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Isolation and diversity analysis of resistance gene analogues (RGAs) from cultivated and wild strawberries

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Abstract Degenerate oligonucleotide primers, designed based on conserved regions of Nucleotide Binding Site (NBS) domains from previously cloned plant resistance genes, were used to isolate Resistance Gene Analogues (RGAs) from wild and cultivated strawberries. Seven distinct families of RGAs of the NBS-LRR type were identified from two related wild species, *Fragaria vesca* and *F. chiloensis*, and six different *Fragaria* × ananassa cultivars. With one exception (GAV-3), the deduced amino acid sequences of strawberry RGAs showed strong similarity to TIR (Toll Interleukin I Receptor)-type *R* genes from *Arabidopsis*, tobacco and flax, suggesting the existence of common ancestors. GAV-3 seemed to be more closely related to the non-TIR type. Further studies showed that the recombination level and the ratio of non-synonymous to synonymous substitutions within families were low. These data suggest that NBS-encoding sequences of RGAs in strawberry are subject to a gradual accumulation of mutations leading to purifying selection, rather than to a diversifying process. The present paper is the first report on RGAs in strawberry.

Keywords Disease resistance genes · Diversity · NBS-LRR · *Fragaria* spp. · Resistance Gene Analogues (RGAs)

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Introduction

During the past decade an increasing number of plant disease resistance (*R*) genes from different species have been identified by map-based cloning or transposon tagging. Sequence comparisons among these genes have revealed a remarkable conservation of structural features, despite the diversity of the pathogens with which their products interact (Hammond-Kosack and Jones 1997; Martin 1999; Ellis et al. 2000). The most prevalent class of cloned genes contains a Leucine Rich Repeat (LRR) and a Nucleotide Binding Site (NBS) domain (Dangl and Jones 2001). The NBS domains of most of the characterized R-gene products contain several highly conserved motifs. Of these motifs, the P-loop or kinase 1-a, the kinase 2 and the kinase 3-a domains also occur in other proteins with ATP- and GTP-binding activity, such as the apoptosis regulators Apaf-1 from human and CEd-4 from nematode (Saraste et al. 1990; Bourne et al. 1991; Traut 1991; Meyers et al. 1999). The NBS-LRR proteins are thought to recognize pathogens and respond by activating signal transduction pathways. Nucleotide triphosphate binding is thought to alter the interaction between resistance proteins and other proteins that act downstream of them (Bent 1996). Baker et al. (1997) reported that mutations of key residues in the P-Loop of the tobacco N gene product led to partial loss of function. An additional motif in the NBS proteins is the GLPL, also named the "hydrophobic domain", which is a putative membrane-spanning domain. LRR domains mediate protein-protein interactions, and are the major determinants of recognition specificity (Fluhr 2001). Based on the crystal structure of porcine ribonuclease inhibitor (RI), the LRR region is thought to form a parallel β-sheet structure. In the xxLxxLxx motif, the conserved leucines project into the hydrophobic core, whereas the other residues remain exposed to the solvent, forming a surface that is involved in ligand binding (Kobe and Deisenhofer 1996). The high ratio of non-synonymous to synonymous substitutions

among the "x" residues is consistent with the idea that they interact with pathogen ligands and are subject to diversifying selection so that they can recognize novel ligands (Hulbert et al. 2001). This kind of rapid evolution, which can be explained both by the neutral theory and by Darwinian evolution, has also been found in another type of plant genes possibly involved in plant defence (Castagnaro et al. 1992).

Plant NBS-LRR proteins can be categorized into TIR and non-TIR classes based on the presence or absence of an N-terminal domain with homology to the receptor domain of the innate immunity factors Toll and Interleukin-1 found in animals (Parker et al. 1997). Non-TIR-NBS-LRR proteins contain N-terminal coiled-coil (CC) or leucine zipper (LZ) motifs, corresponding to helical structures that are thought to play an important role in interactions with other molecules in the signal transduction pathway (Pan et al. 2000). While TIR-type genes have not been found in monocot genomes or ESTs, non-TIR sequences are present in both dicots and monocots (Meyers et al. 1999). TIR and non-TIR proteins also display conspicuous differences in amino acid motifs within the NBS domain: RNBS-A-TIR (LQKKLLSKLL) and RNBS-D-TIR (FLHIACFF) are present exclusively in the TIR class, whereas RNBS-A-nonTIR (FDLxAWVCVSQxF) and RNBS-D-nonTIR (CFLYCALFPED) are found exclusively in the non-TIR class. In addition, the final amino acid in the Kinase 2 domain can also be used to distinguish the two classes with 95% accuracy: tryptophan (W) is found in this position in non-TIRs and aspartic acid (D) in TIRs (Meyers et al. 1999).

Despite the conservation of the motifs mentioned, the overall degree of sequence similarity among R genes is very low, and for that reason cross-hybridization, using cloned genes as probes, is unlikely to be useful for detecting new R genes.

Recently, the use of PCR with degenerate primers targeted to the short conserved regions in the NBS has proven to be an efficient method for identifying resistance gene analogues (RGAs). This approach has been successfully used in potato (Leister et al. 1996), soybean (Yu et al. 1996, Kanazin et al. 1996), lettuce (Shen et al. 1998), *Arabidopsis thaliana* (Aarts et al. 1998; Speulman et al. 1998), *Brassica* spp. (Joyeux et al. 1999), apple (Lee et al. 2003) and in several other plant species. Genetic analysis showed that RGAs often map to known disease resistance loci where they tend to occur in clusters (Leister et al. 1996). Meyers et al. (1999) reported that over 1% of the *A. thaliana* genome may correspond to the NBS type of *R* genes, and genetic mapping studies of RGAs provided evidence that they co-segregate with fungal disease resistance markers (Aarts et al. 1998).

Strawberry is an important horticultural crop in many countries, but yields are strongly affected by fungal diseases and pests, forcing the excessive use of chemical products to control them. Although molecular markers of disease resistance in strawberry have been reported (Haymes et al. 1997; Lerceteau-Kohler et al. 2002), the octoploid genetic structure of commercial strawberry makes it difficult to associate molecular markers with disease resistance genes. Since no reports of the molecular cloning of putative resistance genes from strawberry have yet appeared, the aim of this work was the isolation and characterization of RGAs from wild and cultivated strawberry, and the evaluation of their variability and phylogenetic relationships.

Materials and methods

Plant material and DNA extraction

DNAs from the *Fragaria* × ananassa cultivars Camarosa, Sweet Charlie, Pájaro, Oso Grande, Gaviota and Milsei tudla, the breeding line US159 (Galletta et al. 1993), one accession of the related wild species *Fragaria vesca* and *F. chiloensis*, and two different accessions of *Duchesnea indica,* with different responses to the anthracnose disease (*Colletotrichum* spp.), were used for RGA isolation. Genomic DNA was extracted from young leaflets using the CTAB protocol suggested by Weising et al. (1995).

Isolation of RGAs by PCR with degenerate primers

The degenerate PCR primers used were those designed by Leister et al. (1996) from the P-loop and the GLPLAL motifs of the NBS region of the *R* genes *RPS* 2 from *A. thaliana* and *N* of tobacco. These primers are listed in Table 1.

PCRs were carried out in a total volume of 50 µl containing 100 ng of template DNA, 50 mM KCl, 10 mM TRIS-HCl (pH 8.4), 2.5 mM $MgCl_2$, each primer at 0.5 µM, each dNTP at 0.2 mM, and 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.). PCR was performed in a PTC-100 thermal cycler (MJ Research, Watertown, Conn.). Cycling conditions included initial denaturation for 2 min at 94°C, followed by 35 cycles of

Table 1 Degenerate PCR primers used for RGAs isolation

Primer	Conserved amino acid motif	Primer sequence (5′to 3′) [a]
s1 (forward)	P-loop (GGVGKTT)	GGTGGGGTTGGGAAGACAACG
s2 (forward)	P-loop (GGVGKTT)	GGIGGIGTIGGIAAIACIAC
as1 (reverse)	Hydrophobic domain (GLPLAL)	CAACGCTAGTGGCAATCC
as2 (reverse)	Hydrophobic domain (GLPLAL)	IAAIGCIAGIGGIAAICC
as3 (reverse)	Hydrophobic domain (GLPLAL)	IAGIGCIAGIGGIAGICC

[a] I, deoxyinosine

1 min at 93°C, 1 min at 45°C and 1 min 20 s at 72°C, with a final extension step of 10 min at 72°C. Annealing temperature was selected after testing temperatures ranging from 38 to 48°C. Different concentrations of genomic DNA and primers were also tested to optimize the reactions.

Cloning and sequencing of PCR products

Amplification products were separated by electrophoresis on 1% TAE-agarose gels and stained with ethidium bromide for visualization. Bands of the predicted size (approximately 520 bp) were excised from the gel and purified using the Prep-A-Gene DNA Purification kit (Bio-Rad).

Each purified band, presumably containing different products of similar sizes, was cloned using the pGEM T-easy vector system (Promega) and transformed into *Escherichia coli* DH5α. Ten recombinant bacterial colonies obtained from each ligation event were isolated. Recombinant plasmids were extracted using alkaline lysis, and digested with *Eco* RI to verify the presence of the expected insert.

Sequences of PCR products were determined using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, Calif.). The nucleotide sequences of strawberry RGAs have been deposited in the GenBank Database under Accession Nos. AY607708–AY607729.

Sequence analysis

DNA sequence analysis and translations to the corresponding amino acid sequence were performed using DNAMAN (version 4.03) software, and intron searches (putative splicing sites) were performed with the NETPLANTGENE program (Hebsgaard et al. 1996).

The identity of RGAs was confirmed by comparisons of DNA and amino acid sequences with the GenBank NR database using BLASTX and BLASTP (Altschul et al. 1990) algorithms. Determination of conserved structural motifs in RGA sequences was carried out with the programs MEME (Bailey and Elkam 1994) and PROSITE (Hofmann et al. 1999) and multiple sequence alignment with ClustalX (Thompson et al. 1997). Evolutionary analyses and construction of neighbor-joining phylogenetic trees were performed using the MEGA version 2.1 software (Kumar et al. 2001). The reliability of tree branches was evaluated using the Bootstrap method (Felsenstein 1985) and Kimura's correction was applied (Kimura 1980). The ratio of synonymous to non-synonymous substitutions was calculated with the SNAP program (Nei and Gojobori 1986) and GENECONV software (S. Sawyer, Department of Mathematics, Washington University, St. Louis, Mo.; available at http://www.math.wustl.edu/~sawyer) was used to determine whether genetic variation among strawberry RGAs could have resulted from recombination events between the sequences.

Results

Isolation of RGAs from strawberry by PCR using degenerate primers

Six combinations of degenerate primers, designed from the P-loop and GLPLAL domains within the NBS were used to amplify DNA fragments from strawberry and related species (see Table 1). However, only the primer combination s2-as3 was successful in amplifying products from all templates used except those from the *F. x ananassa* cultivar Pájaro and two different accessions of the wild species *Duchesnea indica*. Attempts to obtain amplification products from DNA of the latter genotypes, with all possible primer combinations, were unsuccessful, although many independent DNA preparations were tested.

Cloning and characterization of the amplicons obtained revealed that each band was made up of many RGA sequences. As expected, considering the sizes of previously published NBS sequences, most amplification products were 510 bp long, but there were also sequences of 486, 498, 504, 519, and 536 bp. Of the 80 putative strawberry RGA clones sequenced, 51 (36 from *F. ananassa*, 10 from *F. chiloensis*, and 5 from *F. vesca*) showed significant homology to *R* gene sequences and RGAs from other species in the GenBank NR Database. BLAST analysis detected homology to the internal conserved motifs typical of the NBS-LRR type of *R* genes (kinase 2 and kinase 3a) in addition to the two motifs targeted by the degenerate primers. Of these 51 RGAs, 23 had potential stop codons and/or frameshift mutations, whereas the other 28 presented uninterrupted ORFs. No putative splicing sites were detected in these clones.

Diversity analysis of strawberry RGAs

The diversity of the strawberry NBS-RGAs was analyzed. Nucleotide sequence identity among strawberry RGAs ranged between 59 and 100%, while amino acid sequence identity ranged from 24 to 100%. The predicted amino acid sequences were also subjected to phylogenetic analysis using the neighbor-joining method (Saitou and Nei 1987). Clones with amino acid identities above 97% were considered to be identical; therefore, only 22 sequences were included in the analysis.

A Neighbor Joining phylogenetic tree grouped strawberry RGAs into seven clusters or families (A–G), all of which were supported by high bootstrap values (Fig. 1). A parsimony analysis produced a similar tree (data not shown) confirming the robustness of the former. Family A is composed of 10 members and includes sequences from the three species studied; family

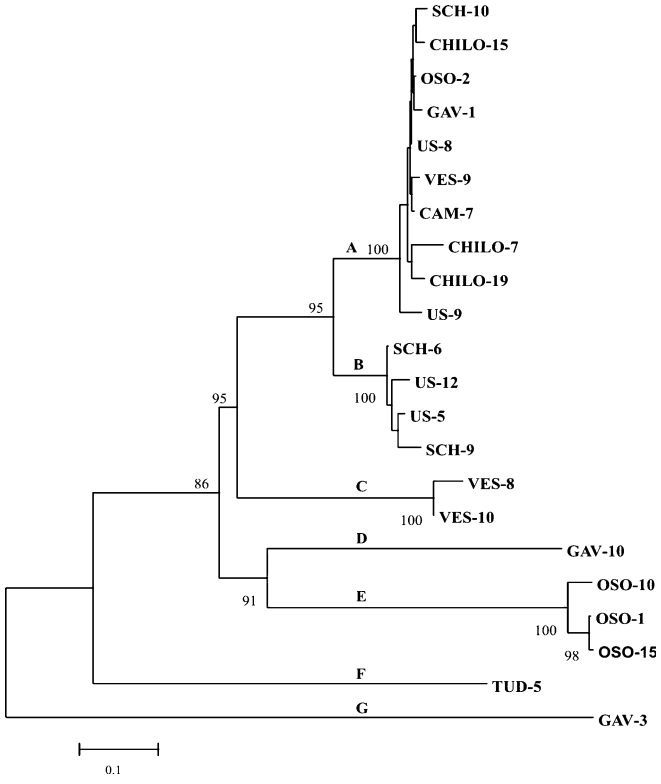

Fig. 1 Phylogenetic tree of the deduced amino acid sequences of strawberry RGAs, based on the Neighbor-Joining method. The *numbers on the branches* indicate bootstrap values (1000 iterations). Branches corresponding to strawberry RGA families are labeled A–G. Sequence sources are indicated as follows: CAM, Camarosa; SCH, Sweet Charlie; GAV, Gaviota; OSO, Oso Grande; TUD, Milsei Tudla; US, breeding line US159 (all *F. x ananassa* cultivars); VES and CHILO indicate accessions of the wild species *F. vesca* and *F. chiloensis*, respectively

B contains four members derived from the *F. ananassa* species (cvs. US-159 and Sweet Charlie) and no sequences obtained from wild species; family C includes two members—both from the wild species *F. vesca*; family E includes three members belonging to the *F. ananassa* cv. Oso Grande, while families D, F and G each contain a single member from the cultivated species (see Fig. 1). Identity scores for members of a given family ranged between 88 and 98%. Inter-family scores ranged from 24 to 70% depending on the RGA considered.

The ratio of non-synonymous to synonymous substitutions (K_a/K_s ratio) among triplets encoding the same amino acids of strawberry RGAs for each family ranged between 0.46 and 0.66.

In order to explore whether genetic variation among RGA sequences from wild and cultivated strawberry is due to genetic recombination, we evaluated the number of potential recombination events among the sequences using the program GENECONV, a statistical test for detecting gene conversion. GENECONV finds the most likely candidates for gene conversion events between pairs of aligned sequences within and outside the alignment. Candidate events are ranked by multiple-comparison corrected P-values based on BLAST-like global scores (10,000 random permutations), and possible recombination events are suggested if the value is lower than 0.05 (Sawyer 1989). Results using the GENECONV algorithm did not detect any recombination event between any two sequences in the alignment (inner fragments). Nevertheless, it did find an outer fragment in VES-8, suggesting that a recombination event may have occurred but the source segment is not represented in the sample nor indicated by the program. At all events, these results indicate a low level of recombination.

Comparative analysis

Phylogenetic relationships among amino acid sequences deduced for the strawberry RGAs and *R* genes from other species were investigated. The analysis was conducted using the NBS region sequences of the following *R* genes: *N* from tobacco, *RPM1* , *RPP1* , *RPS2* and *RPS5* from *A. thaliana*, *Mi-1.2, I2C-1* and *prf* from tomato, *Dm3* from lettuce, and *L6* and *M* from flax (obtained from the GenBank database). A multiple alignment of the amino acid sequences predicted for the 22 RGA clones and the *R* genes mentioned above is shown in Fig. 2. The Neighbor-Joining phylogenetic tree constructed from the amino acid alignment of NBS domains of these *R* genes and strawberry RGAs confirmed that strawberry RGAs can be clustered in seven families as shown in Fig. 1. The two major branches resolved in the phylogenetic tree include groups of *R* genes that can be differentiated based on the presence or absence of TIR regions (Fig. 3). All isolated strawberry RGAs were grouped into the TIR type of NBS-LRR *R* genes, with the exception of GAV-3, which fell on the non-TIR branch. We have confirmed this finding by inspecting the sequences for the presence of aspartic acid (D) at the C-terminus of the Kinase 2 domain (Meyers et al. 1999).

In addition to the phylogenetic analysis, searches in GenBank using the BLAST algorithm (BLASTX and BLASTP) confirmed that strawberry RGA sequences are closely related to the NBS domains of some previously characterized *R* genes. In particular, a high degree of identity was observed among members of the E family and the cloned genes *M, L6* and *N* from the TIR class of *R* genes, whereas OSO-1 shares 40.5% identity with M (flax), GAV-10 and N (tobacco) share 39.3% identity, and OSO-10 is 37.6% identical to L6 (flax). In addition, strawberry RGAs showed even higher identity values than those shown above when they were compared with RGAs identified in other plant species, as shown in Table 2.

Discussion

Isolation of strawberry RGAs

By using the combination of degenerate primers s2 and as3, designed to anneal to two of the motifs that are conserved in the NBS region of *R* genes from different

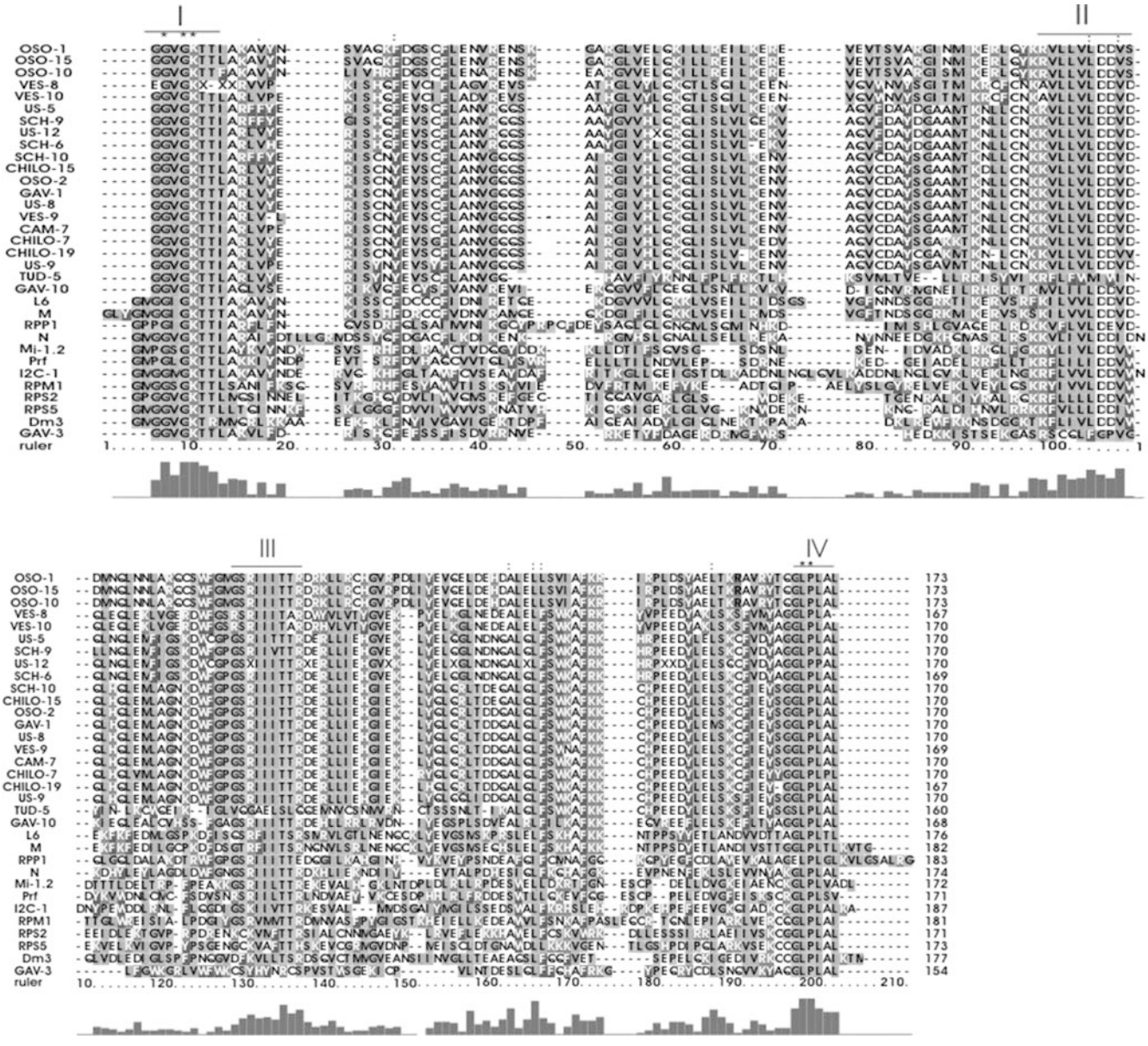

Fig. 2 Multiple amino acid sequence alignment of strawberry RGAs with the NBS domains of other plant resistance genes, constructed with Clustal X. The following sequences were used: tobacco *N* (Accession No. A54810), tomato *Prf* (LEU65391), *Mi-1.2* (AF039682), *I2C-1* (AF004878), *Arabidopsis RPM1* (AAF27008), *RPP1* (AF098962), *RPS5* (AF074916) and *RPS2* (A54809), lettuce *Dm3* (AF113948), flax *L6* (T18546) and *M* (LUU73916). The locations of key conserved domains are shown at the *top* and labeled with Roman numerals (I, kinase 1a or P-Loop; II, kinase 2; III, kinase 3a; IV, hydrophobic domain or GLPL). The alignment quality curve is displayed *below* the alignment. *Asterisks*, *colons* and *dots* indicate completely, strongly and weakly conserved positions, respectively

species, we were able to isolate and clone seven distinct families of RGAs from wild and cultivated strawberry species.

In the *Arabidopsis* genome, approximately 114 genes code for TIR-NBS-type resistance proteins (http://nib-lrrs.ucdavis.edu). Our results indicate that a large number of TIR-NBS like sequences is also present in the strawberry genome, although we could not find in the literature or in GenBank any other reports about them. Consequently, this present study represents the first report on RGAs in strawberry. From these results, it is not possible to decide whether these sequences originate from different alleles spread over the octoploid genome or from paralogous genes in each haploid genome set. Future research will be directed towards elucidating this issue.

The PCR-derived sequences were identified as RGAs or portions of *R* genes as they contain the conserved motifs characteristic of NBS-LRR *R* genes; 28 of them contained uninterrupted ORFs. All the strawberry RGAs detected were closely related to sequences of known *R* genes and RGAs from other species. Thus, some of them may encode resistance gene products of unknown specificity.

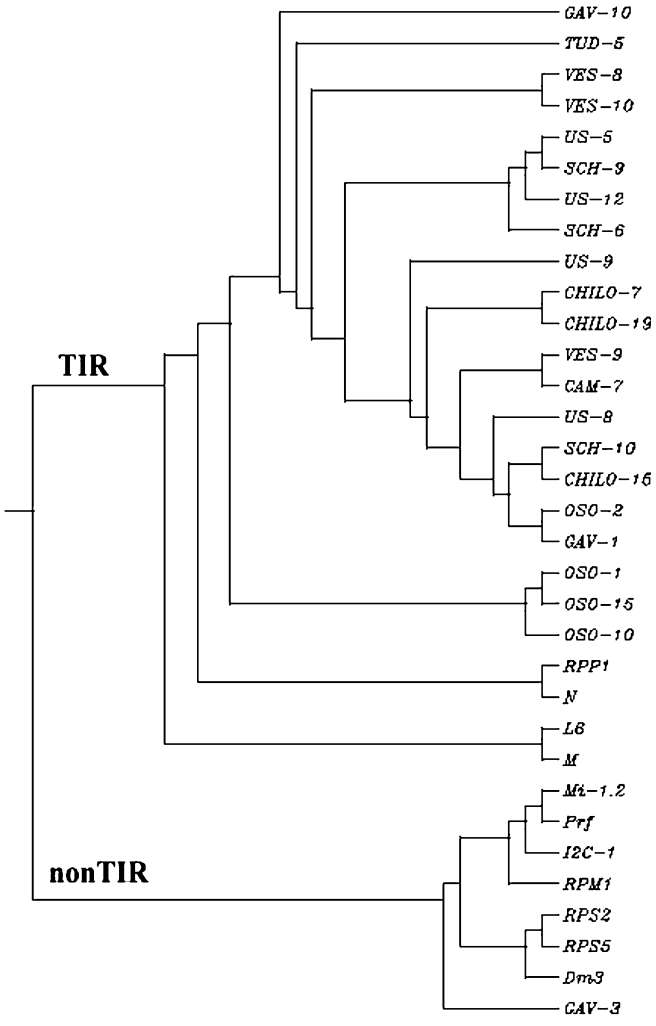

Fig. 3 Neighbor Joining phylogenetic tree based on an alignment of amino acid sequences of strawberry RGAs and NBS domains of previously characterized *R* genes from tobacco (*N*), tomato (*Prf*, *Mi-1.2* , and *I2C-1*), *Arabidopsis* (*RPM1, RPP1, RPS5* and *RPS2*), lettuce (*Dm3*), and flax (*L6*and *M*). TIR and non-TIR subclasses are indicated

Although we can not rule out the possibility that RGAs belonging to these families may also be present in the *D. indica* and *F. ananassa* cv. Pájaro genomes, repeated attempts to amplify such sequences from different preparations of genomic DNA, using all possible combinations of primers, failed to generate any visible products.

Diversity and evolution of strawberry RGAs

The high degree of similarity between strawberry RGAs and NBS sequences derived from *R* genes cloned from *Arabidopsis*, flax, tobacco, and RGAs from apple and pear, among others, suggests an ancestral relationship. Most of the strawberry RGA clones were grouped into the TIR type, except for the clone GAV-3. However, we can not completely rule out the possibility that the latter clone might also belong to the TIR type, because the presence of stop codons and frameshift mutations makes it difficult to identify the internal motifs of each type. Furthermore, analysis of the sequence of the GAV-3 clone suggests that it may be a pseudogene.

The use of a pair of non-exhaustive degenerate primers matching the P-Loop and GLPL motifs from NBS domains similar to those used in strawberry has permitted the identification of TIR and non-TIR types of RGAs in apple (Lee et al. 2003), alfalfa (Cordero and Skinner 2002) and chickpea (Huettel et al. 2002), among others. But in soybean, Kanazin et al. (1996), using a similar pair of primers, detected only sequences that clearly belong to the TIR type of *R* genes, as we have detected in strawberry. Conversely, Tian et al. (2004) did not detect any sequences belonging to the TIR type of NBS-LRR *R* genes in sugar beet, by using an exhaustive set of primers targeted to the five amino acid motifs of the NBS.

Although our results suggest that RGAs in strawberry are of the TIR type, we can not decide whether this

Table 2 Similarity among RGAs of strawberry and other species

Strawberry RGA[a]	GenBank Accession with highest similarity (source)	Identity	E-value	Other accessions with high similarity (source)	Identity	E-value
CHILO-15 (A)	Resistance protein (*Rosa roxburghii*)	74%	2e-59	Putative disease resistance gene analog NBS-LRR (*Malus prunifolia*)	50%	3e-36
SCH-9 (B)	resistance protein (*R. roxburghii*)	73%	1e-60	Putative disease resistance gene analog NBS-LRR (*M. prunifolia*)	46%	3e-35
VES-8 (C)	Resistance protein (*R. roxburghii*)	52%	3e-40	NBS-LRR disease resistance protein (*Pyrus communis*)	49%	7e-34
GAV-10 (D)	NBS-LRR resistance gene-like protein ARGH12 (*Malus x domestica*)	50%	3e-31	Unknown (*Cicer arientinum*)	50%	2e-30
OSO-15 (E)	Resistance-gene protein (*Vigna unguiculata*)	53%	2e-34	Putative resistance gene analogue protein (*Lens culinaris*)	48%	2e-35
TUD-5 (F)	Resistance protein (*R. roxburghii*)	85%	8e-48	NBS-LRR disease resistance protein (*P. communis*)	54%	6e-26
GAV-3 (G)	Resistance protein (*R. roxburghii*)	54%	2e-18	Resistance-gene protein (*V. unguiculata*)	45%	2e-08

[a]A–G correspond to different strawberry RGAs family found (see Fig. 1)

reflects a specific genetic trait of strawberry or an unforeseen experimental artifact. We may speculate that the unequal distribution of TIR and non-TIR RGAs in strawberry could be attributable to processes of evolutionary loss that have occurred in this genus over time, as was suggested by Tian et al. (2004) for the TIR class in sugar beet, but further investigation is required to test this hypothesis. The presence of non-TIR type of RGAs will be investigated by using primers that anneal to a conserved motif specific to this class (RNBS-D) that should allow us to discriminate them from the TIR class. The experimental evidence from our laboratory did not show any particular correlation between the degree of resistance/susceptibility to a particular pathogen and the presence or absence of any particular class of RGAs.

The source of genetic variation within *R* genes has been the subject of much discussion (Noel et al. 1999; Noir et al. 2001). Different genetic mechanisms have been proposed for the evolution of *R* genes, such as recombination, unequal crossing over, gene conversion or point mutations (Michelmore and Meyers 1998).

The NBS sequences of strawberry species showed considerable variation. The analysis of strawberry RGAs suggested that the gradual accumulation of point mutations is the primary force generating variability. Nevertheless, sequence exchange cannot be excluded, as a possible recombination event was also detected in the wild species *F. vesca*.

Furthermore, the estimated ratios of non-synonymous to synonymous substitutions are similar of those observed in other NBS domains (0.44–0.66), suggesting that these sequences are subject to purifying rather than diversifying selection. The latter is consistent with the proposed function of these domains, as any inappropriate sequence alteration will negatively affect its signaling properties.

Currently, experiments are being conducted in our laboratory to evaluate the presence of the non-TIR type of RGAs in the genotypes under study using a class specific set of primers, to clone the corresponding genes, study the RGAs segregation in different crosses and their expression in different genotypes.

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