Acta Biologica Hungarica 62(2), pp. 171–181 (2011)
DOI: 10.1556/ABiol.62.2011.2.7

NON-TIR-NBS-LRR RESISTANCE GENE ANALOGS IN APRICOT (*PRUNUS ARMENIACA* L.)

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(Received: July 7, 2010; accepted: August 17, 2010)

Genes encoding for proteins with nucleotide-binding site and leucine-rich repeat motifs (NBS-LRR) have been suggested to play a general role in plant defence mechanism. In *Prunus* species, many TIR (Toll / Interleukin-1 Receptor), and only very few non-TIR sequences were identified, which was explained either by the unequal distribution of TIR/non-TIR sequences in the *Prunus* genome or by the incapability of primers in the amplification of non-TIR RGAs. The objective of this work was to check whether a new *semi-nested* PCR strategy can be developed for the targeted isolation of non-TIR-NBS-LRR Resistance Gene Analog (RGA) sequences from apricot. Three primers (CUB-P-loop F, CUB-Kin2 F and CUB-HD R) were designed, from which CUB-Kin2 F and CUB-HD R were constructed to anneal selectively to the non-TIR sequences. A colony Polymerase Chain Reaction (PCR) indicated that out of the 96 clones tested 28 showed amplification using the newly developed primers, while no amplification occurred when using the formerly described primers. Half of the 28 positive clones were sequenced and they turned out to represent 11 different non-TIR RGA sequences. A phylogenetic analysis was carried out based on an alignment containing 293 *Rosaceae* and 21 non-*Rosaceaa* sequences. A significantly higher ratio (91%) of non-TIR sequences were arranged in multi-genera clades than that of (57%) the TIR groups confirming that non-TIR sequences might be of more ancient origin than TIR sequences.

Keywords: Apricot – *Prunus armeniaca* L. – resistance gene analogs – RGA – non-TIR-NBS-LRR

INTRODUCTION

Over 60 plant disease resistance (R) genes have been cloned from model and crop species. Of these, 40 genes encode for proteins containing a nucleotide-binding site and leucine-rich repeat motifs (NBS-LRR) [16]. Consistent identification of this class of proteins across diverse plant species was suspected to indicate a general role for NBS-LRR genes in plant defence mechanism. Genome sequencing projects also revealed that NBS-LRR genes are abundant in plant genomes. Based on resistance domain analyses, the grape genome was found to contain 459 NBS-LRR-encoding genes, whereas 147, 330 and 464 NBS-LRR genes were found in *Arabidopsis*, poplar and rice, respectively [5, 18].

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The NBS motif is part of a conserved domain acting as a molecular switch for the signal transduction. The LRR is thought to be responsible for the recognition specificity similarly to the antibody-like detector of pathogens. At the N terminus, NBS-LRR proteins carry either the coiled coil (CC) domain or a domain homologous to the Toll/Interleukin-1 Receptor (TIR), allowing classification of NBS genes into two groups, the non-TIR-NBS-LRRs, present in all angiosperms, and the TIR-NBS-LRRs that are specific to dicotyledonous species [11].

TIR- and non-TIR-NBS-LRR sequences can be distinguished based on their amino acid motifs within the NBS domain. While motifs such as the P-loop, kin-1 a, and Gly-Pro-Leu-Pro-Leu-Ala (hydrophobic motif) are present in both classes, motifs RNBS-A-TIR (Leu-Gln-Lys-Lys-Leu-Leu-Ser-Lys-Leu-Leu) and RNBS-D-TIR (Phe-Leu-His-Ile-Ala-Cys-Phe-Phe) are found exclusively in the TIR class. In addition, RNBS-A-non-TIR (Phe-Asp-Leu-x-AlaTrp-Val-Cys-Val-Ser-Gln-x-Phe) and RNBS-D-non-TIR (Cys-Phe-Leu-TyrCys-Ala-Leu-Phe-Pro-Glu-Asp) are found exclusively in the non-TIR class. It is also possible to distinguish the two classes with 95% accuracy identifying the final amino acid in motif Kinase 2; as this amino acid position is frequently occupied by tryptophan in non-TIRs and aspartic acid in TIRs [10]. In dicotyledonous species, the ratios of non-TIR and TIR-NBS-LRR sequences were reported to be 1 : 2 (93 : 54) for the complete *Arabidopsis* genome, 3.5 : 1 (362 : 97) for grapevine and 3 : 1 (252 : 78) for poplar [18]. Also, an important property is that most NBS-LRR genes are unevenly distributed in plant genomes and exist mainly as multi-gene clusters. The majority of NBS-containing genes in the grapevine (83.2%) and the poplar (67.5%) genomes were found in clusters. The TIR and non-TIR-NBS sequences were found to be separately clustered on chromosomes.

Cloning of NBS-LRR Resistance Gene Analogs (RGAs) has been achieved, by using degenerate primers designed on the conserved sequence motifs within the NBS region [6]. This approach has been successfully applied in different plant species. The obtained sequences, if showed high similarity to annotated plant resistance genes, were labelled as Resistance Gene Analogs [6] or Resistance Gene Homologs (RGH) [8] when their common origin seems highly presumable. This method is so popular among the researchers that over 1,600 NBS sequences have been amplified from a diverse array of plant species using degenerate PCR primers [9].

Based of the *Rosaceae* NBS sequences, the ratios between the TIR/non-TIR sequences are 16 : 0; 59 : 17; 11 : 3; 5 : 6; 48 : 30 and 38 : 37 in *Fragaria, Malus, Prunus, Pyrus, Rosa* and *Rubus,* respectively [12]. In the genome-wide RGA isolation of apricot, only members of the TIR family were previously found [2] and mapped [13]. Although the resistance map for *Prunus* was prepared on which 42 loci were defined from four *Prunus* species, the NBS domain analysis showed a ratio of 13 : 2 between TIR and non-TIR sequences [7].

This phenomenon was explained by the authors with the possibility that there is an unequal distribution of TIR/non-TIR sequences in the *Prunus* genome similar to species, or that there is a bias in the amplification of RGAs due to limitations of primer design [7, 13].

In grape NBS sequences obtained with a given combination of degenerate primers showed a tendency to cluster closely together on dendrograms [1]. This topology offers a possibility to get groups of more distantly related sequences by applying more primer combinations.

The objective of this work was to check whether a *semi nested* new PCR strategy can be applied for the targeted isolation of non-TIR-NBS-LRR RGA sequences from the apricot genome. The cloning, sequencing and molecular analysis of such apricot RGAs may shed light on some interesting phylogenetic aspects.

MATERIALS AND METHODS

Plant materials

'Goldrich' was chosen as a well-known PPV resistance apricot *(Prunus armeniaca)* cultivar. Plant material came from the germplasm collection (Szigetcsép, Hungary) of the Corvinus University of Budapest, Department of Genetics and Plant Breeding.

Primer design and PCR strategy

Genomic DNA was extracted from fully expanded young leaves using DNeasy Plant Mini Kit (Qiagen, Germany). Two combinations of degenerate primers were used to amplify the NBS domain from apricot cultivar 'Goldrich' by PCR. PCR was carried out in a PTC 200 thermocycler (MJ Research, Canada). The amplification was carried out using a temperature profile with an initial denaturing of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 48.5 °C for 45 s and 72 °C for 1 min, and a final extension of 72 °C for 5 min. For the colonial PCR we used the next programme: denaturing of 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 56.5 °C for 45 s and 72 °C for 1 min, and a final extension of 72 °C for 5 min. Approximately 20–30 ng of genomic DNA was used for PCR amplification in a 25 μL reaction volume, containing 1 × PCR buffer (Promega, USA) with final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.4 μM of the adequate primers and 0.625 U of GoTaq DNA polymerase (Promega, USA).

Degenerate primers for CUB-P-loop F (5′-RTT GNA TGG GNG GNN TDGG-3′), CUB-Kin2 F (5′-TND TNH TNG ATG AYR TNT GG-3′) and CUB-HD R (5′-GCY ARW GGY AVH CCW YYA CA-3′) were designed (CUB: Corvinus University of Budapest) on the basis of the alignment of 25 sequences (Fig. 1) to anneal within the conserved regions identified at the 5′ and 3′ ends of the NBS domain. The degenerated primers previously used [13] in apricot RGA analysis were also used: forward primer P-loopGent F (5′-GGAATGGGKGGAGTYGGYAARAC-3′) [3] developed from the P-loop [GxGxxGR(T/S)] motif and LM637 R (5′ARIGCTARIGGIARIC C3′) primers [6] was developed to the hydrophobic motif of the NBS domain. The applied nucleotide codes were according to IUPAC nucleic acid notation.

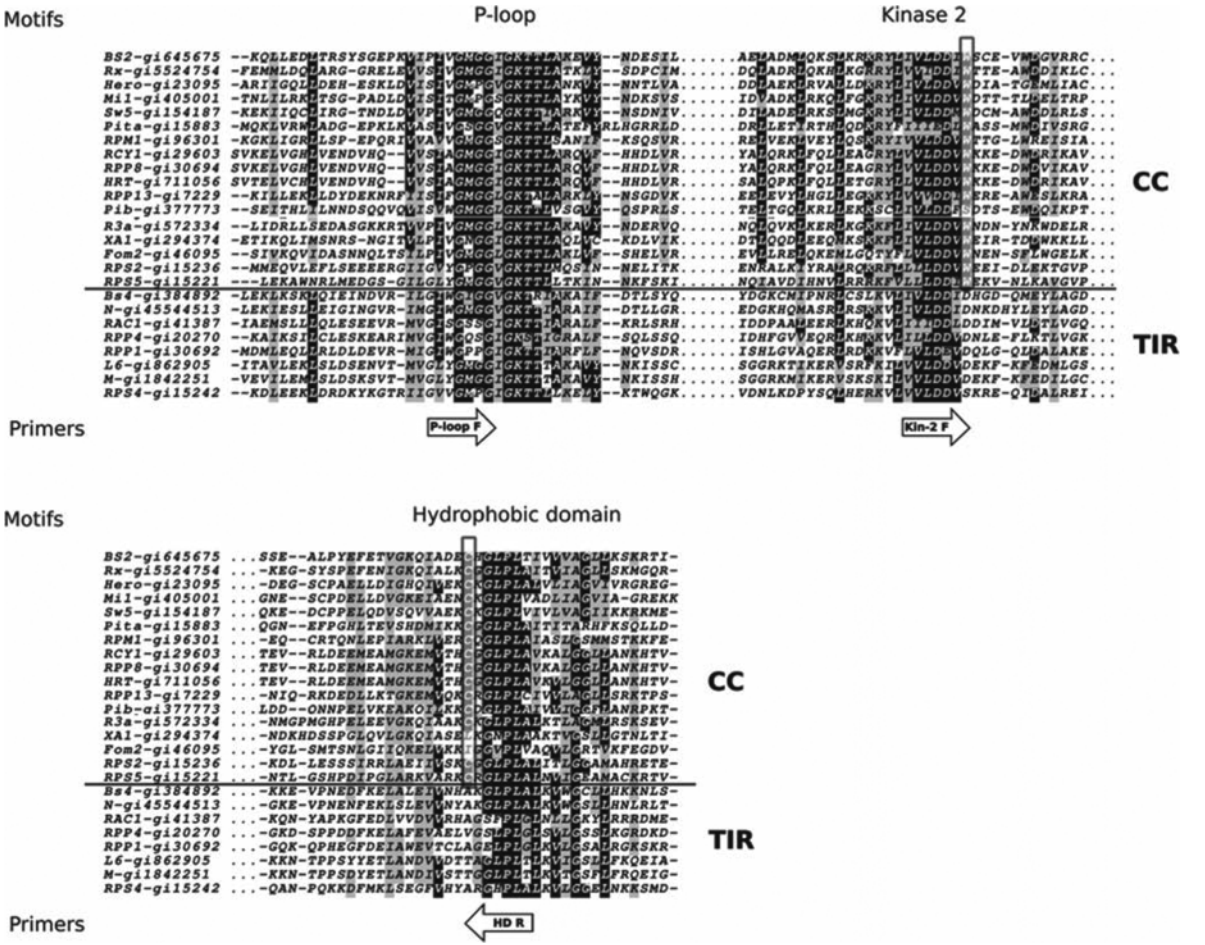

Fig. 1. Multiple sequence alignments of the deduced amino acid sequences of NBS domain from 25 known NBS-LRR resistance genes. The highly specific CC-NBS amino acids (tryptophan in kinase2 and cysteine in the HD motifs) are high lighted. White arrows show the annealing site and the orientation of the primers

Cloning and sequencing of genomic PCR products

PCR products amplified by the CUB-P-loop F and CUB-HD R or the P-loopGent F and LM637 R were extracted and purified from the agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Germany) and cloned into a pGEM-T Easy plasmid vector (Promega, USA). Plasmids were used to transform competent *E. coli* JM109 cells. The white clones were screened by a semi-nested PCR strategy, using the Kin2 F non-TIR selective primer in combination with the above described reverse primers. Plasmid DNA was isolated from those clones which gave the expected size band. Plasmid DNA was isolated with the Rapid Plasmid DNA Daily Miniprep Kit (V-Gene, China) and all clones were determined in both directions in an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). Sequence data from this article have been deposited at the GenBank/NCBI/database under accession numbers GQ336813-GQ336825.

Sequence analyses

Analyses of DNA and deduced amino acid sequence similarity were performed against nucleotide and protein sequence databases at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov). Twenty RGA sequences showing BlastP *E* values lower than $1*10^{-50}$ and 21 others were harvested to represent annotated R gene NBS sequences from various species. In addition, 262 *Rosaceae* NBS sequences used for a previous phylogenetic analysis [12] were also included in the alignment. Protein sequences were aligned by Clustal W [4]. A phylogenetic tree was generated using the Bootstrap neighbour-joining method with a Kimura2-parameter model by MEGA version 4.0 [15]. The stability of internal nodes was assessed by bootstrap analysis with 1,000 replicates.

RESULTS

Targeted identification of non-TIR-NBS-LRR RGAs from apricot by semi-nested PCR

Twenty-five NBS-LRR resistance genes (8 from the TIR-NBS-LRR and 17 from the non-TIR-NBS- LRR class) were retrieved from the NCBI GenBank database and used for the alignment (Fig. 1). The sequences represented 8 species (*Arabidopsis thaliana* 12, *Lycopersicum esculentum* 4, *Oryza sativa* 2, *Solanum tuberosum* 2, *Linum usitatissimum* 2, *Cucumis melo* 1, *Nicotiana tabaccum* 1, *Capsicum chacoense* 1). Based on the characteristic differences between the nucleotide sequences of the TIR and non-TIR classes, three primers were designed. The first forward primer (CUB-P-loop F) was designed to anneal within the P-loop motif and was not selective

between TIR and non-TIR sequences. Two additional primers (CUB-Kin2 F and CUB-HD R) were designed to anneal selectively to the non-TIR sequences (Fig. 1).

The CUB-HD R primer was designed to anneal to the hydrophobic motif where a cystein preferentially occurs in many non-TIR sequences, 15 from 17 sequences (Fig. 1). Although this targeted cystein was present in the great majority of the analyzed *Rosaceae* non-TIR-NBS-LRR hydrophobic motif sequences (*Rosa* 24/24 and *Rubus* 36/37) [18], a cystein also occurred in 2 of the 6 apricot TIR-NBS-LRR sequences determined [13], therefore, it was required to check once again the status of the putative non-TIR-NBS-LRR sequences by using a highly selective primer CUB-Kin2 F in combination of the CUB-HD R.

The primer designed for the Kinase 2 motif (Leu-x-Val-Leu-Asp-Asp-Val-Trp) contained a unique tryptophan amino acid at the C terminus, which is highly specific to the non-TIR-NBS-LRRs. These degenerate primers, CUB-P-loop F and CUB-HD R were used for PCR amplification from the apricot cultivar 'Goldrich'. This primer combination amplified a fragment of the expected size, approximately 500 bp as was seen in case of the previously applied P-loopGent F and LM637 R primers (Fig. 2A–B). The two possible combinations of these primers were not used since they resulted in weak amplification (Fig. 2C) or amplified too many artefacts (Fig. 2D). An approx. 280 bp fragment was amplified by using the CUB-Kin2 F in combination with the CUB-HD R (Fig. 2E) indicating the presence of non-TIR-NBS sequences in the apricot genome.

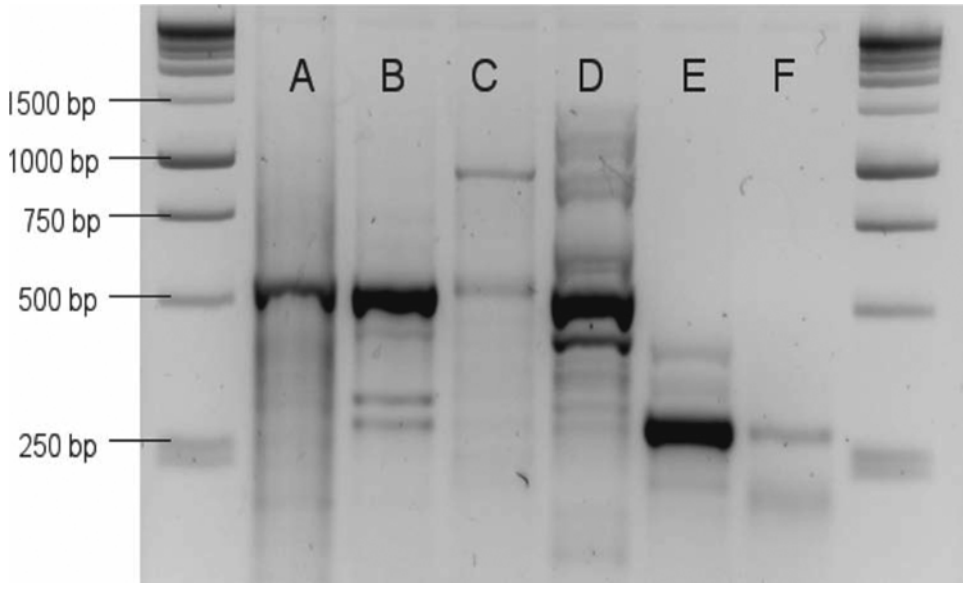

Fig. 2. Polymerase chain reaction (PCR) amplification of the apricot *(Prunus armeniaca)* cultivar '*Goldrich*' resistance gene analogs (RGA) with CUB-P-loop F and CUB-HD R (A), P-loopGent F and LM637 R (B), CUB-P-loop F and LM637 R (C), P-loopGent F and CUB-HD R (D), CUB-Kin2 F and CUB-HD R (E) and CUB-Kin2 F and LM637 R (F). 1-kb DNA ladder was used as fragment size marker

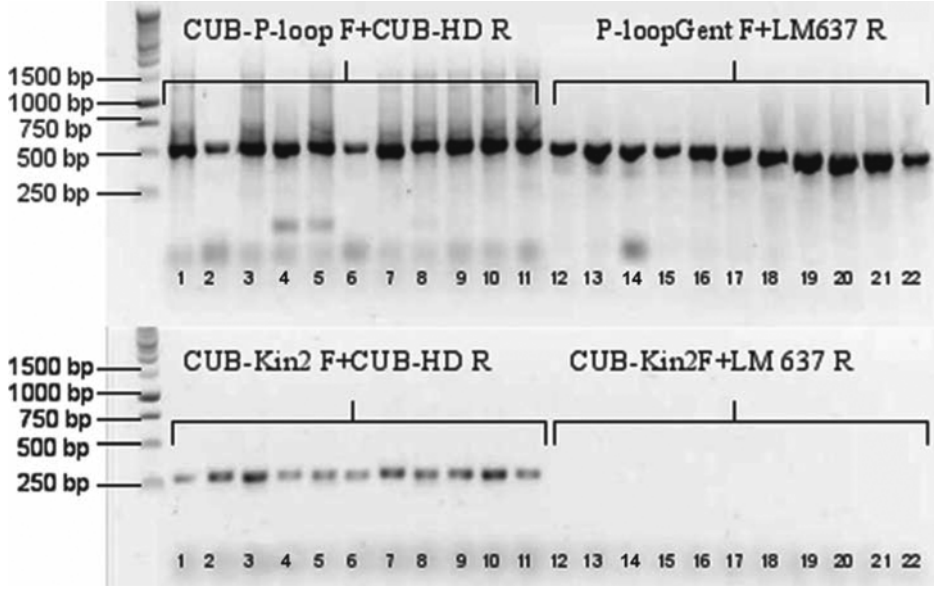

Fig. 3. Colony PCR analysis of the cloned fragments obtained using the primer combinations, CUB-P-loop F and CUB-HD R or P-loopGent F and LM637 R. Upper part: White colonies were checked using the newly developed (1–11) and formerly described (12–22) primer combinations. Lower part: The efficiency of the newly developed PCR primer set (CUB-Kin2 F and CUB-HD R) selective for the non-TIR amplification (1–11) and that formerly used (CUB-Kin2 F and LM637 R) (12–22)

The approximately 500 bp size fragments (Fig. 2A–B) were cloned into a pGEM-T Easy plasmid vector and altogether 96–96 clones were screened by using a semi-nested colony PCR strategy. Out of the 96 clones derived from the newly designed primer application (CUB-P-loop F and CUB-HD R), 28 showed amplification using the combination of the CUB-Kin2 F and CUB-HD R primers (Fig. 3), while positive clones were not observed from the second primer combination (P-loopGent F and LM637 R).

Half of the 28 positive clones were sequenced and they turned out to represent 11 different sequences (Fig. 4). The deduced amino acid sequences contained 152–169 amino acid residues without stop codons. BLASTX 2.2.22+ analysis of the sequences confirmed significant similarity (highest *E* values varied between 7E-47 and 1E-21) to sequences of the non-TIR-NBS-LRR class retrieved from the NCBI Protein Reference Sequences database. In addition to the positive clones, 10 negatives were also sequenced as control. The BLASTX 2.2.22+ analysis of the two unique sequences from the total of 10 negative clones revealed significant homology with sequences of the TIR-NBS-LRR family deposited in the NCBI Protein Reference Sequence database (highest *E* values ranged between 3E-44 to 6E-19). Sequence identity ranged from 0.209 to 0.976 within the group of non-TIR-NBS sequences, while the identity between the two TIR-NBS sequences was 0.408. As expected, sequence

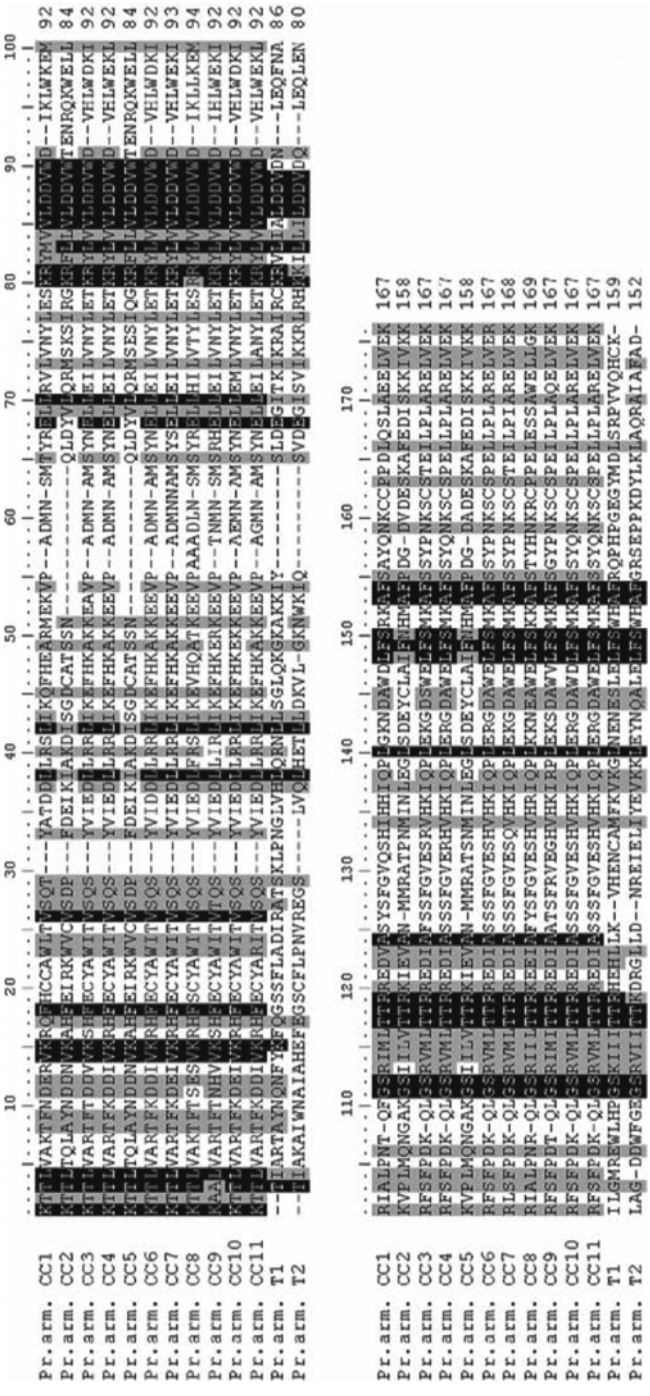

Fig. 4. Protein sequences alignments of the 11 non-TIR-NBS and two TIR-NBS apricot

identity was much restricted between the members of the two groups and ranged from 0.128 to 0.196. These results show the differences between the efficiency of PCR strategies. BLASTN 2.2.22+ analysis of the non-TIR sequences to the *Prunus* EST database revealed similarity to three sequences (BU043646, DW342940 and DW344486) with the highest *E* value of 7E-126, while no similarity was found up to the 1E-50 value when TIR sequences were used as query sequences.

Phylogenetic analysis of apricot non-TIR-NBS-LRR RGAs

A phylogenetic analysis of the newly identified non-TIR- and TIR-NBS-LRR sequences was carried out based on an alignment containing sequences (293 from the *Rosaceae* and 21 from non-*Rosaceae* species) chosen with special consideration. All non-TIR- and TIR-NBS-LRR sequences clustered on different clades (Bootstrap value = 99%) (Fig. 5).

The newly described apricot NBS-LRR sequences clustered in four distant groups on the phylogenetic tree. PaCC 5 and PaCC 2 clustered within the previously established non-TIR I group [12], the PaCC 1, 3, 4, 6, 7, 8, 9, 10 and 11 grouped with the sequences of the non-TIR VI group. The TIR sequences, PaT 1 and PaT 2 clustered with the TIR XII and TIR XI sequences, respectively. Groups occurring in the phylogenetic tree were only accepted as real groups if they had a bootstrap support higher than 70%.

Two types of terminal cluster were set up: clades containing sequences of a single genus, and clades containing more than one genus. A significantly higher ratio (91%) of non-TIR sequences were arranged into multi-genera clades than that of (57%) the TIR groups. The non-*Rosaceae* sequences formed outgroups to the TIR clades, while in the non-TIR clade 25% of the non-*Rosaceae* sequences were clustered in multi-genera groups. Average branch length shows significant differences between the two main clades (Fig. 5). The branch lengths are shorter and diverging branches show terminal location on the TIR clade, while on the non-TIR clade we observed longer branches, and forkings were basally localized excluding two genus-specific clades, *Prunus* IV and *Rosa* IX.

DISCUSSION

Only a limited number of CC-NBS-LRR RGAs were isolated from several *Prunus* species, while from apricot such sequences were not known at all [2, 7, 13]. These studies could not determine whether this phenomenon was attributable to the limited occurrence of these sequences in the apricot genome or the failure of the applied PCR strategy. To reveal the real explanation of this phenomenon, a new approach was worked out in the present study.

Primer designing is a determining step during the isolation of new NBS-LRR gene analogues. Previously, primers were designed based on only one or few available

sequences [2, 7, 13]. Consequently, non-degenerated or slightly degenerated primers were used for the searching of gene analogues from *Prunus* species. To increase PCR efficiency, 25 annotated TIR- and non-TIR-NBS-LRR R gene sequences were aligned from 8 distinct species (Fig. 1). Based on this amino acid alignment, three primers were designed. Two of them were constructed to selectively anneal to the non-TIR-NBS motifs while another was designed having no specificity between the two groups. All primers were characterized by a permutation index higher than that of the primers used before [2, 7, 13].

The 103 NBS-LRR sequences were isolated from grapevine and it was found that the sequences amplified by low degeneracy primers were very similar and tended to form separate clusters on a dendrogram [1]. To compare the performance of the newly designed and previously used [3, 6] primers, the CUB-Kin2 F and LM637 R primers were used in combination (Fig. 2F). The obtained weak amplification may indicate the ineligibility of the LM637 R primer for amplifying non-TIR-NBS sequences and verifies that primer designing is a crucial step in such analyses.

The phylogenetic analysis showed that the newly identified non-TIR-NBS sequences clustered into four distant groups on a previously established phylogenetic tree [12] of 293 and 21 sequences from *Rosaceae* and non-*Rosaceae* species, respectively. The phylogenetic topology of the TIR and non-TIR sequences was different with significantly more multi-genera clades occurring in case of non-TIR sequences. A similar topology was observed on a phylogenetic tree containing all NBS sequences from the poplar, grape, rice and *Arabidopsis* genomes [18]. This pattern is associated with the ancient origin of non-TIR genes, since non-TIR genes have originated even before the divergence of the tested species (including both monocots and dicots). Based on the analysis of more closely related OTUs (operational taxonomic unit), our results support this conclusion.

Since non-TIR and TIR-NBS-LRR sequences are located in separate clusters in the genome [18], polymorphic markers derived from the non-TIR-NBS sequences can explore such parts of the genome that could not be found using markers targeted on the TIR sequences. Hence our results may contribute to deepen our knowledge in *Prunus* resistance genes. Our results also demonstrated that primer design is a limiting step in finding NBS-LRR sequences in *Prunus* [8, 12]. However, the lower abundance of the non-TIR-NBS sequences in the *Prunus* genome due to the stronger dependence of these species on TIR RGAs (as was previously supposed [17]) cannot be ruled out based on our results. However, our results indicate that the previously hypothesized unequal distribution of non-TIR- and TIR-NBS sequences might be an erroneous conclusion based on the limitation of primer design. These doubts may only be fully resolved after the first annotated *Prunus* genome sequence will be available.

ACKNOWLEDGEMENT

This work was supported by the TÁMOP 4.2.1./B-09/01/KMR/2010-0005 grant.

REFERENCES

1. 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.
2. Dondini, L., Costa, F., Tataranni, G., Tartarini, S., Sansavini, S. (2004) Cloning of apricot RGA (Resistance Gene Analogs) and development of molecular markers associated with Sharka (PPV) resistance. *J. Hortic. Sci. Biotech. 79*, 729–734.
3. Gentzbittel, L., Mouzeyar, S., Badaoui, S., Mestries, E., Vear, F., De Labrouhe, D. T., Nicolas, P. (1998) Cloning of molecular markers for disease resistance in sunflower, *Helianthus annuus* L. *Theor. Appl. Genet. 96*, 519–525.
4. Higgins, D., Thompson, J., Gibson, T., Thompson, J. D., Higgins, D., Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic. Acids Res. 22*, 4673–4680.
5. The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature 408*, 796–816.
6. Kanazin, V., Marek, L. F., Shoemaker, R. C. (1996) Resistance-gene analogs are conserved and clustered in soybean. *Proc. Natl. Acad. Sci. 93*, 11746–11750.
7. Lalli, D. A., Decroocq, V., Blenda, V., Shurdi-Levraud, V., Garay, L., Le Gall, O., Damsteegt, V., Reighard, G. L., Abbott, A. G. (2005) Identification and mapping of resistance gene analogs (RGAs) in *Prunus*: a resistance map for *Prunus*. *Theor. Appl. Genet. 111*, 1504–1513.
8. 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.
9. McHale, L., Tan, X., Koehl, P., Michelmore, R. W. (2006) Plant NBS-LRR proteins: adaptable guards. *Genome Biol. 7*, 212.
10. Meyers, B. C., Kaushik, S., Nandety, R. S. (2005) Evolving disease resistance genes. *Curr. Opin. Plant Biol. 8*, 129–134.
11. Meyers, B. C., Dickerman, A. W., Michelmore, R. W., Sivaramakrishnan, S., Sobral, B. W., Young, N. D. (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. *Plant J. 20*, 317–332.
12. Samuelian, S. K., Baldo, A. M., Pattison, J. A., Weber, C. A. (2008) Isolation and linkage mapping of NBS-LRR resistance gene analogs in red raspberry (*Rubus idaeus* L.) and classification among *Rosaceae* NBS-LRR genes. *Tree Genet. Genomes 4*, 881–896.
13. Soriano, J. M., Vilanova, S., Romero, C., Llácer, G., Badenes, M. L. (2005) Characterization and mapping of NBS-LRR resistance gene analogs in apricot (*Prunus armeniaca* L.). *Theor. Appl. Genet. 110*, 980–989.
14. Takken, F. L., Albrecht, M., Tameling, W. I. (2006) Resistance proteins: molecular switches of plant defence. *Curr. Opin. Plant Biol. 9*, 383–390.
15. Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol. 24*, 1596–1599.
16. Xiao, S. (2006) Current perspectives on molecular mechanisms of plant disease resistance. In: Teixeira da Silva, J. A. (ed.) *Floriculture, Ornamental and Plant Biotechnology.* Global Science Books, Isleworth, pp. 317–333.
17. Xu, Q., Wen, X., Deng, X. (2007) Phylogenetic and evolutionary analysis of NBS-encoding genes in Rosaceae fruit crops. *Mol. Phylogenet. Evol. 44*, 315–324.
18. Yang, S., Zhang, X., Yue, J., Tian, D., Chen, J. (2008) Recent duplications dominate NBS-encoding gene expansion in two woody species. *Mol. Genet. Genomics 280*, 187–198.