1 receptor (TIR) and nonTIR classes based on the presence or absence of a TIR domain at the N terminus of the protein (Meyers et al. 1999). Most of the nonTIR NBS–LRR *R* genes have been reported to contain a coiled-coil motif or a leucine zipper structure in the N-terminal region (Pan et al. 2000). TIR and nonTIR NBS–LRR *R* genes can be anticipated on the basis of characteristic motifs present in the NBS domain (Meyers et al. 1999). For example, motifs RNBS-A-TIR (LQKKLLSKLL) and RNBS-D-TIR (FLHIACFF) occur exclusively in the TIR subclass, whereas RNAS-A-nonTIR (FDLxAWCCVSQxF) and RNBS-D-nonTIR (CFLYCELPED) are specific to the nonTIR subclass (Penuela et al. 2002). In addition, the single amino acid residue at the final position of the kinase-2 motif, the tryptophan (W) and asparatic acid (D) are characteristic of the nonTIR and TIR-type proteins respectively (Meyers et al. 1999).

The common motifs within the NBS domain that are highly conserved across plant species, have provided opportunities for isolating resistance gene analogues (RGAs) by use of PCR strategy with degenerate primers in a large number of plant species such as soybean (Kanazin et al. 1996; Yu et al. 1996), potato (Leister et al. 1996), lettuce (Meyers et al. 1999), cereals (Pan et al. 2000), sugar beet (Tian et al. 2004), rape (Tanhuanpaa 2004) and cotton (He et al. 2004; for a review, see Dangl and Jones 2001). The cloned RGAs have been found to cluster in plant genomes and some are closely linked to known resistance loci, or are part of known *R* genes (Kanazin et al. 1996; Yu et al. 1996; Collins et al. 1998; Aarts et al. 1998; Speulman et al. 1998; Ashfield et al. 2003; Radwan et al. 2004). Thus the identification of RGAs represents a potential powerful strategy for the amplification of resistance gene candidate sequences. These sequences can be developed into molecular markers for use in MAS or genetic mapping, or even positional cloning of new *R* genes. For example, Donald et al. (2002) developed, from RGA sequences, a 650-bp cleaved amplified polymorphic sequence (CAPS) marker and an 850-bp sequence characterized amplified region (SCAR) marker that co-segregated with the powdery mildew resistance locus in grapevine. A similar study was reported in citrus in which two RGAs (pt8a, pt9a) were mapped to a region containing *R* genes responsible for citrus tristeza virus resistance and nematode resistance (Deng et al. 2000).

In the present study, we cloned 11 TIR-type and 23 nonTIR-type RGAs from chestnut rose by two PCR strategies, and developed RFLP, CAPS, and sequence-tagged -site (STS) markers from some of these cloned RGAs for the initial mapping of powdery mildew resistance in *R. roxburghii* cv. Guinong no. 6 through QTL analysis.

Materials and methods

Mapping population and resistance phenotyping

An F1 population of 109 plants derived from an intraspecific cross between Guinong no. 6 (highly resistant) and Guinong no. 5 (highly susceptible) was used for genetic mapping of the powdery mildew resistance. Powdery mildew disease phenotypes were evaluated under natural disease pressures in late May of 2003, the peak time of powdery mildew disease outbreak in chestnut rose. The survey was repeated in the same period 2004. A scale with five steps ranging from 0 (no visible symptom of infection) to 4 (full coverage of the whole leaf surface by the fungus) was used to assess the disease severity. Disease index (DI) for each individual was calculated and analyzed according to Liu et al. (1996) and Wen et al. (2005b).

PCR amplification of RGA sequences

The genomic DNA of 'Guinong no. 6' was used as the DNA template for PCR amplification of RGAs. Degenerate primers for amplifying RGA sequence were listed in Table 1. PCR amplifications were carried out

Table 1 Degenerate primers used for PCR amplification from chestnut rose

Primer	Motif (annealing site)	Primer sequence[a]	Reference
F1	P-loop	GGAGTCGGTAAAACAACAATAG	Deng et al.(2000)
F2	P-loop	GGDGTDGGNAARACWAC	Deng et al.(2000)
F3	P-loop	GGDGTDGGNAARACSAC	Deng et al.(2000)
R1	GLPL	GAGGGCTAAAGGAAGGCC	Deng et al.(2000)
R2	GLPL	IARIGCIARIGGIARNCC	Deng et al.(2000)
R3	GLPL	AGIGCHAGNGGNAGNCC	Deng et al.(2000)
R4	GLPL	AGNGCHAGNGGYAANCC	Deng et al.(2000)
R5	GLPL	AGNGCYAANGGYAANCC	Deng et al.(2000)
R6	GLPL	AANGCHAGNGGYAANCC	Deng et al.(2000)
K2F	Kinase-2	ATCNACATCATCNAGHACRA	Gaspero et al. (2003)
K2R	Kinase-2	TYGTKCTNGATGATGTNGA	This paper

[a]The sequences are coded according to the International Units of Biochemistry: D (A, G or T); R (A or G); W (A or T); Y (C or T); S (G or C); H (A, C or T); M (A or C); K(G or T); N (A, G, T or C); I Inosine

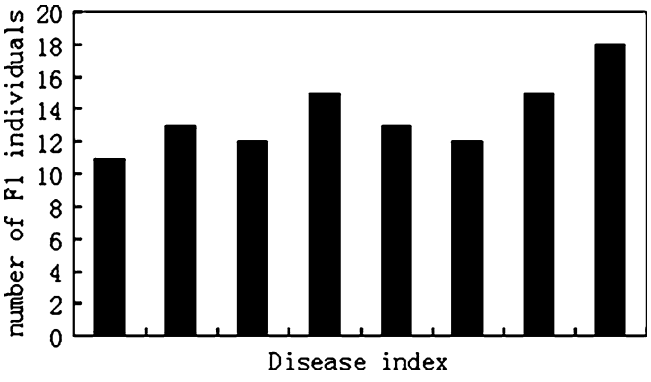


Fig. 1 Frequency distribution of disease index of 109 F1 plants derived from a cross between *R. roxburghii* cv. Guinong no. 6 and *R. roxburghii* cv. Guinong no. 5. The disease severity was evaluated in the field under natural infestation

using two strategies (Fig. 2). One is direct amplification with degenerate primers designed against the P-loop and GLPL motifs. The primer combinations F2 and R1, F2 and R5, F3 and R1, and F3 and R5 were used for such direct amplification. The amplification reactions were performed on a PTC-100 thermal cycler in a total volume of 50 µl consisting of 50 ng DNA, 5 µl 10× buffer (MBI Fermentas), 5 µl of 10 mM dNTP, 4 µl of 25 mM $MgCl_2$, 2 µl of 10 µM primers and 2 units of *Taq* polymerase (MBI Fermentas). The cycling conditions were as follows: 94°C 3 min followed by ten cycles of 94°C 30 s, annealing temperature stepdown each cycle of 0.7°C (from 58°C to 51°C), 72°C 1 min. The following 25 cycles were carried out as described above but with an annealing temperature of 57°C. This strategy only produced nonTIR-type RGAs in chestnut rose.

The other strategy was targeted to amplify TIR-type RGAs from chestnut rose by overlap extension amplification according to Ho et al. (1989). Four primers were used for this strategy. The outer two are F2 and R5 while the inner two are K2R and K2F, both of them were designed based on the kinase-2 motif of TIR-type NBS sequences (Fig. 2). The PCR protocol included two rounds of PCR amplifications. The first round was performed in two separate reaction amplifications: one with the primer combination of F2 and K2R (PCR1); the other with K2F and R5 (PCR2), and both were performed in a 50 µl volume as described above. The denaturing step was carried out at 94°C 3 min, followed by 32 cycles of 95°C 30 s, 45°C 1 min, and 72°C 1 min. The second round PCR (PCR3) was performed in a 50 µl reaction containing 1 pmol of both PCR1 and PCR2 derived DNA products, 5 µl 10× buffer (MBI Fermentas), 5 µl 10 mM dNTP, 4 ul of 25 mM $MgCl_2$, 2 µl of 10 µM primers (F2 and R5), and 2 units of *Taq* polymerase (MBI Fermentas). Cycling conditions were as follows: 94°C 3 min followed by 32 cycles of 94°C 30 s, 55°C 1 min, and 72°C 1 min.

Cloning and sequence analysis

PCR products were separated on a 1.5% agarose gel. Fragments of the expected size were excised from the gel and purified using a gel extraction column (Omega BioTek, USA). The obtained DNA was cloned into pMD18-T vector (Takara Bio Inc.). Recombinant plasmid DNA was extracted by alkaline lysis (Sambrook et al. 1989). Each clone was re-amplified with M13 universal primers and then subject to restriction analysis. Three restriction enzymes (*Taq*I, *Hae*III, and *Hin*fI) were used for digestion of the PCR products according to the manufacturer's instructions (MBI Fermentas). Unique clones were sequenced with the Bigdye Terminator V3.1 cycle sequencing kit on an ABI 3700 sequencer.

DNA sequences were compared with translated sequences using BLASTX (Altschul et al. 1997) in GenBank (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Phylogenetic trees were constructed using Mega 2.0 (Kumar et al. 2000) by comparing with known *R* genes, with the sequences un-trimmed and by full alignment. The bootstrap values for the dendrogram were produced using the "bootstrap" function of Mega2.0 with 1,000 replications.

Development of RGA-based STS, CAPS, and RFLP markers and genetic mapping

Twelve pairs of specific primers were designed from divergent RGA sequences with primer3 software (http://www-genome.wi.mit.edu/cgi-bm/primer/primer3-www.cgi). PCR products were separated by electrophoresis on 1.8% agarose gel or 6% polyacrylamide gel (60 w for 2 h). STS markers were generated if there was a size difference in the RGA products amplified from the two chestnut rose parents. RGA products showing no difference in size were cleaved with suitable restriction endonucleases (Table 3) to generate CAPS markers. Aliquots (6 μl) of the products were incubated for 4 h with 5 units of the enzyme according to the manufacturer's instruction. In addition, RGA DNA products were used as probes for Southern analysis of genomic DNA (10 μg) of both parents digested with six different restriction endonucleases (i.e., *Eco*RI, *Hin*dIII, *Bam*HI, *Eco*RV, *Dra*I, and *Taq*I). Southern hybridization was performed according to Xu et al. (2004). RGAs that revealed RFLPs between the two parental types were then used, along with polymorphic STS and CAPS markers, for identification of markers that were linked to powdery mildew resistance by bulked segregant analysis (BSA, Michelmore et al. 1991). The resistant and susceptible DNA bulks for powdery mildew resistance were prepared by pooling equal amount of DNA from the five most resistant and the five most susceptible individuals from the F1 population of Guinong no. 6 × Guinong no. 5. Two additional resistant bulks were constructed in a similar manner to confirm the polymorphism of the tested markers. Markers that are present in the resistant parent and resistant bulks, but are absent in the susceptible parent and susceptible bulks, were considered as putative powdery mildew resistance-linked markers and were further confirmed in a set of resistant and susceptible individuals that constituted the resistant and susceptible DNA bulks. The confirmed markers were used for genotyping of all the 109 plants in the F1 population.

Linkage analysis

Data and linkage analysis of the F1 population were performed according to Lambert et al. (2004). For a pseudotest-cross population, all the markers were maintained as two separate datasets. One was from the male (Guinong no. 5), and the other was from the female parent (Guinong no. 6). Only those markers derived from the resistant parent (Guinong no. 6) were considered in further analysis. The ratio of presence versus absence patterns of each marker was tested for goodness-of-fit to the expected ratio of 1:1. Markers with a chi square (χ^2) value greater than 3.84 ($a = 0.05$) were excluded from further analysis. Linkage analysis and QTL detection were performed using Map Manager QTXb20 (Meer et al. 2004), with the linkage criterion being set at $P = 0.05$.

Results

Phenotypic analysis of powdery mildew resistance

Genetically, chestnut rose is believed to be highly heterozygous (Wen et al. 2005b) and many traits may segregate in an F1 population of an intraspecific cross. Disease reaction phenotypes in relation to natural powdery mildew infection of 109 F1 plants derived from an intraspecific cross between *R. roxburghii* cv. Guinong no. 6 and *R. roxburghii* cv. Guinong no. 5 were evaluated for two consecutive years (2003 and 2004). Data in Fig. 1 showed that there was a wide segregation of the disease reaction phenotypes in this F1 population, ranging from completely resistant (disease index score 0) to highly susceptible (disease index score 39.5). This result indicated that the mildew-resistant parent Guinong no. 6 is heterozygous at the resistance gene loci. The continuous distribution of the phenotypic variations suggested that the resistance to powdery mildew in Guinong no. 6 might be under control of multiple genes, each of which has only a minor contribution to the resistance phenotype. However, it is also likely that the resistance phenotype is a result of an interaction between one or two major semi-dominant *R* genes and a variable local patho-environment due to the nature of the disease assay. Our QTL analysis indicated the existence of a major locus controlling the resistance phenotype (see later text).

Cloning of the TIR and nonTIR NBS–LRR RGAs from chestnut rose

With the degenerate primer pairs F2 and R1, F2 and R5, F3 and R1, and F3 and R5, a band of the predicted size (~500 bp) was observed after PCR amplification (Fig. 3). This band was excised from the agarose gel, its DNA fragments were cloned into the plasmid vector pMD18-T vector. Forty unique clones were chosen for DNA sequencing. Analysis of the 40 sequences against BLASTX algorithm demonstrated that 24 of the sequences (designated RGA1 to RGA24 respectively) had a high level of homology (*E*-values $< e \times 10^{-5}$) to the corresponding sequences of plant *R* genes and known RGAs in the GenBank database. Peptide sequences were deduced from these 24 clones. Unexpectedly, 23 of them (excluding RGA5) appeared to be nonTIR-type RGAs based on visual assessment according to the criterion reported by Meyers et al. (1999; Fig. 4). This was supported by the fact that all the 23 sequences contained the RNBS-A-nonTIR motif (FDLxKxWVSVSDDF), a characteristic motif specific to the nonTIR-type plant *R* genes (Meyers et al. 1999), and further, they contained a tryptophan (W) at the end of the kinase-2 motif (Fig. 4).

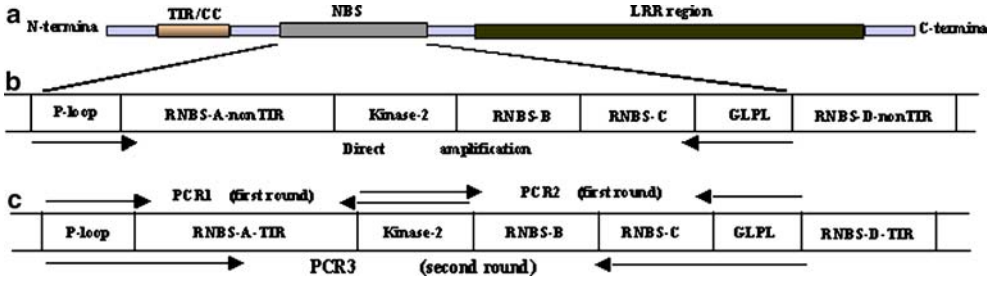


Fig. 2 Schematic model of the structure of NBS–LRR plant *R* genes and the two strategies employed to amplify nonTIR and TIR NBS–LRR resistance gene analogues (RGAs) from chestnut rose. **a** Putative function feature of NBS–LRR plant *R*-proteins; **b** conserved motifs in the nonTIR–NBS–LRR *R*-proteins and a direct amplification strategy with degenerate primers; and **c** conserved motifs in the TIR–NBS–LRR *R* proteins and an overlap extension amplification strategy with two steps

Among the remaining sequences, 11 showed similarity to transposon-like sequences, while the rest five sequences gave no or weak blast hits in the database (data not shown).

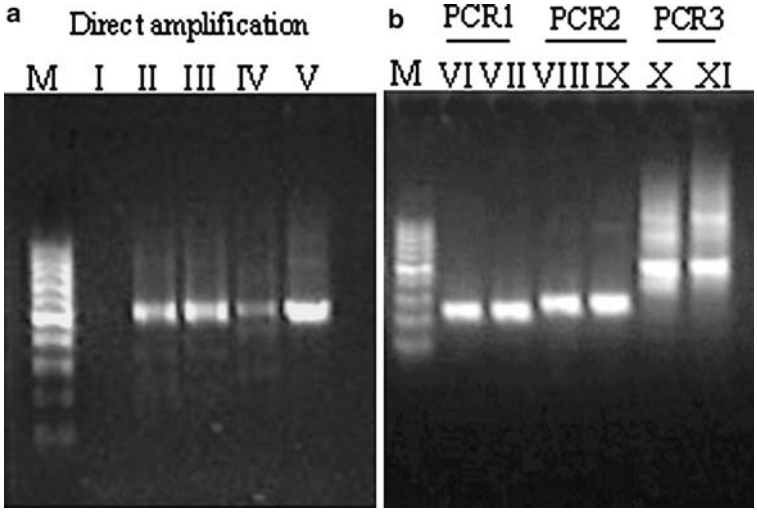
Another PCR strategy called overlap extension was employed to detect whether there is TIR-type RGAs in the genome of chestnut rose (Fig. 2). With the primer pairs of F2 and K2R, and K2F and R5, the first round of the two PCR reactions both generated approximately 300-bp bands. The second round with the first round PCR products as template produced the expected 500-bp band (Fig. 3). After ligating into pMD-18-T vector, 25 clones were used for sequencing. Sequence analysis revealed that ten of them were homologous to known RGAs. There existed TIR-type specific characteristics including an RNBS-A-TIR motif (LQxQLLSxxL) and an aspartic acid (D) at the end of the kinase-2 motif (Fig. 5). It is for this reason that these ten sequences, designated RGA25 to RGA34, were considered to be TIR-type RGAs according to the prediction methods described by Meyers et al. (1999).

Sequence and phylogenetic analysis

Sequence analysis showed that each of the 34 RGAs contained a single continuous ORF. As mentioned earlier, BLAST searches identified homology of these deduced protein sequences to several plant *R* protein sequences and to many known RGAs, i.e., up to 68% in the TIR-type RGA group and up to 65% in the non-TIR-type RGA group. The homology was considerably higher at internal motifs such as P-loop, kinase-2, kinase-3a, and GLPL (Figs. 4, 5). Therefore, we conclude that the 34 RGAs belong to the NBS–LRR superfamily in chestnut rose.

Phylogenetic analysis was conducted to determine the relationships among RGAs and other known plant *R* genes. The amino acid sequences were aligned and analyzed as described in Materials and methods. A consensus phylogenetic tree presented in Fig. 6 indicated that there were two distinct groups of RGAs. One was nonTIR-type RGAs (RGA1 to RGA24, excluding

Fig. 3 PCR products amplified with the two strategies **a** PCR products amplified by a direct amplification strategy. Lane I, negative control; Lanes II, III, IV and V, products of primer combinations of F2 and R1, F2 and R5, F3 and R1, and F3 and R5, respectively; **b** PCR products amplified by an overlap extension strategy. Lanes VI and VII, PCR products amplified by combination of F2 and K2R, Lanes VIII and IX, PCR products amplified by combination of K2F and R5, Lanes X and XI, PCR products based on the first amplification round products as template with the primers F2 and R5 for amplification



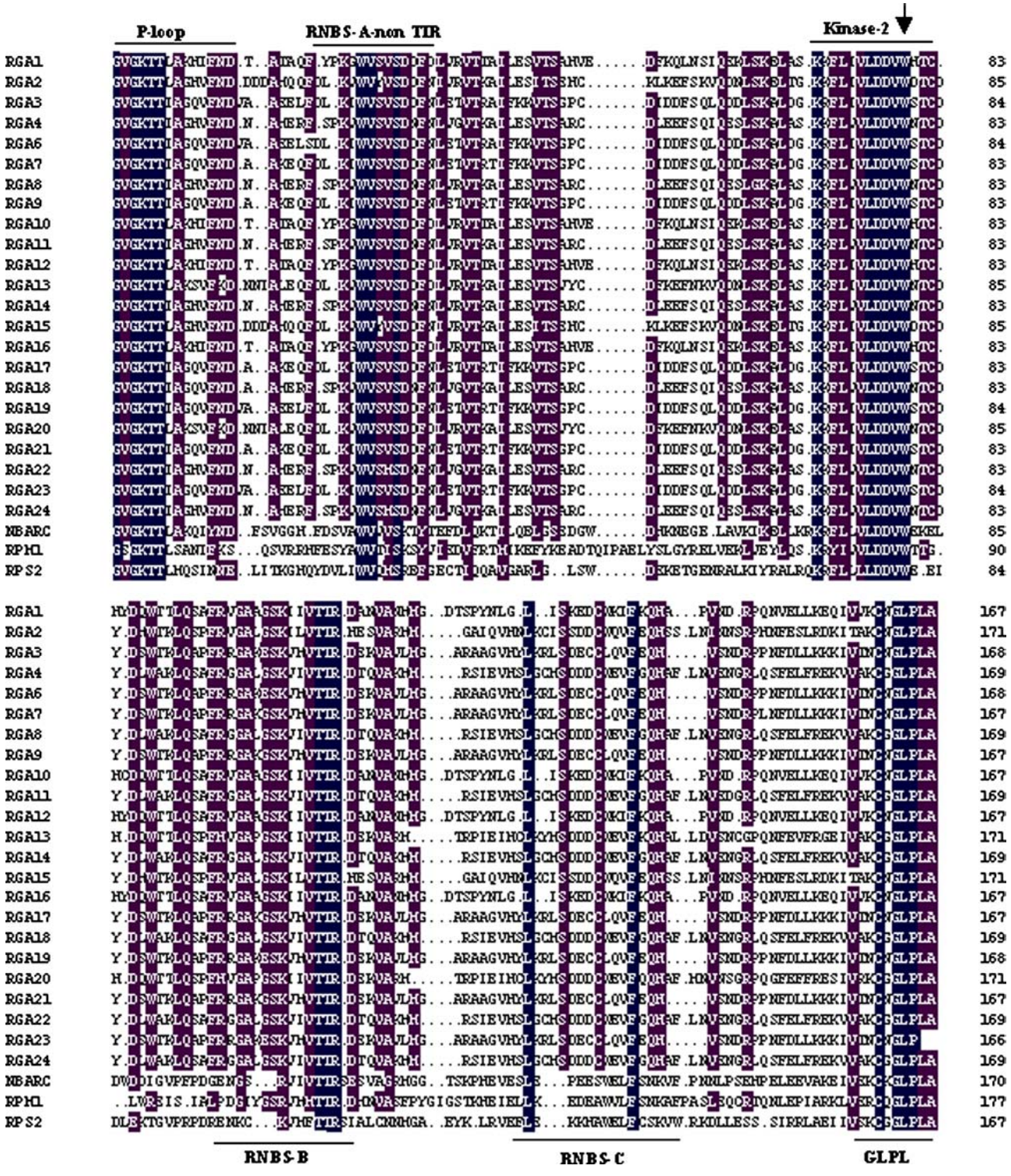


Fig. 4 Amino acid alignment of chestnut rose nonTIR RGAs and known nonTIR-NBS–LRR plant *R* genes. Motifs from P-loop to GLPL were identified according to Meyers et al. (1999). RNBS-A-nonTIR motif and the tryptophan (*W*) residues at the final position of Kinase-2 motif (*arrow indicated*) are exclusively in the nonTIR–NBS–LRR plant *R* genes and RGAs

RGA5); this was consistent with the grouping defined visually according to Meyers et al. (1999) as described in the earlier text. The other was TIR-type RGAs; this group contained RGA25 to RGA34, as also predicted by the visual method, and RGA5 that was not defined as TIR-type RGA by the visual method.

Based on sequence similarity and phylogenetic relationships, these 34 sequences could be divided into 11 subgroups, designated RGAI to RGA XI (Fig. 6). The sequence identity between the subfamilies ranged from 19.5% (between RGAI and RGAV) to 80.1% (between

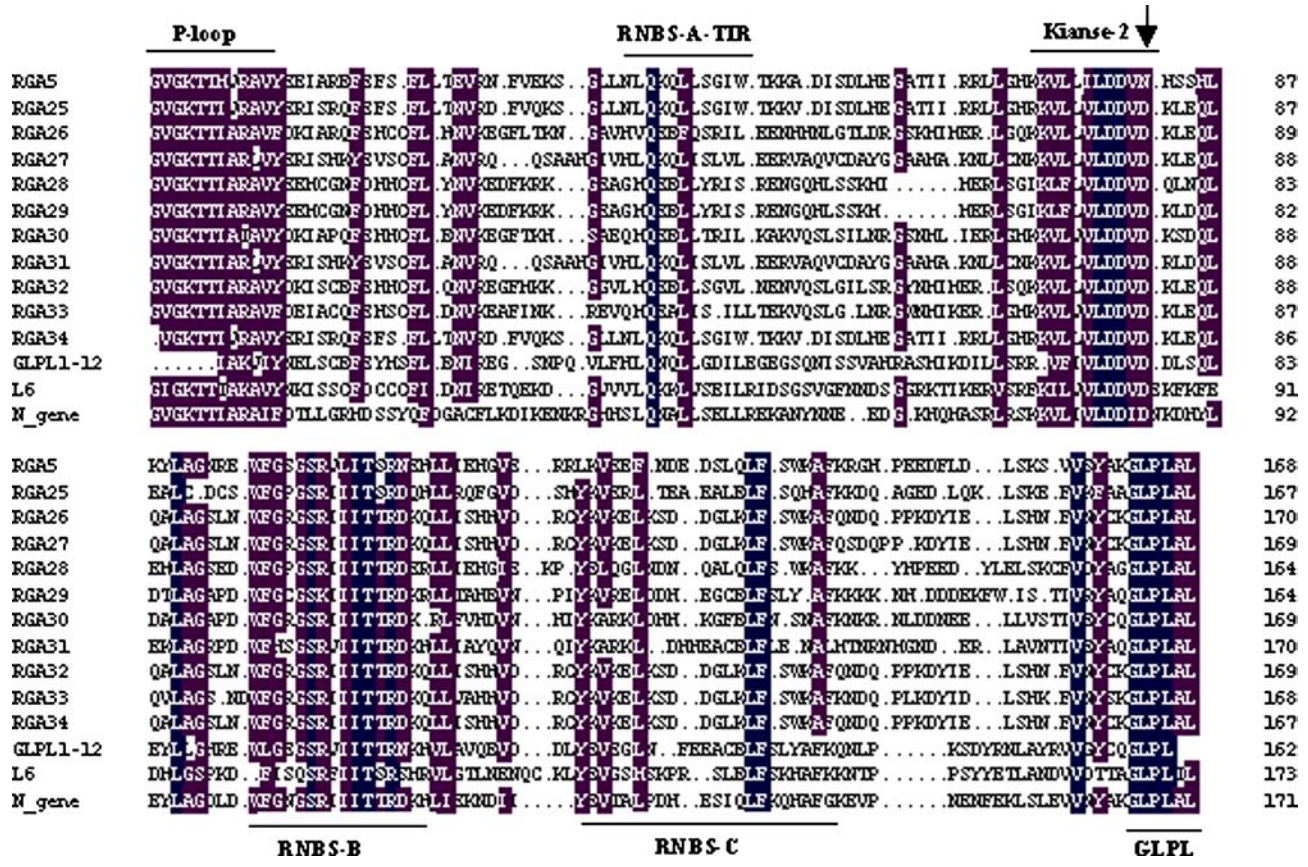


Fig. 5 Alignment of deduced amino acid sequences of TIR–NBS–LRR RGAs and known plant *R* genes belonging to the same class. RNBS-A-TIR motif and the asparatic acid (*D*) at the final position of Kinase-2 (*arrow indicated*) are the characteristics of TIR–NBS–LRR RGAs. GLPL1-12 sequence has been found to associate with the powdery mildew resistance locus (*Run1*) in grapevine (Donald et al. 2002)

RGAVII and RGAVIII) at the amino acid level (Table 2), while the sequence identity within each subfamily was >85%. Subgroups RGAI, RGAIII, and RGAIV consisted of four or more members of RGAs, the rest contained only one or two RGA members, reflecting a difference in abundance for these RGA subgroups in the chestnut rose genome. Moreover, subgroups RGAI, RGAII, RGAIII, RGAIV, RGAVI, RGAVII and RGAVIII were found to be most similar to the *I2C-2* gene from tomato, while RGAV, RGAIX, RGAX, RGAXI and RGAXII were most similar to the *N* gene from tobacco.

Development of RGA-STS, CAPS and RFLP markers

Sequences of 12 RGAs were used to design specific primers (Table 3). The genomic DNA of the parents and the mildew-resistant and susceptible bulks were used to screen the 12 primers for polymorphisms. The PRIMER4 pair detected a 500-bp STS marker that showed presence/absence polymorphism between resistant and susceptible parents/bulks. All the other primer pairs generated a monomorphic band (approximately 500-bp). To test if polymorphic CAPS markers could be developed, the PCR products amplified by these primers were subjected to enzymatic digestion with a panel of 17 restriction endonucleases. Results showed that five PCR product/enzyme combinations, i.e., PRIMER13/*Mbo*I, PRIMER1/*Dpn*I, PRIMER3/*Hin*fI, PRIMER22/*Dde*I, and PRIMER25/*Alu*I revealed polymorphisms between the resistant parent/bulks and the susceptible parent/bulks (Table 3).

Restriction fragment length polymorphic analysis was carried out to determine the copy number of certain RGA. Eleven representative RGAs (Fig. 6) were used as probes for Southern hybridization. Except for RGA1, RGA5 and RGA27, only a few bands were detected by the RGAs tested, suggesting that the majority of the RGAs had a low copy number in the chestnut rose genome (Fig. 7). RFLP analysis was also employed to identify markers associated with powdery mildew resistance using the BSA strategy. A total of seven loci were detected. Three RGAs, i.e., RGA22, RGA7 and, RGA4 were found to be associated with powdery mildew resistance. The RGA22/*Eco*RI combination revealed a 10-kb DNA fragment (RGA22C marker) that was present in resistant individuals, but absent in the susceptible ones (Fig. 7). The RGA7/*Eco*RV and RGA4/*Bam*HI combinations detected, respectively, a 14-kb and a 11-kb fragment that was polymorphic between the

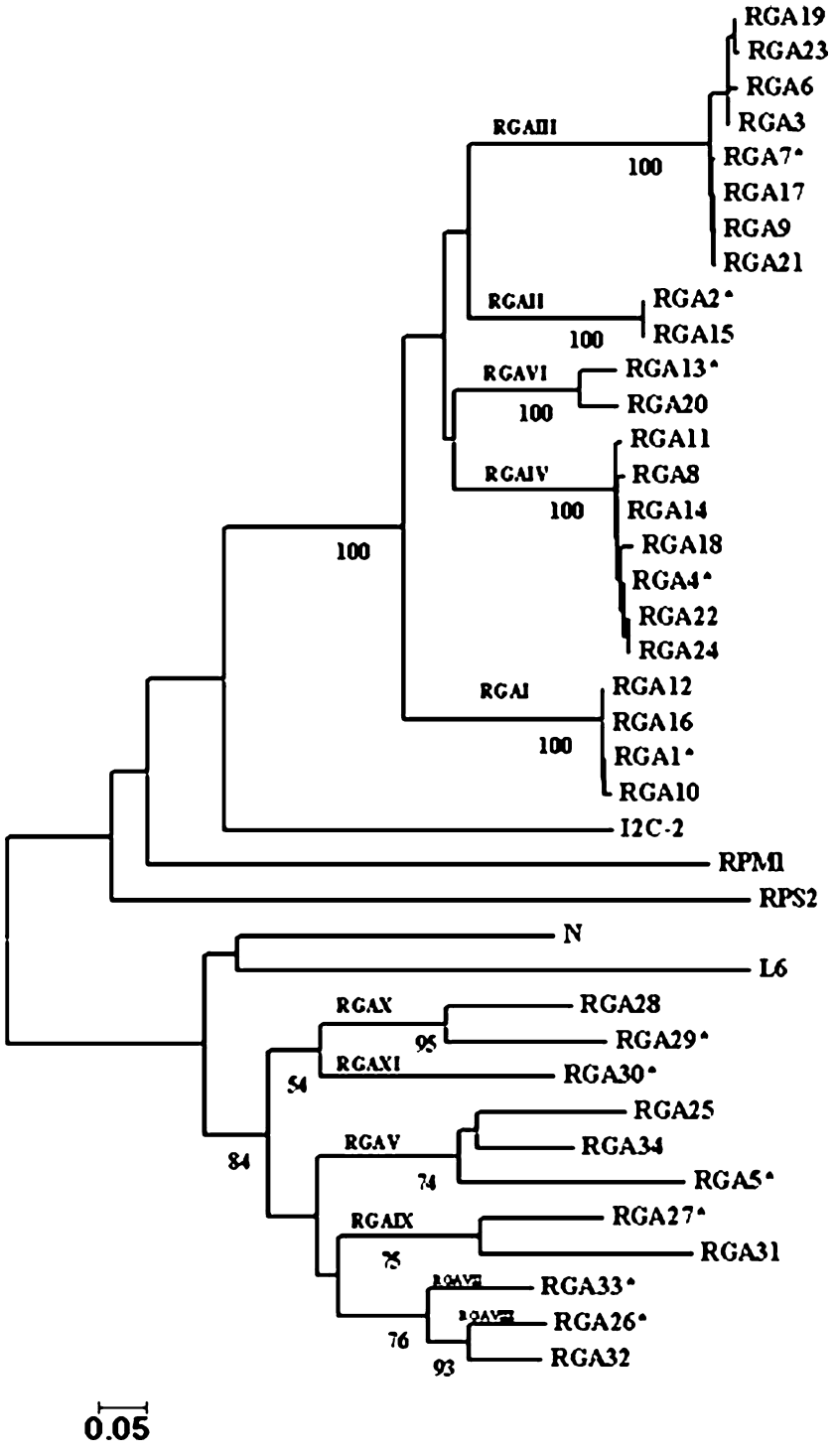


Fig. 6 Phylogenetic tree based on alignment of the deduced amino acid sequences of chestnut rose RGAs and known plant *R* genes. The tree was constructed using the neighbor-joining method provided in the Mega2.0 software. The 34 RGA sequences were grouped into 11 subgroups: RGAI to RGAXI (labeled over the branches *in bold*), and asterisks indicate the representatives of each group. Bootstrap values (1,000 replicates) are given below the branches. Known *R* genes with NBS domain and used in this study were tobacco *N* gene, flax *L6* gene (U27081), tomato *I2C-2* gene (T06404), *Arabidopsis RPM1* gene (X87851) and *RPS2* gene (U14158)

resistant parent/bulks and susceptible parent/bulks. However, of all the TIR-type RGAs subjected to RFLP analysis, none revealed polymorphism between parents.

Linkage analysis

The three RGA-based RFLP markers, one STS marker, and five CAPS markers were applied to genotyping of 109 F1 plants. Three RFLP markers, RGA22C, RGA7B, and RGA4A and the STSP4 marker segregated in a pattern of either presence or absence at the ratios of 52 (presence):50 (absence), 47:55, 54:51, and 51:56, respectively. These segregation ratios fitted a 1:1 ratio according to chi square analysis. Thirteen recombination events were detected between RGA22C and STSP4 marker, while eight were detected between STSP4 and RGA7B, and 27 between RGA7B and

Table 2 Similarity percentage of amino acid sequence from the P-loop motif through GLPL motif (167 residues) among the 11 representative sequences of the 11 RGA subgroups from chestnut rose and three known NBS–LRR plant *R* genes

	RGAI	RGAII	RGAIII	RGAIV	RGAV	RGAVI	RGAVII	RGAVIII	RGAIX	RGAX	RGAXI	I2C-2	RPM1
RGAII	63.0												
RGAIII	56.4	64.6											
RGAIV	63.6	66.3	61.6										
RGAV	19.5	21.0	26.3	23.9									
RGAVI	67.3	69.0	61.0	69.2	21.7								
RGAVII	27.9	26.1	25.7	29.7	49.1	26.1							
RGAVIII	27.4	25.0	25.2	29.1	47.3	25.6	80.1						
RGAIX	25.6	22.0	24.7	26.8	48.2	23.9	70.7	74.9					
RGAX	24.0	24.2	26.3	26.5	48.8	26.8	55.3	57.4	48.5				
RGAXI	26.9	25.8	27.9	30.6	40.2	27.7	64.0	59.5	47.6	49.7			
I2C-2	43.6	42.8	39.3	42.4	24.8	42.8	29.7	28.6	26.9	27.8	30.0		
RPM1	33.1	25.7	27.6	30.9	20.9	28.1	28.1	26.4	25.3	24.4	27.2	30.6	
N	27.5	25.6	25.8	29.9	38.3	29.5	47.5	47.8	43.2	45.0	44.1	30.7	24.9

Values were calculated using the Multiple Sequence Alignment Program with full alignment method in the DNAman software (a gap creation penalty = 10, and a gap extension penalty = 2)

RGA4A. By QTL analysis, a major locus explaining 72% variation of the powdery mildew resistance, was detected with a LOD = 10.7 in the interval defined by markers RGA22C and RAPDAL09. This locus was tentatively named *CRPM1*. RAPDAL09 is a previously developed RAPD marker segregating in a ratio of 1 (presence):1 (absence) in this F1 population (Data unpublished). Further linkage analysis showed that the RGA22C marker was about 3.6 cM from the *CRPM1* locus (Fig. 8).

Discussion

Targeted isolation of TIR-type and nonTIR-type RGAs by different PCR strategies

Here the successful isolation of RGAs from a perennial indigenous fruit crop—chestnut rose has been reported. With the intent of characterizing a broad set of NBS–LRR candidate genes, two PCR-based strategies were employed. By direct amplification, with several degenerate primer combinations including F2 and R1, F2 and R5, F3 and R1, and F3 and R5, only nonTIR-type RGAs were isolated. It is somewhat surprising because the primers F2/F3 and R1/R5 were designed to anneal to the motifs of the P-loop and GLPL respectively, which were conserved in both TIR and nonTIR-type NBS–LRR *R* genes. In addition, the same degenerate primers used to amplify citrus genomic DNA, yielded both TIR-type RGAs and nonTIR-type RGAs (Deng et al. 2000). One explanation for this phenomenon is that because of likely biased codon usage within the conserved motif, these primer pairs probably led to preferential amplification of the nonTIR-type sequences. Similarly, Zhu et al. (2002) reported that in *Medicago* different primer combinations could only amplify either TIR NBS–LRR RGAs or nonTIR NBS–LRR RGAs, but both types of RGAs could be cloned from soybean (*Glycine max*) using anyone of the primer combinations. The biased sampling due to preferential amplification now becomes a main concern for the use of a PCR-based strategy to survey a multigene family (Di Gaspero and Cipriani 2003).

According to previous studies, TIR-type NBS–LRR genes appeared to be "lost" in cereals (Pan et al. 2000) and in coffee trees (Noir et al. 2001). It is not known if TIR–NBS–LRR *R* genes are present in the chestnut rose genome. By using an overlap-extension PCR strategy, we successfully isolated TIR-type RGAs from chestnut rose. Previous studies only documented isolating targeted subsets of RGAs by choosing highly selective primers that are specific to either TIR-type or nonTIR-type NBS–LRR *R* genes (Penuela et al. 2002). Our success in amplifying targeted RGA sequences with the overlap-extension strategy suggested that this method is efficient for isolating group-specific RGAs with high fidelity and may be applied on other plant species.

Diversity and evolutionary histories of *R* genes in chestnut rose

RGAs encoding a NBS motif have been successfully isolated from a broad range of plant species. To our knowledge, results from this investigation provide the first view of the complexity and diversity of RGAs in chestnut rose. Sequence homology among the 34 RGA nucleotide sequences ranged from 26.9% to 99.8%, with the highest value for RGA3 and RGA6 (99.8) and the lowest for RGA25 and RGA5 (26.9). It is likely that RGA3 and RGA6 might have arisen from a recent duplication of a common ancestor gene and that RGA25 and RGA5 may represent divergent origins. It is believed that duplicated RGA sequences in a given plant genome are probably subject to unequal crossing-over events for generation of diversity (Meyers et al. 1998; Song et al. 1997; Ellins et al. 1999; Cana et al. 2003). However, so far clear evidence is lacking for genetic

Table 3 Specific primers, product sizes, and polymorphisms detected by RGA-based STS or CAPS marker

RGA class	Name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	T_m (°C)[a]	Product size[b]	polymorphism[c]
NonTIR–NBS–LRR	PRIMER1	TTTTGGGGTCTGTCGTTTACGG	GGGGTGGGGAAGACAACACTC	57	446	*Dpn*I
NonTIR-NBS–LRR	PRIMER2	GGGTAATCCATTGCATTTTGCAG	TGCCATGCAACAGTTTGACCTT	55	457	-
NonTIR-NBS–LRR	PRIMER3	GTAATCCACCGCATTTGGCAAC	TAAGACAACGATTGCGGGACAA	58	424	*Hin*fI
NonTIR–NBS–LRR	PRIMER4	GGGTAACCCACCACATTTTGCT	CAATGGAACGGTTTAGTCCCAAAG	55	455	SSCP
NonTIR–NBS–LRR	PRIMER7	GGGCTAAGGGTAATCCATTGCAG	AATGATGCCGCAAAGGAACAGT	55	466	-
NonTIR–NBS–LRR	PRIMER11	GGTAAGCCACCACATTTTGCT	CAATGGAACGGTTTAGTCCCAAAG	55	454	-
NonTIR–NBS–LRR	PRIMER13	GCCCTGGAACAGTTCGATCTTA	GTAATCCACCGCATTTGGCAAC	60	462	*Mbo*I
NonTIR–NBS–LRR	PRIMER17	AGACAACGATTGCGGGACAAGT	AATTTGGTGGCCTGTCATTGCT	58	438	-
NonTIR–NBS–LRR	PRIMER22	CTCCCGAAACAACTCAAAGC	CGCAGGTCATGTCTTCAATG	60	451	*Dde*I
TIR–NBS–LRR	PRIMER5	TCGACGATTGAGGGCTAAAGGA	GAGGAAATCGCTCGTGAATTTGA	54	476	-
TIR–NBS–LRR	PRIMER25	GGGGTGGGCAAGACTACTAT	CCAGCAGCAAATTTCACAAA	51	485	*Alu*I
TIR–NBS–LRR	PRIMER27	GCTAAAGGAAGGCCCTTACAA	GGGGTTGGGAAGACTACGAT	51	503	-

[a]Annealing temperature used in the amplifications with genomic DNA of resistant parent (Guinong no. 6)
[b]Based on the template RGA sequences
[c]Polymorphisms detected between resistant parent (Guinong no. 6) and susceptible parent (Guinong no. 5)

events or mechanisms that are responsible for the complexity and diversity of RGAs in plant genomes.

Genetic and genomic studies have provided insights into the evolution of *R* genes and the mechanisms generating variation in NBS–LRR *R* genes (for a review, see Meyers et al. 2003). Two main important genetic mechanisms including a slowly evolving divergence hypothesis (Michelmore and Meyers 1998; Stahl et al. 1999), and a rapidly evolving process (Leister et al. 1998; McDowell et al. 1998) have been proposed to account for *R* gene evolution. Molecular data of this investigation also provides clues for the putative mechanisms that have contributed to the evolution of *R* genes in chestnut rose. Point mutations, small insertions or deletions in the regions between the conserved motifs were observed and these kinds of genetic variations constitute the primary source for RGA cluster divergence in chestnut rose. Taking these into consideration, the evolution of NBS–LRR-encoding genes in chestnut rose appeared to adopt a slowly evolving divergence mechanism rather than a rapidly evolving process, as previously suggested by Michelmore and Meyers (1998) and Tian et al. (2004). A greater degree of divergence was observed among TIR-type RGAs compared to nonTIR-type RGAs, suggesting that TIR-type RGAs may have been evolving more rapidly than nonTIR types. However, in the absence of molecular data for the complete set of NBS–LRR *R* genes from chestnut rose, it cannot be inferred with certainty what kind of evolutionary mechanisms are responsible for *R* gene evolution in chestnut rose.

Utility of chestnut rose RGAs as *R* gene-linked markers

Previous studies indicated that some RGAs might be genetically located at, or near known resistance loci (Kanazin et al. 1996; Yu et al. 1996; Collins et al. 1998; Donald et al. 2002; Ashfield et al. 2003; Radwan et al. 2004). For example, two RGAs were found in close linkage to the nematode resistance locus *Gro1* in potato (Leister et al. 1996) and three RGA markers were linked to the CTV and nematode resistance in citrus (Deng et al. 2000). In this study, 4 RGA-derived markers (three RFLP markers and one STS marker) were found to be associated with *CRPM1*, a major R locus contributing to powdery mildew resistance in chestnut rose cultivar Guinong no. 6, further demonstrating the utility of RGAs in *R* gene mapping. However, more accurate tests of the disease reaction phenotypes of the existing F1 plants in response to powdery mildew attack and an increase of the F1 population size are required to define the genetic map of the *CRPM1* locus. Nevertheless, the closest flanking markers, RGA22C and RAPDAL09 identified in this study should be useful for screening for more recombinant F1 plants for further mapping of *CRPM1* and for MAS breeding programs for powdery mildew-resistant cultivars of chestnut rose.

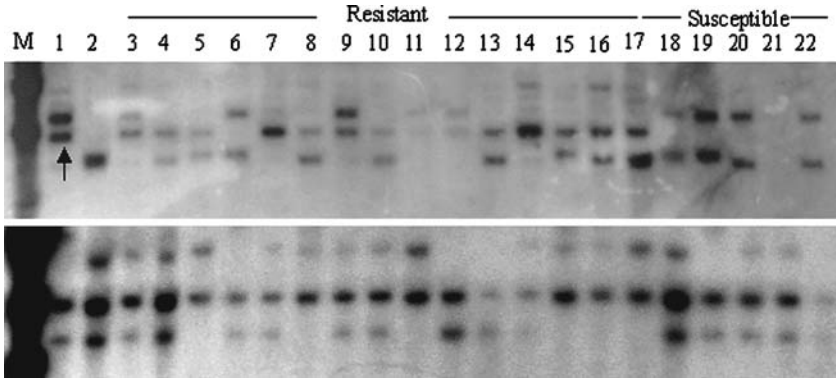


Fig. 7 NonTIR and TIR-type RGAs were used as probes for Southern hybridization. **a** With the probe/enzyme combination of RGA22 (nonTIR-type RGA)/*Eco*RI, a 10-kb marker (*arrow indicated*) tightly linked to the powdery mildew resistance was detected in the 15 individuals from the three resistant bulks but was absent in the five individuals from the susceptible bulk. **b** Analysis with the combination of RGA28/*Bam*HI. RGA28 belongs to TIR-type RGA. M, λDNA/*Hin*dIII marker; Lane 1, resistant parent (Guinong no. 6); Lane 2, susceptible parent (Guinong no. 5); Lanes 3–17, typical resistant individuals from the F1 population; Lanes 18–22, typical susceptible individuals from the F1 population

Acknowledgements This project was supported by the National Natural Science Foundation of China (NSFC) (Nos.30260070 30123001 and 30471201), the 863 Project of China, and Natural Science Foundation of Guizhou Province. The authors are grateful to Dr. S. Xiao from Center for Biosystems Research of University of Maryland Biotechnology institute, Dr. Z. Deng from Gulf Coast Research and Education Center of University of Florida, and Dr. W.W. Guo from our laboratory for critical review and helpful advice, and to Prof. K. Watanabe from Plant Genetic Diversity Gene Research Center of Tsukuba University (Japan) for his reading the manuscript. Our appreciations also extend to Mr. Y.Z. Liu, J.K. Song, X.D. Cai and S.H. Zeng in our laboratory for their assistance with the experiments.

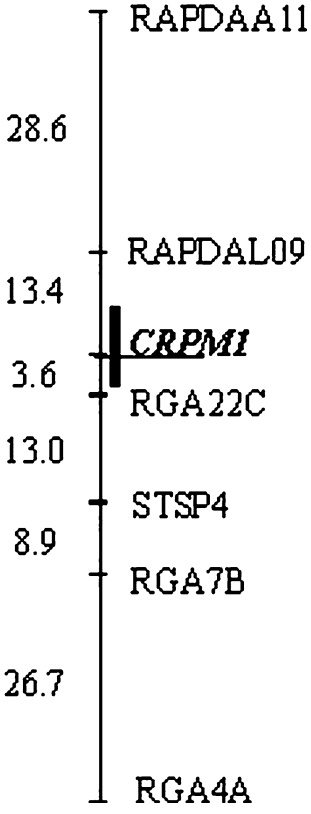


Fig. 8 A portion of the linkage map showing the four RGA-derived molecular markers that were associated with powdery mildew resistance (RGA22C, RGA7B, RGA4A and STSP4) and the major QTL (*CRPM1*). The confidence interval of the major QTL was indicated with a *solid box*. The peak of LOD value was indicated with a *wide line*. Genetic distances were calculated using the Kosambi centi-Morgans

References

Aarts MG, Hekkert BL, Holub EB, Beynon JL, Stiekema WJ, Pereira A (1998) Identification of *R*-gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. Mol Plant Microbe Interact 11:251–258

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programmes. Nucleic Acids Res 25:3389–3402

Ashfield T, Bocian A, Held D, Henk AD, Marek LF, Danesh D, Penuela S, Meksem K, Lightfoot DA, Young ND, Shoemaker RC, Innes RW (2003) Genetic and physical localization of the soybean *Rpg1-b* disease resistance gene reveals a complex locus containing several tightly linked families of NBS–LRR genes. Mol Plant Microbe Interact 16:817–826

Cana EF, Geffroy V, Macadre C, Creusot F, Imbert-Bollore P, Sevignac M, Langin T (2003) Characterization of expressed NBS–LRR resistance gene candidates from common bean. Theor Appl Genet 106:251–261

Collins NC, Webb CA, Seah S, Ellis JG, Hulber SH, Pryor A (1998) The isolation and mapping of disease resistance gene analogs in maize. Mol Plant Microbe Interact 11:968–978

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature 411:826–833

Deng Z, Huang S, Ling P, Chen C, Yu C, Weber CA, Moore GA, Gmitter FG (2000) Cloning and characterization of NBS–LRR class resistance-gene candidate sequences in citrus. Theor Appl Genet 101:814–822

Di Gaspero G, Cipriani G (2003) Nucleotide binding site/leucine-rich repeats, Pto-like and receptor-like kinases related to disease resistance in grapevine. Mol Gen Genomics 269:612–623

Donald TM, Pellerone F, Adam-Blondon AF, Bouquet A, Thomas MR, Dry IB (2002) Identification of resistance gene analogs linked to a powdery mildew resistance locus in grapevine. Theor Appl Genet 101:301–308

Ellins JG, Lawrence GJ, Luck JE, Dodds N (1999) Identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-for-gene specificity. Plant Cell 11:495–506

He LM, Du CG, Covaleda L, Xu ZY, Robinson AF, Yu JZ, Kohel RJ, Zhang HB (2004) Cloning, characterization, and evolution of the NBS–LRR-encoding resistance gene analogue family in polyploid cotton (*Gossypium hirsutum* L). Mol Plant Microbe Interact 17:1234–1241

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51–59

Huang CC, Cui YY, Weng CR, Zabel P, Lindhout P (2000) Development of diagnostic PCR markers closely linked to the tomato powdery mildew resistance gene *Ol-1* on chromosome 6 of tomato. Theor Appl Genet 101:918–924

Jones DA, Jones JDG (1997) The role of leucine-rich repeat proteins in plant defences. Adv Bot Res 24:90–167

Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc Natl Aca Sci USA 93:11746–11750

Kumar S, Tamura K, Jakobsen I, Nei M (2000) Mega 2.0. http://www.megasoftware.net

Kunoh H (1995) Host-parasite specificity in powdery mildews. In:Singh US, Kohmoto K, Singh RP (eds) Pathogenesis and host specificity in plant diseases: histopathological, biochemical, genetic and molecular bases, vol. 2 Eukaryotes. Elsevier, Oxford, pp 239–250

Lambert P, Hagen LS, Arus P, Audergon JM (2004) Genetic linkage maps of two apricot cultivars (*Prunus armeniaca* L.) compared with the almond Texas×peach Earlygold reference map for *Prunus*. Theor Appl Genet 108:1120–1130

Leister D, Ballvora A, Salamini F, Gebhardt C (1996) A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nat Genet 14:421–429

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Graner A, Schulze-Lefer P (1998) Rapid reorganization of resistance gene homologues in cereal genomes. Proc Natl Acad Sci USA 95:370–375

Linde M, Mattiesch L, Debener T (2004) *Rpp1*, a dominant gene providing race-specific resistance to rose powdery mildew (*Podosphaera pannosa*): molecular mapping, SCAR development and confirmation of disease resistance data. Theor Appl Genet 109:1261–1266

Liu L, Kloepper JW, Tuzun S (1996) Induction of systemic induced resistance in cucumber against *Fusarium* wilt by plant growth-promoting rhizobacteria. Phytopathology 85:695–698

Ma YX, Zhu Y, Wang CF (1997) The aging retarding effect of 'Long-Life CiLi'. Mech Ageing Dev 96:171–189

McDowell JM, Dhandaydham M, Long TA, Aatrs MG, Goff S, Holub EB, Dangl JL (1998) Intragenic recombination and diversifying selection contribute to the evolution of downy mildew resistance at the *RPP8* locus of *Arabidopsis*. Plant Cell 10:1861–1874

Meer J, Cudmore R, Manly KF (2004) Map Manager QTX. http://www.mapmanager.org/mmQTX.html

Meyers BC, Chin DB, Shen KA, Sivaramakrishnan S, Lavelle DO, Zhang Z, Michelmore RW (1998) The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. Plant Cell 10:1817–1832

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

Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW (2003) Genome-wide analysis of NBS–LRR-encoding genes in Arabidopsis. Plant Cell 15:809–834

Michelmore RW, Paran l, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130

Monosi B, Wisser R J, Pennill L, Hulbert SH (2004) Full-genome analysis of resistance gene homologues in rice. Theor Appl Genet 109:1434–1447

Noir S, Combes M-C, 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

Pan Q, Wendel J, Fluhr R (2000) Divergent evolution of plant NBS–LRR resistance gene homologues in dicot and cereal genomes. J Mol Evol 50:203–213

Penuela S, Danesh D, Young ND (2002) Targeted isolation, sequence analysis, and physical mapping of nonTIR NBS–LRR genes in soybean. Theor Appl Genet 104:261–272

Radwan O, Bouzidi MF, Nicolas P, Mouzeyar S (2004) Development of PCR markers of the *PI5*/*PI8* locus for resistance to *Plasmopara halstedii* in sunflower, *Helianthus annuus* L. from complete CC-NBS–LRR sequences. Theor Appl Genet 109:176–185

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbour Laboratory, New York, pp 19–22

Song WY, Pi LY, Wang GL, Gardner J. Olsten T, Ronald PC (1997) Evolution of the rice Xa21 disease resistance gene family. Plant Cell 9:1279–1287

Speulman E, Bouchez D, Holub EB, Beynon JL (1998) Disease resistance gene homologs correlate with disease resistance loci of *Arabidopsis thaliana*. Plant J 14:467–474

Stahl EA, Dwyer G, Mauricio R, Kreitman M, Bergelson J (1999) Dynamics of disease resistance polymorphism at the *Rpm1* locus of *Arabidopsis*. Nature 400:667–671

Tameling WIL, Elzinga SDJ, Darmin PS, Vossen JH, Takken FLW, Harling MA, Cornelissen B (2002) The tomato *R* gene products I-2 and Mi-1 are functional ATP binding proteins with ATPase activity. Plant Cell 14:2929–2939

Tanhuanpaa P (2004) Identification and mapping of resistance gene analogs and a white rust resistance locus in *Brassica rapa ssp. Oleifera*. Theor Appl Genet 108:1039–1046

Teruel MN, Meyer T (2000) Translocation and reversible localization of signaling proteins: a dynamic future for signal transduction. Cell 2:181–184

Tian Y, Fan L, Thurau T, Jung C, Cai D (2004) The absence of TIR-type resistance gene analogues in the sugar beet (*Beta vulgaris* L.) genome. J Mol Evol 58:40–53

Van der Biezen EA, Sun J, Coleman MJ, Bibb MJ, Jones JDG (2000) *Arabidopsis* RelA/SpoT homologs implicate (p)ppGpp in plant signaling. Proc Natl Acad Sci USA 97:3747–3752

Wen XP, Pang XM, Deng XX (2004) A comparative study of morphological, RAPD, AFLP approaches to characterize relationships of *Rosa roxburghii* Tratt and its relatives. J Hortic Sci Biotechnol 79:189–196

Wen XP, Deng XX (2005a) Micropropagation of chestnut rose (*Rosa roxburghii* Tratt) and genetic stability assessment of the in vitro plants using RAPD and AFLP markers. J Hortic Sci Biotechnol 80:54–60

Wen XP, Xu Q, Fan W, Deng X (2005b) A preliminary study on inheritance tendency of the resistance to powdery mildew using F_1 progenies of chestnut rose (*Rosa roxburghii* Tratt). Acta Horticulturae Sinica 320: 304–306

Xiao SY, Ellwood S, Callis O, Patrick E, Li TX, Coleman M, Turner JG (2001) Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by *RPW8*. Science 291:118–120

Xu Q, Wen XP, Deng XX (2004) A simple protocol for isolation of genomic DNA from chestnut rose (*Rosa roxburghii* Tratt) for PCR and RFLP analysis. Plant Mol Biol Reptr 22:301–302

Yu YG, Buss GR, Maroof MA (1996) Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proc Natl Acad Sci USA 93:11751–11756

Zhu H, Cannon SB, Young ND, Cook DR (2002) Phylogeny and genomic organization of the TIR and nonTIR NBS–LRR resistance gene family in *Medicago truncatula*. Mol Plant Microbe Interact 15:529–539