

Characterization of Resistance Gene Analogues (*RGAs*) in Apple (*Malus × domestica* Borkh.) and Their Evolutionary History of the Rosaceae Family

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Abstract

The family of resistance gene analogues (*RGAs*) with a nucleotide-binding site (NBS) domain accounts for the largest number of disease resistance genes and is one of the largest gene families in plants. We have identified 868 *RGAs* in the genome of the apple (*Malus × domestica* Borkh.) cultivar 'Golden Delicious'. This represents 1.51% of the total number of predicted genes for this cultivar. Several evolutionary features are pronounced in *M. domestica*, including a high fraction (80%) of *RGAs* occurring in clusters. This suggests frequent tandem duplication and ectopic translocation events. Of the identified *RGAs*, 56% are located preferentially on six chromosomes (Chr 2, 7, 8, 10, 11, and 15), and 25% are located on Chr 2. *TIR-NBS* and non-*TIR-NBS* classes of *RGAs* are primarily exclusive of different chromosomes, and 99% of non-*TIR-NBS* *RGAs* are located on Chr 11. A phylogenetic reconstruction was conducted to study the evolution of *RGAs* in the Rosaceae family. More than 1400 *RGAs* were identified in six species based on their NBS domain, and a neighbor-joining analysis was used to reconstruct the phylogenetic relationships among the protein sequences. Specific phylogenetic clades were found for *RGAs* of *Malus*, *Fragaria*, and *Rosa*, indicating genus-specific evolution of resistance genes. However, strikingly similar *RGAs* were shared in *Malus*, *Pyrus*, and *Prunus*, indicating high conservation of specific *RGAs* and suggesting a monophyletic origin of these three genera.

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Introduction

When a genome sequence is available, the analysis of large gene families can contribute to the understanding of major events responsible for molecular evolution. This is the case for resistance gene analogues (*RGAs*) with a nucleotide-binding site (NBS) domain [1–5]. The NBS domain is part of the larger NB-ARC domain that hydrolyses ATP and GTP and functions as a molecular switch for signal transduction after pathogen recognition [6]. Many resistance proteins encoded by *RGAs* contain a leucine-rich repeat (LRR) domain [7,8], involved in protein-protein interactions and in pathogen recognitions [9]. Proteins codified by *RGAs* can be further classified according to the presence of the toll/interleukin-1 receptor (TIR) or other N-terminal features, such as coiled-coil (CC) and BED finger (Bed) [3,10,11]. The N-terminal features are involved in downstream specificity and signaling regulation [12]. *RGAs* evolved for pathogen recognition and frequently matched with specific pathogen avirulence factors to trigger signal transduction cascades and defense responses [9].

The genome sequencing of model plants has enabled the study of *RGA* families in monocots and dicots, including *Arabidopsis*

italiana [11,13], *Brassica rapa* [14], *Carica papaya* [15,16], *Cucumis sativus* [17], *Glycine max* [18,19], *Zea mays* [20,21], *Medicago truncatula* [22], *Oryza sativa* [23–25], *Populus trichocarpa* [26], *Sorghum bicolor* [27], *Vitis vinifera* [2,5,28,29], *Brachypodium distachyon* [30,31], *Solanum tuberosum* [32], and *Solanum lycopersicum* [33]. According to these studies, approximately 0.2–1.3% of genes predicted in plant genomes corresponds to *RGAs*, which occur at a density of 0.3–1.6 per mega base (Mb). The genome of apple (*Malus × domestica* Borkh.) also contains a large number of *RGAs* [34]. Apple is characterized by recent whole genome duplication (WGD) [34]. The role and relevance of such radical genomic changes in plant evolution was largely demonstrated, but the number and timing of WGDs in the different plant species was only partially understood [35,36]. Polyploidy is common in angiosperms [22,37], and most if not all extant species are thought to be ancient polyploids [38]. However, ancestral genomes are in most cases dispersed on multiply rearranged chromosomes, having also suffered wholesale gene losses [5,39]. Given that synonymous substitutions are immune to selection pressure [40], the per-site synonymous substitution rate (*K*_s) is widely used to infer the time of WGD and to describe the relationships among chromosomes [2,34].

In this study, cluster organization of *RGAs* and their distribution across chromosomes were analyzed in terms of recent duplication of the apple genome. In addition, the phylogenesis of *RGAs* from the domesticated and wild *Malus* species, including also other Rosaceae, *P. trichocarpa*, and *V. vinifera* *RGAs*, was considered to clarify the evolutionary history of apple and its related species.

Results

Classes of *RGAs* in *Malus* × *domestica*

Based on the presence of the NBS domain, 868 *RGAs* were identified in the genome of the *M. domestica* cultivar ‘Golden Delicious’, and all of them showed a significant (more than 90%) protein similarity with *RGAs* of *A. thaliana*, *P. trichocarpa*, and *V. vinifera*. In addition, 124 putative *RGA* alleles were found, and they were not further analyzed. By domain analysis, *RGAs* were assigned to *TIR-NBS-LRR* (*TNL*) and *CC-NBS-LRR* (*CNL*) classes. In particular, 505 *RGAs* were classified as *NBS-LRR* (*NL*), including *CNL* subclass, and 231 *RGAs* were classified as *TIR-NBS* (*TN*), including *TIR-NBS-LRR* (*TNL*), *NBS-LRR-TIR* (*NLT*), *TIR-CC-NBS-LRR* (*TCNL*), *TIR-CC-NBS* (*TCN*), and *TIR-NBS* (*TN*) subclasses (Table 1). In addition, 132 *RGAs* were characterized only by the presence of the NBS (N) or CC-NBS (CN) domains.

The 868 *RGAs* accounted for 1.51% of *M. domestica* predicted genes, a percentage slightly higher than that in other plant genomes (Table 1). The density of *RGAs* per Mb was similar for *M. domestica* and other genomes with the exception of *Z. mays*, *C. papaya*, *C. sativus*, and *S. bicolor*.

The mean exon number detected in apple *RGAs* was 4.51, and the number of exons of *CNL* class (3.46) was lower than the number of *TNL* class (6.41; $P < 0.001$). Thus, the number of exons in *RGAs* of *M. domestica* was consistent with the number in *A. thaliana* and *B. rapa* but higher than the number in *V. vinifera*, *P. trichocarpa*, and *O. sativa* (Table 1). Moreover, 23% of *CNL* *RGAs* are encoded by a single exon, while all *TNL* have at least three exons.

Genome Organization and Phylogeny of *RGAs* in *Malus* × *domestica*

Contigs anchored to the genome were used to assess the distribution of *RGAs* in the apple genome [34]. Of the *RGAs*, 778 (90%) were located across the 17 apple chromosomes (Figure 1). Among the anchored *RGAs*, 435 (56%) were assigned to six chromosomes: Chr 2, 7, 8, 10, 11, and 15 (Figure 1 and Table 2). Conversely, Chr 4, 6, 13, 14, and 16 had a low content of *RGAs* (27, 9, 17, 22, and 14 *RGAs*, respectively). *RGAs* were mainly (80%) grouped in clusters, 152 clusters included the majority (622) of the *RGAs* (Figure 1, Table 2 and Table S1). On average, four *RGAs*

Table 1. Classification and organization of resistance gene analogues (*RGAs*) with a nucleotide-binding site (NBS) domain in different plant genomes.

Characteristic	<i>Malus</i> × <i>domestica</i>	<i>Arabidopsis thaliana</i>	<i>Populus trichocarpa</i>	<i>Vitis vinifera</i>	<i>Oryza sativa</i>	<i>Cucumis sativus</i>	<i>Carica papaya</i>	<i>Sorghum bicolor</i>	<i>Brassica rapa</i>	<i>Brachypodium distachyon</i>	<i>Glycine max</i>	<i>Zea mays</i>
Number of total predicted genes	57,524	27,228	45,654	33,514	41,911 (28,236 [30])	26,682	28,591	27,640	nd	25,532	46,430	32,540
Genome size (Mb)	750	125	485	487	389	243	372	730	529	272	1,115	2,500
N° of <i>RGAs</i>	868	178	402	391	535	61	54	211 (245 [30])	92	178 (238 [30])	429 [30]	129 [80]
NBS-LRR class ^a	505 (58%)	57 (32%)	236 (59%)	194 (57%)	480 (89%)	nd	31 (57%)	184 (74%) [30]	17 (18%)	212 (89%) [30]	236 (55%) [30]	95 (74%) [30]
TIR-NBS class ^b	231 (27%)	115 (64%)	94 (23%)	42 (13%)	3 (1%)	nd	7 (13%)	2 (1%) [31]	42 (46%)	nd	154 (36%) [30]	nd
Other <i>RGAs</i> ^c	132 (15%)	6 (4%)	72 (18%)	103 (30%)	52 (10%)	nd	16 (30%)	61 (25%) [30]	33 (36%)	27 (11%) [30]	39 (9%) [30]	34 (26%) [30]
<i>RGAs</i> /total genes (%)	1.51	0.65	0.88	1.01	1.27	0.23	0.19	0.76 (0.88 [30])	nd	0.69 (0.9 [30])	0.92 [30]	0.39 [30]
<i>RGAs</i> per Mb	1.16	1.42	0.82	0.7	1.5	0.25	0.15	0.28 (0.33 [30])	0.92	0.65 (0.87 [30])	0.38 [30]	0.056 [30]
Average number of exons in <i>RGAs</i>	4.51	4.19	2.35	3.96	3.72	nd	nd	nd	4.2	nd	nd	nd
Number of Single <i>RGAs</i>	156	46	135	55	104	20	12	nd	18	nd	nd	nd
Number of Clusters	152	39	75	52	157	11	13	nd	24	nd	nd	nd
Maximum Number of <i>RGAs</i> in clusters	21	11	19	26	11	9	7	nd	5	nd	nd	nd
Average Number of <i>RGAs</i> in cluster	4.11	3.21	3.75	5.78	3.48	3.72	2.92	nd	2.54	nd	nd	nd
Source	this paper and [34]	[11]	[26]	[2,29]	[24,25]	[17]	[16]	[27]	[14]	[31]	[19]	[21]

^aNBS-LRR class includes: NBS-LRR (NL) and CC-NBS-LRR (CNL). Percentage (%) of this class relative to the total number of *RGAs* reported in brackets.

^bTIR-NBS class includes: TIR-NBS-LRR (TNL), NBS-LRR-TIR (NLT), TIR-CC-NBS-LRR (TCNL), TIR-CC-NBS (TCN), and TIR-NBS (TN). Percentage (%) of this class relative to the total number of *RGAs* reported in brackets.

^cClass of other *RGAs* includes: NBS (N) and CC-NBS (CN). Percentage (%) of this class relative to the total number of *RGAs* reported in brackets.

nd: not declared by the authors.

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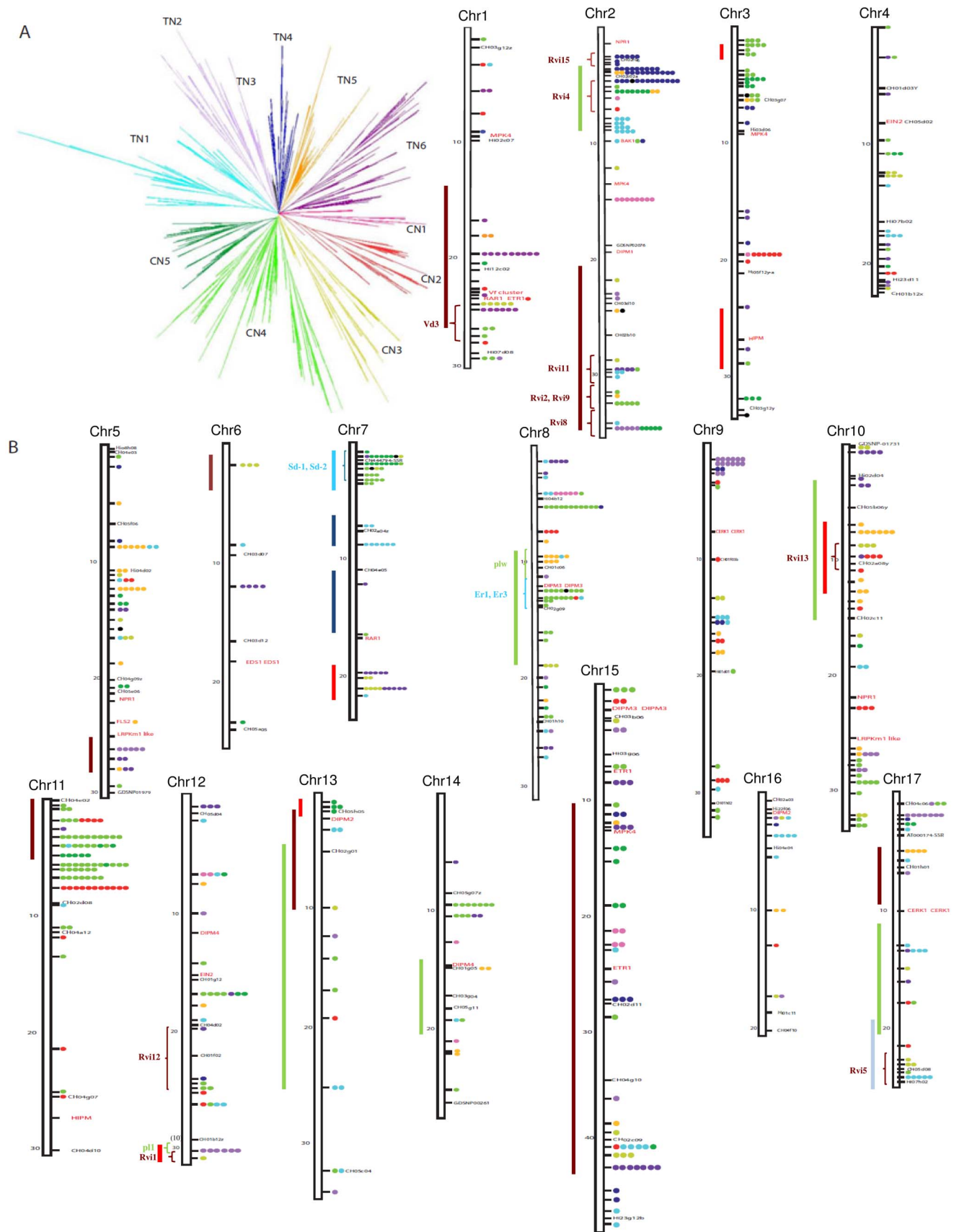


Figure 1. Chromosomal organization of RGAs in *Malus × domestica*. **A:** Phylogenetic analysis of NBS domain was carried out by neighbor-joining method [65] on RGAs protein sequences from *M. domestica* cultivar ‘Golden delicious’. Major phylogenetic clades (from CN1 to CN5 and from

TN1 to TN6) correspond to the classification based on protein domains. TN1 (light blue): TIR-NBS-LRR; TN2 (light purple): TIR-NBS-LRR and TIR-NBS; TN3 (black): TIR-NBS-LRR; TN4 (blue): TIR-NBS-LRR, CC-TIR-NBS, and TIR-NBS; TN5 (orange): TIR-NBS-LRR, and TIR-NBS; TN6 (dark purple): TIR-NBS-LRR; CN1 (pink): CC-NBS-LRR; CN2 (red): CC-NBS-LRR and NBS-LRR; CN3 (light green): CC-NBS-LRR, NBS-LRR, NBS; CN4 (green): CC-NBS-LRR, NBS-LRR, NBS; CN5 (dark green): CC-NBS-LRR, NBS-LRR, NBS. **B:** *RGAs* assigned to chromosomes (Chr) are represented by dots with colors corresponding to major phylogenetic clades. The size of each chromosome is given in megabase (Mb, on the left side), whereas the markers of the genetic map are shown in black (on the right side). Resistance-related genes different from *RGAs* are shown in red. Known quantitative trait loci (QTL) for resistance to apple scab (brown), powdery mildew (green), aphids (light blue), fire blight (red) and rust mite (blue) are shown by bars on the left side of chromosomes [67–73], together with the major resistance genes to apple scab (*Vd3* and *Rvi* genes) [74–76], powdery mildew (*P11*) [77], and aphids (*Sd-1*, *Sd-2*, *Er1*, *Er2*) [78,79].

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were present in a cluster, and the largest cluster contained 21 *RGAs* (located on Chr 2). Several clusters of *RGAs* can be associated with QTLs affecting disease resistance of *Malus* genotypes (Figure 1).

As previously shown in *Arabidopsis* [6,11], *RGAs* of *TIR-NBS* and non-*TIR-NBS* classes had different topologies in the phylogenetic analysis (Figure 1A). In particular, six major *TIR-NBS* clades (numbered from TN1 to TN6) and five non-*TIR-NBS* major clades (numbered from CN1 to CN5) were identified in apple. *RGAs* of *TIR-NBS* class were mainly located on Chr 2, 5, 9, 12, 15, 16, and 17, with Chr 16 hosting the *TIR-NBS* class almost exclusively (Figure S1A and Table S1). Chr 3, 4, 8, 11, 13, and 14 were mainly characterized by non-*TIR-NBS* class, and Chr 11 had almost exclusively *RGAs* of non-*TIR-NBS* class. Considering *TIR-NBS* and non-*TIR-NBS* phylogenetic clades, the major clade TN6 represented more than one-third of the *RGAs* on Chr 1 and 6, while the major clade CN4 included more than half of the *RGAs* on Chr 11 and 14 (Figure S1A). Moreover, the major clade TN4 was located preferentially (63%) on Chr 2 (Figure S1B).

Phylogeny of *RGAs* in Domesticated and Wild *Malus* Species

Twenty-four wild *Malus* species (Table S2) were considered, and PCR fragments were amplified from germplasm. After sequence comparison, unique fragments were translated in to amino acid sequences (Table S1), and 115 of them matched NBS sequences of known resistance proteins with an E-value lower than $1E^{-10}$. Phylogenetic analysis indicated that *RGAs* of wild *Malus* species grouped mainly in clades that included sequences of the domesticated apple (Figure 2). A significant fraction of phylogenetic clades contained only a few *RGAs*, probably due to the short sequence of the NBS domain used for this analysis. Some clades consisted mainly of sequences from wild species and contained only few *RGAs* of the domesticated apple.

Phylogeny of *RGAs* among Rosaceae Species

A total of 693 Rosaceae *RGA* sequences at NCBI were downloaded (75 from *Rubus*, 293 from *Prunus*, 16 from *Fragaria*, 125 from *Rosa*, 34 from *Pyrus*, and 150 public sequences from *Malus* species) and compared to the 868 *RGAs* of *M. domestica* and the 210 sequences obtained from wild *Malus* species (Table S1). In

Table 2. Organization and distribution of resistance gene analogues (*RGAs*) with a nucleotide-binding site (NBS) domain in the apple (*Malus × domestica*) chromosomes.

Chromosome	Number of <i>RGAs</i>	Genome organization of <i>RGAs</i>		
		Number of single <i>RGAs</i>	Number of Clusters	Average Number of <i>RGAs</i> /cluster
1	43	10	7	4.7
2	109	14	15	6.3
3	47	12	11	3.2
4	27	12	6	2.5
5	48	11	11	3.4
6	9	2	2	3.5
7	57	4	11	4.8
8	76	11	14	4.6
9	40	7	10	3.3
10	56	14	14	3.0
11	79	7	10	7.2
12	37	11	6	4.3
13	17	9	4	2.0
14	22	6	4	4.0
15	58	14	14	3.1
16	14	3	4	2.8
17	39	9	8	3.8
Not anchored <i>RGAs</i>	90	–	–	–
Total	868	156	152	4.1

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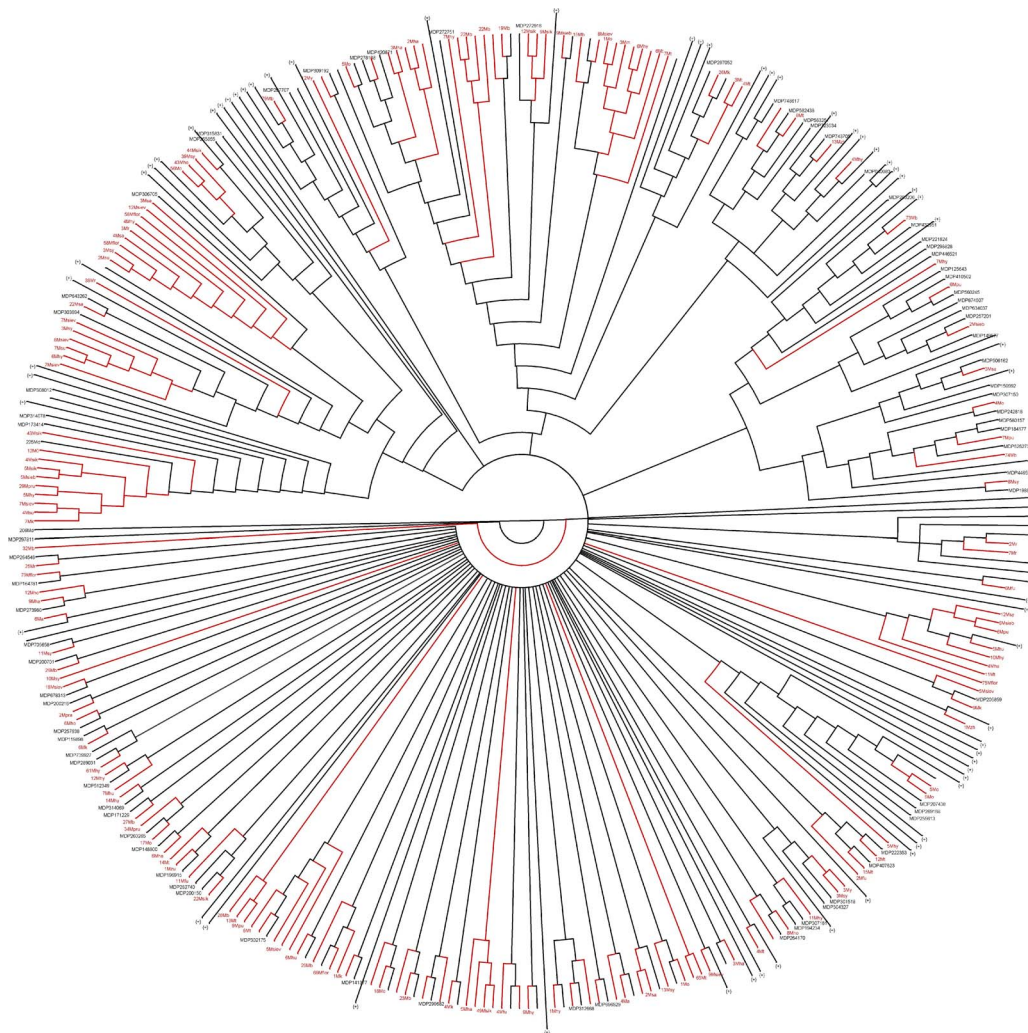


Figure 2. Phylogenesis of *RGAs* from *Malus* × *domestica* and from wild *Malus* species. Phylogenetic analysis of the NBS domain was carried out by the neighbor-joining method [65] using *RGA* sequences of *M. domestica* cultivar 'Golden delicious' (black) and wild *Malus* species (red). Proteins present in contiguous positions on the tree and belonging to the same species are merged (collapsed branches are indicated by the + sign). Phylogentic tree reveals 18 clades specific to *M. domestica*, six clades specific to wild *Malus* species, and 49 clades that include *RGAs* sequences of both domesticated and wild apple species.
doi:10.1371/journal.pone.0083844.g002

the phylogenetic tree of Rosaceae species (Figure 3), 49 clades were specific to the genus *Malus*, and included sequences from two or more *Malus* species. Most of the remaining clades were represented by *RGAs* from two or more Rosaceae genera. In particular, three clades comprised *RGAs* of *Malus*, *Pyrus*, and *Prunus*, indicating a monophyletic origin of the three genera and strong conservation of some *RGA* sequences in these plants. Few clades were represented by non-apple *RGAs*, and clades specific to *Fragaria* or *Rosa* were also present.

Comparison of *RGAs* among *Malus* × *domestica*, *Populus trichocarpa*, and *Vitis vinifera*

RGA sequences can also be compared across different plant families, and a phylogenetic tree of *RGAs* from *M. domestica*, wild *Malus* species, *V. vinifera*, and *P. trichocarpa* (Table S1) was obtained (Figure 4). Several clades included sequences from two or three species, and two major clades, named Md1 and Md2, comprised only sequences of *M. domestica* (Figure 4). However, sequences of the Md1 clade were grouped in three subclades in the phylogenetic

tree of *RGAs* from Rosaceae species (Figure S2). *RGAs* of subclades Md1 sc2 and Md1 sc3 did not show similarity with any Rosaceae *RGAs*, whereas sequences of Md1 subclade 1 (Md1 sc1) shared significant similarity with four *RGAs* of *Pyrus* (Figure S2). Clade Md2 included one and two *RGAs* from *Rubus* and *Rosa*, respectively. Most of the *RGAs* of the clade Md2 are located on Chr 2, 3, 7, 11, 12, and 15.

Duplication of *RGAs* in *Malus* × *Domestica*

To study the recent duplication of *RGAs* in the *M. domestica* genome, Ks values were determined, and results from recent gene duplications were highlighted (Figure S3). Links among different *RGAs* helped to describe the relationships among the duplicated apple chromosomes [34]. Homologous apple chromosomes had more than 10 links, except for Chr 13 and 16, which hosted only a low number of *RGAs*. Chr 6 was not included in this analysis because it contains only nine *RGAs*, six of them derived from the recent WGD. Moreover, the duplicated chromosomes had *RGAs* belonging to the same phylogenetic clades (Figure S4).

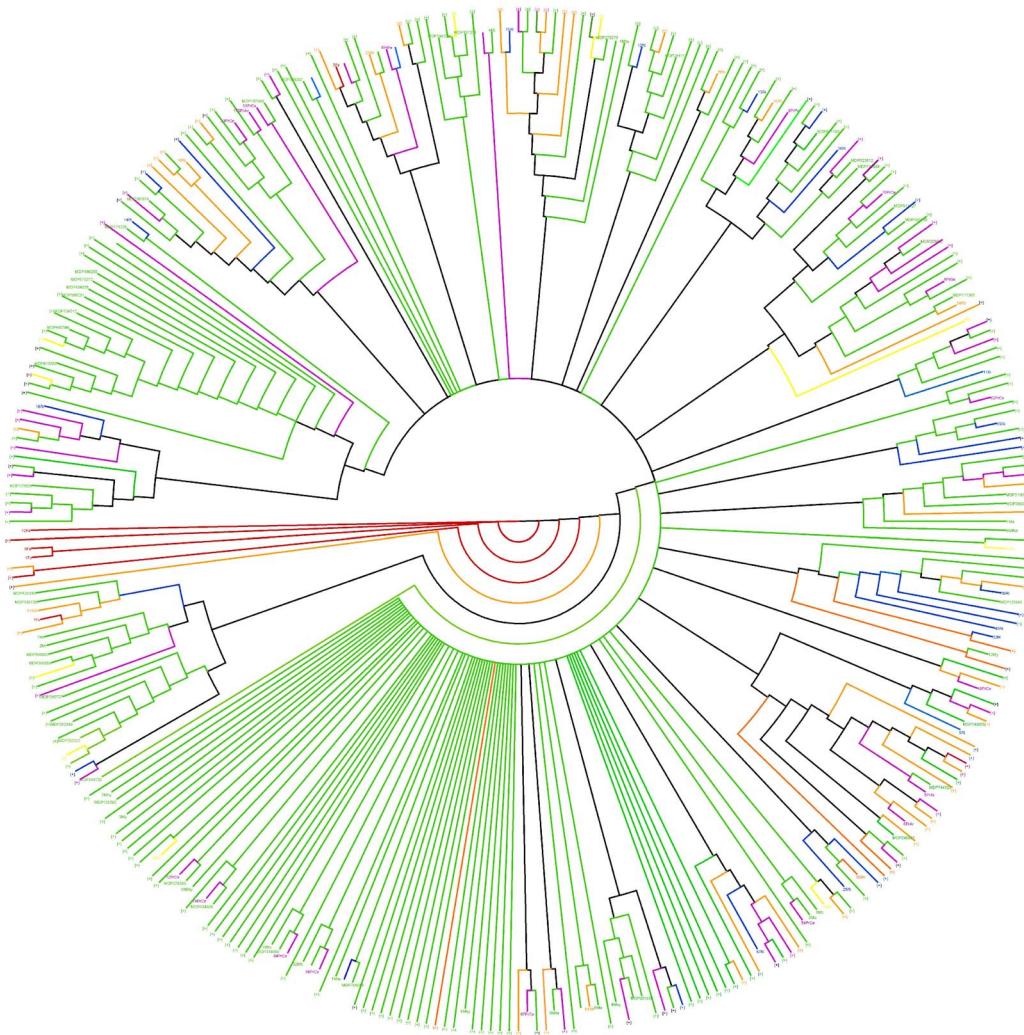


Figure 3. Phylogenesis of *RGAs* from *Malus* species (wild and domesticated apple), *Pyrus communis*, *Prunus* species, *Fragaria ananassa*, *Rubus idaeus*, and *Rosa* species. Phylogenetic analysis of the NBS domain was carried out by the neighbor-joining method [65] using *RGA* sequences of domesticated and wild *Malus* species (green), *Pyrus* spp. (yellow), *Prunus* spp. (purple), *Fragaria* spp. (red), *Rosa* spp. (orange), and *Rubus* spp. (blue). Proteins present in contiguous positions of the tree are merged (collapsed branches are indicated by the + sign). Phylogentec tree indicates 49, three and one clades specific to *Malus* spp., *Fragaria* spp. and *Rosa* spp., respectively. Clades with *RGAs* of different genera: three clades of *Malus* spp. and *Prunus* spp.; seven clades of *Malus* spp. and *Pyrus* spp.; two clades of *Malus* spp. and *Rubus* spp.; four clades of *Malus* spp. and *Rosa* spp.; two clades of *Fragaria* spp. and *Rosa* spp.; two clades of *Malus* spp., *Rosa* spp., and *Rubus* spp.; three clades of *Malus* spp., *Pyrus* spp., and *Rosa* spp.; three clades of *Malus* spp., *Prunus* spp., and *Rubus* spp.; four clades of *Malus* spp., *Prunus* spp., *Rosa* spp., and *Rubus* spp.; three clades of *Malus* spp., *Prunus* spp., *Pyrus* spp., *Rosa* spp., and *Rubus* spp.; two clades of *Malus* spp., *Fragaria* spp., *Prunus* spp., *Rosa* spp., and *Rubus* spp., one clade of *Malus* spp., *Fragaria* spp., *Pyrus* spp., *Rosa* spp., and *Rubus* spp.
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Discussion

To counteract pathogens, plants rely on the innate immunity of their cells and on systemic signals emanating from infection sites [9,41]. Pathogen effectors from very diverse organisms are recognized by resistance proteins encoded by *RGAs* and activate plant defense responses [6,9]. NBS-mediated disease resistance is effective against obligate biotrophic and hemibiotrophic pathogens but not against necrotrophs, which kill host tissues during colonization [42].

In apple, the abundance of *RGAs* is only partly related to genome size (750 Mb), which is much smaller than in maize (2300 Mb; [21]) or soybean (1115 Mb; [19]). The *TIR-NBS* class accounts for the largest group of *RGAs* in *A. thaliana* (64%; [11]) and *B. rapa* (64%; [14]). In *P. trichocarpa* [26], *V. vinifera* [2,5,28,29],

and *C. papaya* [16,30], the percentage of *TIR-NBS* class is much lower than in the previously mentioned species. The *TIR-NBS* class is present at a very low frequency in *O. sativa* (1%; [24]) and *S. bicolor* (1%; [27]) and is absent in *B. distachyon* and *Z. mays* [30], supporting the conclusion that this class is specific for dicotyledons. In apple, 231 *RGAs* of *TIR-NBS* class have been identified, and they are mainly located on Chr 2, 5, 9, 12, 15, 16, and 17. However, the number of *RGAs* belonging to non-*TIR-NBS* class in apple (505) is greater than in all other species considered, and these *RGAs* are mainly located on Chr 3, 4, 8, 11, 13, and 14. The existence of chromosome-specific *RGAs* classes suggests that groups of chromosomes evolved separately, but further analyses are required to test this hypothesis. In grapevine, the existence of two chromosome groups has been inferred based on *RGAs* cluster similarity, and the two groups seem to have evolved independently

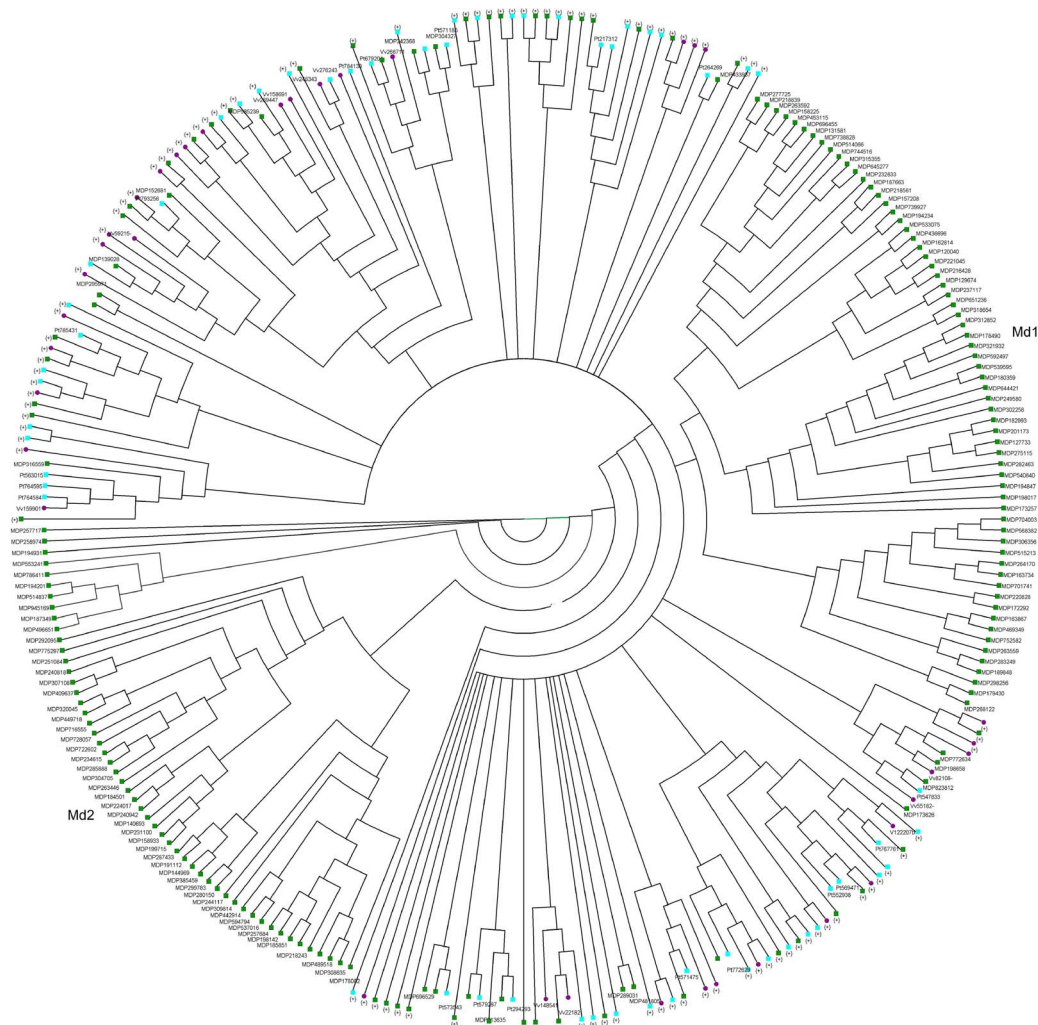


Figure 4. Phylogenesis of *RGAs* from *Malus* species (wild and domesticated apple), *Populus trichocarpa* and *Vitis vinifera*. Phylogenetic analysis of the NBS domain was carried out by the neighbor-joining method [65] using *RGA* sequences of domesticated and wild *Malus* species (green), *P. trichocarpa* (cyan), and *V. vinifera* (purple). Proteins present in contiguous positions on the tree and belonging to the same species are merged (collapsed branches are indicated by the + sign). Two phylogenetic clades comprise only sequences of *M. domestica* (Md1 and Md2). doi:10.1371/journal.pone.0083844.g004

[2]. Moreover, the TIR-NBS class is specific for only one of the two components of *V. vinifera* genome, suggesting an independent evolution of the *RGA* classes [2].

In apple, 56% of *RGAs* (435 of 778 anchored) are located preferentially on six chromosomes, with 14% located on Chr 2. In large gene families, genes are commonly organized in clusters and superclusters [4,5,11,14,16,25,26], as demonstrated here for the apple genome. Of the *RGAs* clusters in apple, 71% (108 of 152) include *RGAs* from the same phylogenetic clade, and 29% *RGAs* from two to three different clades. Clusters frequently consist of tandem duplications of the same gene [5,43]. Heterogeneous clusters, in which sequences belong to different phylogenetic lineages, are also present, most probably as a result of different molecular mechanisms like ectopic recombination, chromosomal translocation, and gene transposition, as has been recently highlighted for the grapevine genome [2]. This kind of genome evolution could be explained in terms of a positive selection for cluster complexity, which could serve as the basis for the generation of new resistance specificities [4,44]. The role of tandem duplication in the apple genome is supported by low Ks

values among *RGAs* of the same cluster, as is already known for other species [2,5,14,22,43]. Gene duplication in a position different from the original cluster has to be preceded by gene transposition, as predicted for *A. thaliana* and *V. vinifera* *RGAs* [1,2]. Thus, a successful transposition is the starting point for the creation of a new *RGA* cluster, and the selection for disease resistance could favor the process [5,45]. Moreover, analysis of *RGA* transposition has indicated that *V. vinifera* putative component genomes may have evolved independently and later fused and evolved together in the same nucleus [2].

Velasco *et al.* [34] have shown that recent WGD has increased the chromosome number in apple from nine in the putative ancestor to the current 17. The recent duplication of *RGAs* due to a WGD event supports the existence of i) a tetraploid state of the genome in which a pair of chromosomes exists with a second homologous pair; ii) duplications inside chromosomes, particularly for Chr 11 where recent duplications can be observed; and iii) duplications in different chromosomes, suggesting recent events of gene transposition. Eight of the 17 chromosomes (Chr 3 and 11, 5 and 10, 9 and 17, and 13 and 16) represent a direct duplication of

four ancestral chromosomes, and each of the extant Chr 4, 6, 12, and 14 derives from translocation between two ancestral chromosomes [34]. More complex events have generated the remaining five chromosomes that are derived from starting three ancestral chromosomes. The different clades of *RGAs* along duplicated chromosomes indicate a similar position of orthologous *RGAs* along each chromosome doublets (Chr 3 and 11, 5 and 10, 9 and 17, and 13 and 16). These results strongly support the origin of the apple chromosomes as described by Velasco *et al.* [34] and indicate that *RGA* distribution might be used to dissect plant genome evolution [2]. As is the case for other species, the process of gene duplication has shaped the apple genome in different ways, including the selective retention of paralogs associated with specific biological processes, the amplification of specific gene families, and an extensive subfunctionalization of paralogs. Both the major WGD event and small-scale duplications could be responsible for the high number of the apple *RGAs*. A remarkable feature of gene duplication in apple is the high proportion of paralogs showing divergent expression patterns [46]. Extensive subfunctionalization could have contributed to the acquisition of new traits specific to apple or to the Pyrinae lineage [47]. Sequences of Eurosid genomes provide evidence of ancient genome duplications that occurred early in evolution, suggesting a polyploid origin for most Eudicots [28,48].

Most of the *RGAs* of wild *Malus* species are closely related *RGAs* of the domesticated apple. Whereas *RGAs* sequencing from wild *Malus* species was partial and could include alleles of the same gene, phylogenetic analysis revealed specific clades of wild *Malus* species, indicating, as expected, the potential to enlarge the genetic variation of *RGAs* in domesticated apple. Moreover, the comparison of apple *RGAs* with those of other Rosaceae indicates the existence of specific clades for apple. In addition, several clades include a mixture of *RGAs* from *Malus*, *Pyrus*, and *Prunus*, indicating that similar resistance genes are still shared in different genera of the Rosaceae. While these results support the monophyletic origin of the three genera, clades specific for each genus were also found. The existence of genus- or species-specific clades indicates the existence of mechanisms for cluster conservation, as reported by Plocik *et al.* [49].

Phylogenetic relationships within the Rosaceae inferred from *RGAs* are consistent with phylogenies based on chloroplast and other nuclear genes [50,51]. The phylogenetic analysis of the *RGAs* from *Malus*, *Vitis*, and *Populus* shows that *Malus* contains two large non-*TIR-NBS* clades that are specific to *Malus*. This inference should be considered with caution, because the *RGA* sequences used in our analysis are from only a few species. Several other reasons could explain the variation of *RGAs* in Rosaceae species, such as the inter-specific variation of the *RGA* family size observed in dicotyledonous plants. Similar situations were reported for other gene families in the *Archeae* [52], bacteria [52,53], and mammals [54,55]. The variation of *RGA* family size between species could be attributed to gene duplication, deletion, pseudogenization, and functional diversification [56–58]. The last case is supported by the necessity of a species to adapt to rapidly changing pathogen populations.

Concluding Remarks

This paper analyses the *RGAs* of *Malus* spp. and other Rosaceae species to reveal specific evolutionary features of *M. domestica*. *RGAs* of *M. domestica* are mainly located in clusters and are mapped preferentially on six chromosomes. *TIR-NBS* and non-*TIR-NBS* classes of *RGAs* are located in different chromosome groups. Phylogenetic reconstruction in the Rosaceae family revealed specific clades of *RGAs* for *Malus* spp., *Fragaria* spp., and *Rosa*

spp., indicating genus-specific evolution of resistance genes. However, strikingly similar *RGAs* were shared in different species of *Malus*, *Pyrus*, and *Prunus* highlighting a monophyletic origin of these three genera and the high conservation of some *RGA* sequences in these plants.

Materials and Methods

Identification of *RGAs* in the Apple Genome

The *RGA* sequences were identified from the predicted proteins of *M. domestica* cultivar ‘Golden Delicious’ [34] based on their NB-ARC domain profile (PF00931 [59]) using HMMER [60]. Putative *RGA* alleles were identified as predicted genes that have more than 90% of sequence similarity and overlap with another *RGA* along each scaffold of the heterozygous apple genome. Apple *RGAs* were validated by BLAST-N analysis (more than 90% protein sequence similarity) against known *A. thaliana*, *P. trichocarpa*, and *V. vinifera* genes. *RGAs* were grouped in different classes based on the presence of the domains TIR, LRR, CC, and BED finger [43]. The motifs were derived from the domain profiles retrieved from PFAM (<http://pfam.janelia.org>), PANTHER (<http://www.pantherdb.org/>), and SMART (<http://smart.embl-heidelberg.de>) databases and from the COILS program; a stringent threshold of 0.9 was used so that CC domains were specifically detected [61]. Resistance-related proteins were also identified based on kinase domains (IPR000719, PF07714, PF00069). Additional putative apple resistance genes were selected using BLAST and *Arabidopsis* proteins as reference sequences, based on a 60% similarity threshold.

Identification of *RGA* Clusters in the Apple Genome

The *Arabidopsis* definition of *RGA* cluster [4] was adopted: two or more *RGAs* in a cluster should be located within an average of 250 Kb and should not be interrupted by more than 21 open reading frames different from *RGAs*, as previously adopted for grapevine *RGA* clusters [2].

Isolation of *RGAs* from Wild Species

Four pairs of degenerate primers targeting the NBS domain [62,63] were used to amplify *RGA* sequences from 26 different *Malus* accessions present in the USDA apple germplasm collection at Geneva (NY, USA) (www.ars-grin.gov/npgs/index.html; Table S2). The homologous sequences represent the following species: *M. baccata*, *M. florentina*, *M. floribunda*, *M. fusca*, *M. halliana*, *M. honanensis*, *M. hupehensis*, *M. kansuensis*, *M. micromalus*, *M. orientalis*, *M. prattii*, *M. prunifolia*, *M. pumila*, *M. robusta*, *M. sargentii*, *M. sieboldii*, *M. sieversii*, *M. sikkimensis*, *M. sublobata*, *M. sylvestris*, *M. transitoria*, and *M. yunnanensis* (Table S2). PCR fragments were cloned in pGEMT easy (Promega), and two clones for each fragment were sequenced. Sequences were screened, cleaned, and compared with resistance genes previously identified in Rosaceae and in other Angiosperms. BLAST DNA similarity searches were performed against the *RGA* sequences of the apple genome using a collection of established *RGAs*. The *RGAs* were translated using tBLAST-N. Clones were filtered based on hit quality, because most of the *RGA* clones encoded between 24 and 40 amino acid residues. Queries having only a single hit below 90% identity were removed, and those with multiple smaller hits were annotated manually. *RGA* sequences from wild *Malus* species were submitted to the NCBI database (www.ncbi.nlm.nih.gov) under the accession numbers reported in Table S1.

Phylogenetic Analyses

Public *RGA* sequences from Rosaceae, *P. trichocarpa*, and *V. vinifera* Release 2 were downloaded from GenBank (<http://www.ncbi.nlm.nih.gov>; Table S1). *RGA* sequences from wild *Malus* species were also included (Table S1). Protein sequences of NBS domain of *RGAs* from *M. domestica* were aligned together with NBS sequences of wild *Malus* species, *P. trichocarpa*, *V. vinifera* and with the other Rosaceae species using hidden Markov models with the Sequence Alignment and Modeling Software System (SAM-T2K [64]); the sequences were formatted for analysis with the Phylip phylogenetic inference package [65].

The SEQBOOT tool of the Phylip package was used to generate 500 bootstraps of the data set, and the PROTDIST tool was used to construct 500 bootstrapping distance matrices using the Dayhoff PAM matrix [65]. These matrices were jumbled twice and processed with the FITCH tool to create a phylogenetic tree. A neighbor-joining tree of the 500 bootstraps was also constructed (jumbling the sequence input order twice), and a majority-rule consensus tree was assembled.

Determination of the Ks Value

Based on a CLUSTALW nucleotide alignment of *M. domestica* *RGAs* sequences, a total of 302253 Ks values were obtained [66]. The connections between chromosomes were defined on the basis of the number of *RGAs* and Ks values. A connection between two chromosomes was accepted if at least ten *RGAs* had a Ks value lower than or equal to the first quartile of 0.25 [34].

Supporting Information

Figure S1 A: Distribution (percentage) of the major phylogenetic clades of apple *RGAs* (Figure 1A) on the 17 *M. domestica* chromosomes (Chr). **B:** Percentage of chromosome (Chr) assignment to the major phylogenetic clades. Colours of major phylogenetic clades and chromosomes are listed below each chart. (TIF)

Figure S2 Phylogenesis of *RGAs* from Rosaceae species. Phylogenetic analysis of the NBS domain was carried out by the neighbor-joining method [65] using *RGA* sequences of domesticated and wild *Malus* species (green), *Pyrus* spp. (yellow), *Prunus* spp. (purple), *Fragaria* spp. (red), *Rosa* spp. (orange), and *Rubus* spp. (blue). The composition of the phylogenetic clades (Md1 and Md2; Figure 4) and subclades (sc) of sequences mainly from *M. domestica* is highlighted. Proteins present in contiguous

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positions on the tree are merged (collapsed branches are indicated by the + sign).

(TIF)

Figure S3 Connections between apple chromosomes based on Ks values from pairwise comparisons of *RGAs*.

Joining lines represent connections between two *RGAs* among duplicated chromosomes [35] (blue, red, pink, green), among not duplicated chromosomes (yellow), and within the same chromosome (gray). Each line represents a connection between two *RGAs* with a Ks value lower than 0.25 [35]. A connection between two chromosomes was accepted if at least ten pairwise comparisons had a Ks value lower than 0.25.

(TIF)

Figure S4 Distribution of *RGAs* among chromosome (Chr) doublets derived from the recent whole genome duplication of apple [34]. Colours of major phylogenetic clades (Figure 1A) are indicated.

(TIF)

Table S1 List of accession numbers and abbreviations of resistance gene analogues (*RGAs*) with a nucleotide-binding site (NBS) domain from *Malus* × *domestica*, *Populus trichocarpa*, *Vitis vinifera*, wild *Malus* species, *Fragaria* spp., *Prunus* spp., *Pyrus communis*, *Rubus idaeus*, and *Rosa roxburghii*. Chromosome location, code, and class based on protein domain analysis are indicated for each of the *RGAs* of *M. domestica*.

(XLS)

Table S2 List of wild *Malus* species accessions (USDA apple germplasm collection at Geneva, NY, USA; www.ars-grin.gov/npgs/index.html) used for the isolation of *RGAs*.

(DOCX)

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Author Contributions

Conceived and designed the experiments: RV MM. Performed the experiments: A. Baldo A. Bailey PF. Analyzed the data: GM MP LR MM. Contributed reagents/materials/analysis tools: RV MM. Wrote the paper: GM MP A. Baldo MM.

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