Investigation of orthologous pathogen recognition gene-rich regions in Solanaceous species

Journal:	<i>Genome</i>
Manuscript ID	gen-2016-0217.R2
Manuscript Type:	Article
Date Submitted by the Author:	04-Jul-2017
Complete List of Authors:	Di Donato, Antimo; University of Naples, Department of Agricultural Science Andolfo, Giuseppe; University of Naples, Department of Agricultural Science Ferrarini, Alberto; Università degli Studi di Verona, Department of Biotechnology Delledonne, Massimo; University of Verona Ercolano, Maria Raffaella; University of Naples,
Is the invited manuscript for consideration in a Special Issue? :	This submission is not invited
Keyword:	Pathogen recognition genes, Capsicum annuum, Solanum melongena, gene arrangements, target sequencing

SCHOLARONE™
Manuscripts

Investigation of orthologous pathogen recognition gene-rich regions in Solanaceous species

Di Donato A.¹, Giuseppe A.¹, Ferrarini A.², Delledonne M.², Ercolano M.R.^{1*}

¹Dipartimento di Agraria, Università di Napoli ‘Federico II’, Via Università 100, 80055 Portici, Italy.

²Dipartimento di Biotecnologie, Università degli Studi di Verona, Strada le Grazie, 15, 37134 Verona, Italy

AD: antimo.didonato3@gmail.com

GA: giuseppeandolfo@hotmail.com

AF: alberto.ferrarini@univr.it

MD: massimo.delledonne@univr.it

MRE: ercolano@unina.it

***Corresponding author:**

Ercolano Maria Raffaella

Dipartimento di Agraria, Università di Napoli ‘Federico II’, Via Università 100, 80055 Portici, Italy.

Tel/Fax +39 081 2539431

Abstract

Pathogen receptor proteins such as receptor-like protein (RLP), receptor-like kinase (RLK) and nucleotide-binding leucine-rich repeat (NLR) play a leading role in the plant immunity activation. The genome architecture of such genes was extensively investigated in several plant species. However, we still know little about their elaborate reorganization arose during the plant speciation process. Using recently released pepper and eggplant genome sequences we were able to identify 1097 pathogen recognition genes (PRGs) in pepper Zunla-1 and 775 in eggplant Nakate-Shinkuro. The retrieved genes were analysed for their tendency to cluster, using different methods to infer the means of grouping. Orthologous relationships among clustering loci were found, and interesting reshuffling within given loci was observed for each analysed species. The information obtained were integrated in a comparative map to highlight the evolutionary dynamics in which the PRG loci were involved. Diversification of 14 selected PRG-rich regions was also explored using a DNA target-enrichment approach. A large number of gene variants was found as well as rearrangements of single protein domain encoding sequences and changes in chromosome gene order among species. Gene duplication and transposition activity have clearly influenced plant genome R-gene architecture and diversification. Our findings contribute to addressing several biological questions concerning the parallel-evolution occurred between genomes of Solanaceae family. Moreover, the integration of different methods proved a powerful approach to reconstruct the evolutionary history in plant families and to transfer important biology findings among plant genomes.

Keywords: Pathogen recognition genes; *Capsicum annuum*; *Solanum melongena*; gene arrangements; target sequencing.

55 Introduction

56 The Solanaceae family comprises more than 3,000 species and includes major food crops such as
57 tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), eggplant (*Solanum melongena*) and
58 pepper (*Capsicum annuum*). In recent years, several important Solanaceae species have been
59 sequenced (Fernandez-Pozo et al. 2015) and wild species genomes investigated by sequencing and
60 resequencing (Qin et al. 2014, Aflitos et al. 2014, Aversano et al. 2015). Solanaceae species have
61 also served as ideal systems for studying the genetics and molecular basis of plant resistance
62 mechanisms (Ercolano et al. 2012). Given the lack of extensive studies on the eggplant and pepper
63 genomes regarding pathogen recognition genes (PRGs: RLP - receptor-like protein, RLK - receptor-
64 like kinase and NLR - nucleotide-binding leucine-rich repeat), information gathered from other
65 species can be used to steer investigation in such species.

66 According to comparative genomic studies, Solanaceous species share extensive syntenic regions
67 (Wang et al. 2008): many of the loci involved in disease resistance in tomato have putative
68 orthologues, in potato, eggplant and pepper in corresponding positions (Grube et al. 2000, Vossen
69 et al. 2014). Further characterisations have demonstrated that tomato PRG homologues in potato,
70 tobacco and pepper are subject to dramatic reshuffling (Andolfo et al. 2013a, Seo et al. 2016, Wei et
71 al. 2016).

72 Comparison of the spatial arrangement of genes in different genomes raises important questions on
73 how complex biological systems evolve and function. Spatial analyses of the orthologous genomic
74 region can unravel selection processes and species history (Hoberman and Durand 2005). Over time
75 the large- and small-scale rearrangements occurring in orthologous loci have shaped the specific
76 genomic architecture of different species (Yeaman 2013). To reconstruct the direction and
77 magnitude of evolutionary trajectories of a given gene family, it is critical to detect the ancient loci
78 that can lead to the formation of gene clusters (Baumgarten et al. 2003, Luo et al. 2012).

In previous studies PRG clusters were identified using approaches based both on identifying a genomic region containing a number of PRGs or on a set of genes that delimit an interspace (Richly et al. 2002; Hoberman and Durand 2005). A more rigorous investigation of well-known Solanaceae PRG clustered loci dynamics can help to understand how their arrangement can impact disease-specific responses, since all functional resistance genes found in tomato and potato are included in a cluster (Andolfo et al. 2014). The recently developed target-enriched strategies for next-generation sequencing could facilitate the analysis of PRG-rich regions (Grover et al. 2012). It has already been demonstrated that targeted sequencing can expand our knowledge of PRGs (Andolfo et al. 2014). Furthermore, targeted sequencing of selected PRG loci combined with availability of high-quality reference genome sequences can offer insights into the mechanisms of PRGs evolution (Gasc et al. 2016, Steuernagel et al. 2016, Witek et al. 2016). In this work, the annotation of NLRs, RLPs and RLKs coding genes in eggplant and pepper genomes was performed. The PRG clustered loci rearrangement arose was reconstructed using multiple and combined methods, and a genome-wide comparative map in the three Solanaceae species was also realised. Finally, Solanaceae loci containing functionally characterised PRGs in such species were explored by targeted sequencing and microsynteny analysis.

Materials and methods

Pathogen recognition gene family annotation

A script developed in-house to identify tomato and potato PRG proteins by Andolfo et al. (2013b) was implemented in this study. The HMM profiles were used to screen the pepper and eggplant proteomes (<http://peppersequence.genomics.cn>; <http://eggplant.kazusa.or.jp>) to identify pathogen recognition proteins. This proteins set was further analysed using InterProScan v5 (Jones et al. 2014) to verify the presence of characteristic domains of pathogen recognition proteins (CC: coiled coil; NB: nucleotide binding; LRR: leucine rich repeat; TIR: Toll/interleukin-1 receptor; Kin: kinase; eLRR: extracellular-leucine rich repeat; TM: transmembrane).

104

105 **Cluster analysis**

106 The calculation of local gene enrichment was conducted using two methods: an arbitrary max gap
 107 approach (MG) (Hoberman et al. 2005), for identifying the spatial arrangement of genes with
 108 similar functions separated by a gap of no more than eight non-R genes (Richly et al. 2002, Luo et
 109 al. 2012); and a sliding window (SW) approach using REEF software
 110 (<http://telethon.bio.unipd.it/bioinfo/reef/>), that is able to identify chromosome regions of a given
 111 size that contain a number of adjacent PRGs based on a statistical test on genomic distribution
 112 (Coppe et al. 2006). Both analyses were conducted on ITAG Tomato Genome 2.3
 113 (<http://solgenomics.net>) and Zunla-1 Pepper Genome 2.0 (<http://peppersequence.genomics.cn>). In
 114 particular, sliding window scanning was conducted with a setting size of 0.5 and 1 Mb, a Q-value of
 115 0.05 and a shift length of 50 Kb. For both methods, we also varied the minimum gene number cut-
 116 off from two to four genes to highlight the clustering tendency of specific regions.

117

118 **Targeted sequencing**

119 Targeted sequencing workflow is illustrated in Figure S1. The experiment was executed on four
 120 plant species: *S. lycopersicum* var. Pyrella (Sl-Pyrella), *S. peruvianum* 10543 (Sp-10543), *S.*
 121 *melongena* var. Cima Viola (Sm-Cima Viola) and *C. annuum* 1014 (Ca-1014). Fully expanded
 122 leaves were detached from three-week-old greenhouse-grown plants of each accession. Genomic
 123 DNA was extracted from young leaf tissue of the same plants, using the DNeasy Plant Mini kit
 124 (Qiagen Valencia, USA), following the manufacturer's instructions.

125 A custom-designed SureSelect Target Enrichment Kit (Agilent Technologies, Santa Clara, CA) was
 126 used to capture selected regions, according to the manufacturer's instructions. The probe library was
 127 designed on 14 selected regions of the ITAG v2.3 genome, identified by Andolfo et al. (2013) for a
 128 total of 5.7 Mb (see supplementary material). Library quality was determined using the Agilent
 129 High Sensitivity DNA kit on the Agilent 2100 Bioanalyzer. Libraries were pooled at equimolar

concentrations and sequenced with TruSeq PE Cluster Kit v3 and TruSeq SBS Kit v3 (Illumina, San Diego, CA) on Illumina HiSeq 1000 sequencer (Illumina, San Diego, CA) generating 100-bp paired-end reads.

Mapping and variant calling

Read mapping was performed using BWA [24] on the reference genomes: ITAG Tomato Genome 2.3 (<http://solgenomics.net/>) for samples of SI-Pyrella and Sp-10543; draft genome of eggplant v2.5.1 (<http://eggplant.kazusa.or.jp>) for the Sm-Cima Viola sample; Zunla-1 Pepper Genome 2.0 (peppersequence.genomics.cn) for the Ca-1014 sample. This procedure was executed for all samples with $t = 10$ and default setting. Adapter sequences were removed from sequence reads using Scythe software with default parameters (v. 0.994; <https://github.com/vsbuffalo/scythe>). Low quality read ends were trimmed using Sickle software (<https://github.com/vsbuffalo/scythe>). All raw sequences were deposited in the NCBI database with the project number SRP075801.

Variants between four genotypes (*S. lycopersicum* var. Pyrella, *S. peruvianum* 10543, *S. melongena* var. Cima Viola and *C. annuum* 1014) and reference genomes relative were called using SAMtools software (Li and Durbin 2009) with a minimum read depth threshold of 20. Identified variants were annotated using SnpEff v3.4 (Cingolani et al. 2012) to predict their effect on the genes, using reference genomes annotations. Finally, polymorphisms were subsequently filtered for position, considering only those that were in a syntenic block at the region was used for probe design indicated in eggplant and pepper sequencing work (Qin et al. 2014, Hirakawa et al. 2014).

Comparative analysis

Orthology analysis was conducted on proteomes of eggplant (SME_r2.5.1_pep_ip), tomato (ITAG2.3_proteins) and pepper (Capsicum.annuum.L_Zunla-1_v2.0_PEP) using Inparanoid Software and Multiparanoid Software with default parameters (Remm et al. 2001, Alexeyenko et al.

2006). We used a confidence score threshold = 1 to directly estimate orthology relationships between the identified PRGs.

A comparative map was constructed, merging results obtained from PRG prediction, cluster dataset and orthology results using the Circos package (Krzywinski et al. 2009). Eggplant chromosomes were assembled using information reported in Hirakawa et al. (2014). Position and IDs of isolated and studied loci were reported by Andolfo et al. (2013b) and in the Sol Genomics Network portal (<https://solgenomics.net/>).

Nucleotide sequences of clustered PRGs in genomic sequenced regions 4 and 10 were extracted from genomes and aligned using EINS-i algorithm of MAFFT (Katoh and Standley 2013) software. The procedure was performed separately for receptor-like proteins (RLP and RLK) and NLR genes coding; sequences with low global alignment identity (< 25%) were discarded. The phylogenetic relationships of predicted Solanaceae PRGs were inferred separately for each structural class (e.g. NLRs, RLPs) using MEGA 6 software with the maximum likelihood method general time reversible model. The bootstrap consensus tree of 100 replicates was taken to represent the evolutionary history of the sequences analysed (Tamura et al. 2013). Transposable elements were found using BLASTn search (Camacho et al. 2009) on library of transposable element reported on Pepper Genome Database (<http://peppersequence.genomics.cn/page/species/download.jsp>), and they were aligned aligned. Specific gene and protein alignments were generated in Geneious R6 platform (Kearse et al. 2012).

Results

Chromosome distribution of pepper and eggplant pathogen recognition genes

A total of 1097 and 775 pathogen recognition genes were identified in *C. annuum* Zunla-1 (Zun) and *S. melongena* Nakate-Shinkuro (Nak) genomes, respectively (Supplementary Table1). The chromosome distribution of PRG classes retrieved in the two analysed species in comparison with the tomato PRGs profile obtained by Andolfo et al. (2013b) is shown in Figure 1. The total number

of genes varied along the chromosomes of each species. Chromosome 4 was enriched in PRGs both in tomato and pepper (94 vs 129), whilst the highest number of genes in eggplant was observed on chromosome 11. A large variation in genome arrangement was observed for the assessed classes in pepper and eggplant. The RLK class showed the largest variation in pepper, ranging from the 78 members on chromosome 4 to 9 members on chromosome 10. Instead, eggplant showed a similar RLP (3 to 24) and RLK (8 to 25) trend distribution. NLR path distribution showed marked differences between the two species: in pepper a conspicuous number of NLR-related genes were identified on chromosomes 3 (43) and 5 (34), whilst in eggplant they are enriched on chromosome 11 (45). Eggplant and pepper genomes also displayed a large number of PRGs (157 and 173 respectively) located on chromosome 0, which could lead to a bias in genome distribution (Supplementary Table 2).

Identification of resistance orthologous groups

To translate the resistance gene information from the tomato model species to other Solanaceae crops, we performed an orthology prediction analysis (Supplementary Table 3). The three species shared a core set of 320 selected as *bona fide* resistance orthologous groups (ROGs) (Figure 2), including a total of 1076 PRG proteins, of which 182 were RLP, 297 NLR and 498 RLK. In common ROGs a larger number (89, 95) of PRG paralogues were shared between *C. annuum* and *S. lycopersicum*. Furthermore, 91 ROGs were only shared between tomato and pepper genomes and 67 between tomato and eggplant. The Orthology analysis also allowed ortho-groups containing homologues of cloned genes. Six very important Solanaceae *R*-gene loci (Cf9, Hero, Prf, Tm2 and Mi1.2) showed a highly confident orthologue in each three species analysed (Supplementary table 3). Two orthologues to tomato LeEIX1 and LeEIX2 homologues (Soly07g008620 and Soly07g008630) were identified in pepper and eggplant genomes (Capana00g004962 and Sme2.5_01783.1_g00006). The tomato RpiBlb1 homologous gene (Soly08g076000) presented two and one orthologues in pepper eggplant genomes, respectively (Capana01g000864 and

207 Capana01g000870; Sme2.5_11213.1_g00002.1). Ve2 gene presented only two orthologues in
208 tomato and pepper genomes (Soly09g005080; Capana09g001153). Interestingly, a large
209 diversification of I2 homologous genes was found in tomato and eggplant genomes. The I-2 gene
210 homologues (Soly011g071430- Soly011g071420), located on chr11, showed several paralogues,
211 including a tomato LRR gene (Soly011g065800) similar to Capana11g000417 and
212 Sme2.5_15564.1_g00001.

213 Other tomato genes implicated in the resistance process, but not included in the principal four
214 pathogen recognition protein classes, showed an orthologous relationship in pepper and eggplant
215 genome. For example, ASC-1, located on chromosome 3, (locus name Soly03g114600) is in the
216 same group as Capana03g000932 and Sme2.5_04467.1_g00005, and the Mlo1 gene (locus name
217 Soly04g049090) showed orthologues both in pepper and eggplant (Capana05g002411,
218 Capana06g001935, Capana11g000102, Sme2.5_00266.1_g00009, Sme2.5_12945.1_g00001).

219

220 **Detection of genomic regions rich in pathogen recognition genes**

221 In order to investigate the genomic arrangement of pathogen recognition loci and to evaluate the
222 grouping tendency of such genes in tomato and pepper genomes, we performed both a sliding-
223 window (SW) scan and max-gap (MG) analysis. The eggplant genome was not included in this
224 analysis since its genome is assembled partially.

225 Taking into account the structural differences of the compared genomes, SW scanning was
226 performed on a window of 0.5 or 1 Mb, varying the minimum gene number cut-off from two to four
227 genes (Table 1). The window of 0.5 Mb was set up based on size of super-clusters detected from
228 Andolfo et al. (2013b) and it has been extended up to 1 Mb considering the larger size of pepper
229 genome.

230 Tomato displayed from 39 to 66 clusters, with a number of genes varying from 5.6 to 6.9. The
231 number of PRG clusters, as well as the number of genes included in a cluster, showed a higher
232 variation in pepper, ranging from 106 to 59 and to 7 to 10, respectively. Using the MG method, we

were able to identify from 40 to 146 clusters in tomato, with the number of genes per cluster varying from 3.2 to 5.7, and from 201 to 72 in pepper, with the number of genes per cluster varying from 3.9 to 6.6 (Table 1). The SW and MG analyses differ in the number of clusters identified and the number of genes included in a single cluster. In general, the average size (Kb) of tomato clusters using MG was lower than in SW. The higher data point match was obtained by using three genes per cluster as a cut-off both for MG and SW (with a sliding window setting of 0.5 Mb for tomato and 1 Mb for pepper). In particular, 23 and 57 clusters in tomato and pepper, respectively, showed exactly the same matching genes. Total MG and SW cluster-datasets were filtered for the presence of at least two orthologues among the analysed species. Following this criterion, we detected four tomato clusters shared with pepper identified only by the MG approach and three clusters identified only by the SW approach. Therefore, to avoid data loss, we merged MG and WM data to obtain a list of conserved Solanaceae PRG clusters (Table 2 and Supplementary Table 4).

Comparative analysis of Solanaceae PRG groups

A comparative genomic map was obtained by merging the results obtained by PRG prediction (2632 PRGs), with cluster analysis results filtered for orthology (176 clusters) (Supplementary Table 2; Figure 3). PRG arrangement data of eggplant draft genome were computed by looking at least at two adjacent genes on a single scaffold. In *S. melongena* 86 PRG groups with an average size of ~22 Kbp were recorded. Seventeen tomato, 21 pepper and 22 eggplant clusters shared at least one PRG orthologue among all three species. Furthermore, 23 tomato clusters shared PRGs exclusively with 34 pepper clusters, and exclusively with five eggplant clusters. In particular, the Cf4/Cf9 locus (tomato chromosome 1) showed orthologous genes in eggplant and pepper syntenic regions. Four PRG clusters detected on tomato chromosome 4 shared orthologous-PRG located on chromosome 5 and 11 in pepper and eggplant, respectively. In particular, Hero cluster share orthologous genes with a pepper cluster on chromosome 5. Two tomato clusters, located on chromosome 5 and containing BS4 gene and homologue R1 genes (Soly05g007350,

259 Solyc05g007610, Solyc05g007630 and Solyc05g007640), shared orthologous clustered genes on
260 pepper chromosome 5. A tomato cluster on chromosome 5 (Solyc05g006570, Solyc05g006620,
261 Solyc05g006630 and Solyc05g006670) is highly conserved in the pepper and eggplant genome.
262 Clusters Pto and Prf shared orthologous genes with clusters located on pepper chromosomes 9 and
263 11. Clusters LeEix1 and LeEIX2 located on chromosome 7 showed orthology with a cluster on the
264 corresponding chromosome in pepper. A tomato cluster containing Rblb1 homologues on
265 chromosome 8 shares orthologous genes with clusters located on pepper chromosome 1 and
266 eggplant chromosome 3. PRG organised in clusters on chromosome 9 have orthologues on pepper
267 chromosome 3. Clusters Tm2 and Sw5 showed orthologous clusters on the corresponding
268 chromosome in pepper. The I2 clusters located on tomato chromosome 11 showed orthologous
269 clusters on chromosome 11 of eggplant and pepper. The tomato cluster flanked by Gpa2 markers
270 showed orthologous clustered genes on pepper chromosome 9.

271 **Sequence diversity in selected PRG orthologous loci**

272 Fourteen orthologous loci of tomato, eggplant and pepper cultivated species (*S. lycopersicum* var.
273 Pyrella: Sl-Pyrella; *S. melongena* var. Cima Viola: Sm-Cima Viola; *C. annuum* 1014: Ca-1014) and
274 of a wild tomato species (*S. peruvianum* 10543: Sp-10543), were re-sequenced using a targeted
275 sequencing approach (Figure S1). A total of 33542210, 30949590, 32242713 and 33888476
276 sequencing reads were generated for Sl-Pyrella, Sp-10543, Sm-Cima Viola and Ca-1014,
277 respectively (Supplementary Table 5). Using this approach, we analysed the evolutionary dynamics
278 of 14 selected regions containing PRGs putatively implicated in plant disease resistance.
279 The size of genomic regions captured and the number of reads mapping to the reference genomes
280 are reported in Table S5. In each region, a large number of homologous genes to cloned resistance
281 genes was found. The reads mapped on the respective genomic regions ranged from 17487130 to
282 9500772 in cultivated and wild tomatoes and from 11005089 to 1651126 in Ca-1014 and Sm-Cima
283 Viola. All variants obtained with respect to the reference gene annotations were filtered for genome

position, taking into account only those that were in the regions used for the probe design in pepper and eggplant syntenic regions. The number of variants identified in the analysed regions ranged from 101579 to 1484. The *S. peruvianum* (Sp-10543) sample showed the highest number of variants (Supplementary Table 6), since the reads are mapped to the heterologous *S. lycopersicon* “Heinz 1706” genome. Ca-1014 also showed a larger quantity of variants (6,930), perhaps due to the greater length of syntenic regions (Supplementary Table 5). The I2 region showed a high level of diversification in all species. In tomato regions 1, 2 and 9 also showed a conspicuous variation. In Sp-10543, five regions (1, 3, 4, 5 and 13) showed a number of variants up to 8000. In Sl-Pyrella and in eggplant, detected variants were nearly all homozygotes (88.8 % and 97.7 %) (Figure S2), while most of the wild tomato variants were heterozygotes (58.1 %). Sl-Pyrella showed the highest percentage of INDELs with 33.83% of insertions and 9.55% of deletions (Figure S3). When compared to the corresponding annotated genomes, we observed a significantly higher polymorphism frequency in intergenic regions than in genic regions for all species except for Sm-Cima Viola (9.23%), which was the only sample with reads mapped on a draft genome (Table 2). In all, 72, 9468, 80 and 243 variants of different types were detected on PRG genomic loci in Sl-Pyrella, Sp-10543, Sm-Cima Viola and Ca-1014, respectively (Table 3).

Microsyntenic PRG region reshuffling

The tomato PRG target region 4, located on chromosome 5, containing one RLP, one RLK and two TNLs, is highly conserved in pepper and eggplant (Figure 4). Orthologous genes in pepper show the same tomato order and orientation, except for an inversion occurring between Capana11g001503 and Capana11g001504 (Figure 4). By contrast, in eggplant a first inversion was observed between genes 1 and 3 (7 and 8 in tomato) and a second inversion between genes 1 and 2 (6 and 8 in tomato). Pepper genes 12 and 13 were orthologous to tomato loci 7 and 8, but unlike the latter, do not encode the TIR domain (Figure 4, panel a).. The Solyc05g006620 gene in pepper was divided into Capana11g001502 and Capana11g001503, probably due to a transposable element insertion, as

showed in Figure 4, panel d. In eggplant changes in the protein structure of Sme2.5_04766.1_g00001 were recorded. Moreover, three missense SNP variants on gene Sme2.5_04766.1_g00001 and one gene Sme2.5_04766.1_g00005 were identified by targeted sequencing in the Sm-Cima Viola accession (Figure 4, panel a). The coding region of Sme2.5_05725.1_g00001 displays six non-synonymous mutations. Non-synonymous mutations were also identified in coding regions of Capana11g001503 and Capana11g001504 in the *C. annuum* 1014 genome. Instead, PRGs in this region were highly conserved in tomato (Pirella vs Heinz 1706).

The tomato target region 10, including a PRG cluster, located on chromosome 8 also showed a good level of collinearity with the pepper and eggplant genome. Figure 5 (panel a) presents the region flanked by the marker est_ae501f12. This cluster in tomato consists of three RLKs, three CNLs and one NL, genes that are split into two clusters in pepper, separated by a third cluster without orthologous genes. An inversion was observed between tomato genes 5, 6, 11 and pepper genes 14, 19 and 20. The tomato gene 7 was originated by tandem duplication of gene 6 (Figure 5, panel a and c). Discordant results were indicated in phylogenetic analysis and nucleotide sequence alignment due to a large TE insertion on the intron of Capana01g00870 (Figure 5, panel d). Gene 20 coding an RLP protein showed partial orthology with an RLK gene in tomato. A similar occurrence was found for the NLR gene 14, lacking the CC domain and proving orthologous to full gene 11 (CC-NB-LRR). Several synonymous mutations were identified on gene 4 and gene 16 in Cima Viola (eggplant) as well as on tomato genes 8 and 9.

Discussion

The plant kingdom exhibits a large variation in PRG repertoires among species but also among single individuals (Shao et al. 2016). In our scanning of eggplant (Nakate-Shinkuro) genome showed more or less the same number of tomato PRGs. By contrast, the PRGs of pepper Zunla-1 was contracted respect that pepper CM334 genome (Kim et al. 2014), in terms of NLRs and RLKs.

Genotype loci rearrangements could occur in response to specific phytopathogens. Indeed, CM334 pepper is resistant to *Phytophthora spp.* and potyviruses, whereas Zunla-1 is resistant to Fusarium wilt, *Phytophthora spp.* and Anthracnose (Qin et al. 2014). The variation in PRG number observed in Solanaceae was also found in other taxa, such as Rosaceae (Jia et al. 2015), Graminaceae (Li et al. 2010), Brassicaceae (Peele et al. 2014) and Fabaceae (Shao et al. 2014).

In our analysis the highest NLR concentration in pepper was evidenced on chromosome 5, that characterized by presence of QTL for resistance to *Phytophthora capsici* (Rehrig et al. 2014), whilst the eggplant genome showed the highest concentration of NLR genes on chromosome 11. On this chromosome in tomato, the I2 gene is located, as are other important resistant gene loci. The approach involving target-sequencing and co-localization with candidate *R*-genes may help to identify putative genes for major diseases also in this species (Andolfo et al. 2013b).

Our results confirm that most Solanaceae PRGs tend to be physically clustered. However, to explore the clustering tendency of such genes in several species it was necessary to perform a first analysis with a dynamic setting in order to identify the most suitable method. Indeed, the partitioning of genes into clusters could be hampered by genome architecture, including gene density and gene order (Hoberman and Durand 2005).

In some studies, following an ordinal data strategy, a locus with two or more PRGs separated by a number of non-PR genes (Richly et al. 2002, Luo et al. 2012) was identified as a cluster. In other studies, a gene cluster was defined as a physical region that contains more than three or more genes within 200 Kb or less (Holub 2001). We noted that by looking at least for three adjacent PRGs of 0.5 Mb in tomato and of 1 Mb in pepper most well-known Solanaceae clusters were identified. The task became more challenging when homologous regions were scrambled by rearrangement events that modified the global genome architecture (Joshi and Nayak 2013). Therefore, we combined the results obtained by using different methods to refine the annotation for size and number of genes included in a cluster because the differences in the size and organisation of the two genomes analysed made the comparison difficult.

361 The size of *R*-gene clusters can vary significantly, from a few genes to several hundred genes, e.g.
362 *Ve* (Kawchuk et al. 2001) and *Dm3* loci (Meyers et al. 1998). Most isolated resistance genes
363 occurring in clusters evidenced genome rearrangement that could be triggered by plant-pathogen
364 interaction (Spoel and Dong 2012). Recently, it was observed that increased pathogen pressure
365 induces epigenetic changes and promotes PRGs rearrangements (Molinier et al. 2006, Boyko et al.
366 2007, Alvarez et al. 2010). Indeed, the PRGs clusters play a leading role in new functional
367 resistance gene generation, since they represented a localized island of genetic variability in the
368 plant genome.

369 Orthology prediction analysis performed by comparing tomato, eggplant and pepper proteomes
370 evidenced more than 1000 pathogen recognition orthologues and related paralogues conserved
371 across the three cultivated Solanaceae spp. Some cloned tomato *R*-genes showed a putative
372 orthologue in pepper and eggplant, whilst other tomato PRGs were shared just with one species.
373 Merging orthology-related data and cluster data, we confirmed that PRGs comprise one of the most
374 plastic gene families in plants, associated with gene loss, gene conservation and gene clustering
375 (Zhang et al. 2014). Tomato chromosome 4 showed the highest number of conserved PRG clusters
376 even if some clusters were fragmented into different chromosomes in other species (Destefanis et
377 al. 2015). The putative *Gpa2* locus in tomato, located on chromosome 12, shares an orthologous
378 relationship with two clusters located on chromosome 9 in pepper. PRG clusters on tomato
379 chromosome 9, including genes *Sw5* and *Tm2*, showed orthologous clustering on pepper
380 chromosome 3 (Grube et al. 2000, Djian-Caporalino et al. 2007), where some genes involved in
381 resistance to viruses are located (Caranta et al. 1997).

382 Targeted capture sequencing allowed the detection of a big set of variations in genes located in
383 important resistance loci. Polymorphisms in the genomic region containing *R*-genes were
384 pronounced in several species (McHale et al. 2012, González et al. 2013). This technique displayed
385 its usefulness for sequencing large genomic regions, offering a simple method to analyse gene
386 polymorphism in a relatively efficient and economic manner (Grover et al. 2012). The sequenced

syntenic regions in eggplant and pepper were larger than those in tomato, possibly due to the difference in genomic size reported for the species analysed (Tomato and Consortium 2012, Qin et al. 2014, Hirakawa et al. 2014). In particular, pepper showed a massive genomic insertion of transposable elements (Kim et al. 2014). Moreover, a lower number of reads was mapped in eggplant since its genome was not assembled in pseudomolecules. In general, the efficiency of target sequencing was similar to that observed in cross-species microarray experiments (Bar-Or et al. 2007, Lu et al. 2009, Nazar et al. 2010). The highest number of variants was obtained in *S. peruvianum*, confirming the level of polymorphism reported in the literature for this species (Aflitos et al. 2014).

The cataloguing of genes and the concerted use of genomic information (clustering tendency; orthology relationship and variant detection) showed to be a valuable strategy for identifying important genes or alleles and for exchanging information related to coding protein function across plant species. Comparative analysis of two selected PRG loci showed a high level of genome rearrangement (gene losses, duplicated genes, genome shuffling and transposable element insertions). PRG polymorphisms (SNPs, IN/DEL, domain loss or insertions) that can play an important role in gene recombination (Baumgarten et al. 2003, Meyers et al. 2003, McHale et al. 2012, Sanseverino and Ercolano 2012). Even if the mechanisms underlying enhanced recombination at these loci have not been clearly established, pathogen recognition protein structure changes can have a great impact in specific disease response (Wang et al. 1998, Zhang et al. 2004, Nandety et al. 2013). PRG architecture seems to be modified by the interplay of large-scale gene organisation that determines global conservation of locus order genome-wide and extensive local genome rearrangements mediated by tandem duplication, transposons and other shuffling elements that lead to distinct local arrangements (Zhang et al. 2014, Aversano et al. 2015). Regions including genes involved in defence responses have been shown to be hot-spots of genomic variability across genomes (Spoel and Dong 2012). Extant local arrangements of Solanaceae pathogen recognition

genes within a genome are indicative of biological and environmental factors influencing genotype adaptation, and have significant influence on phenotypic resistance diversity (Aversano et al. 2015).

Funding

This work was supported by the Italian Ministry of University and Research (GenHORTH project).

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

We thank Prof. Luigi Frusciante for his useful and constructive comments that contributed to improving the manuscript.

References

Aflitos, S., Schijlen, E., de Jong, H., de Ridder, D., Smit, S., Finkers, R., Wang, J., Zhang, G., Li, N., Mao, L., Bakker, F., Dirks, R., Breit, T., Gravendeel, B., Huits, H., Struss, D., Swanson-Wagner, R., van Leeuwen, H., van Ham, R.C.H.J., Fito, L., Guignier, L., Sevilla, M., Ellul, P., Ganko, E., Kapur, A., Reclus, E., de Geus, B., van de Geest, H., Hekkert, B.T.L., van Haarst, J., Smits, L., Koops, A., Sanchez-Perez, G., van Heusden, A.W., Visser, R., Quan, Z., Min, J., Liao, L., Wang, X., Wang, G., Yue, Z., Yang, X., Xu, N., Schranz, E., Smets, E., Vos, R., Rauwerda, J., Ursem, R., Schuit, C., Kerns, M., van den Berg, J., Vriezen, W., Janssen, A., Datema, E., Jahrman, T., Moquet, F., Bonnet, J., and Peters, S. 2014. Exploring genetic variation in the tomato (*Solanum section Lycopersicon*) clade by

- whole-genome sequencing. *Plant J.* (January): 136–148. doi:10.1111/tpj.12616.
- Alexeyenko, A., Tamas, I., Liu, G., and Sonnhammer, E.L.L. 2006. Automatic clustering of orthologs and inparalogs shared by multiple proteomes. *In Bioinformatics*. doi:10.1093/bioinformatics/btl213.
- Alvarez, M.E., Nota, F., and Cambiagno, D. a. 2010. Epigenetic control of plant immunity. *Mol. Plant Pathol.* **11**(4): 563–576. doi:10.1111/j.1364-3703.2010.00621.x.
- Andolfo, G., Jupe, F., Witek, K., Etherington, G.J., Ercolano, M.R., and Jones, J.D.G. 2014. Defining the full tomato NB-LRR resistance gene repertoire using genomic and cDNA RenSeq. *BMC Plant Biol.* **14**(1): 120. doi:10.1186/1471-2229-14-120.
- Andolfo, G., Sanseverino, W., Aversano, R., Frusciante, L., and Ercolano, M.R. 2013a. Genome-wide identification and analysis of candidate genes for disease resistance in tomato. *Mol. Breed.* **33**(1): 227–233. doi:10.1007/s11032-013-9928-7.
- Andolfo, G., Sanseverino, W., Rombauts, S., Van de Peer, Y., Bradeen, J.M., Carputo, D., Frusciante, L., and Ercolano, M.R. 2013b. Overview of tomato (*Solanum lycopersicum*) candidate pathogen recognition genes reveals important *Solanum* R locus dynamics. *New Phytol.* **197**(1): 223–37. doi:10.1111/j.1469-8137.2012.04380.x.
- Aversano, R., Contaldi, F., Ercolano, M.R., Grosso, V., Iorizzo, M., Tatino, F., Xumerle, L., Dal Molin, A., Avanzato, C., Ferrarini, A., Delledonne, M., Sanseverino, W., Cigliano, R.A., Capella-Gutierrez, S., Gabaldón, T., Frusciante, L., Bradeen, J.M., and Carputo, D. 2015. The *Solanum commersonii* Genome Sequence Provides Insights into Adaptation to Stress Conditions and Genome Evolution of Wild Potato Relatives. *In The Plant Cell*. doi:10.1105/tpc.114.135954.
- Bar-Or, C., Czosnek, H., and Koltai, H. 2007. Cross-species microarray hybridizations: a developing tool for studying species diversity. doi:10.1016/j.tig.2007.02.003.
- Baumgarten, A., Cannon, S., Spangler, R., and May, G. 2003. Genome-level evolution of resistance genes in *Arabidopsis thaliana*. *Genetics* **165**(1): 309–319.
- Boyko, A., Kathiria, P., Zemp, F.J., Yao, Y., Pogribny, I., and Kovalchuk, I. 2007. Transgenerational changes in the genome stability and methylation in pathogen-infected plants: (Virus-induced plant genome instability). *Nucleic Acids Res.* **35**(5): 1714–1725. doi:10.1093/nar/gkm029.
- Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T.L. 2009.

- BLAST plus: architecture and applications. *BMC Bioinformatics* **10**(421): 1. doi:Artn 421\nDoi 10.1186/1471-2105-10-421.
- Caranta, C., Lefebvre, V., and Palloix, A. 1997. Polygenic Resistance of Pepper to Potyviruses Consists of a Combination of Isolate-Specific and Broad-Spectrum Quantitative Trait Loci. *Mol. Plant-Microbe Interact.* **10**(7): 872–878. doi:10.1094/MPMI.1997.10.7.872.
- Cingolani, P., Platts, A., Wang, L.L., Coon, M., Nguyen, T., Wang, L., Land, S.J., Lu, X., and Ruden, D.M. 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w 1118; iso-2; iso-3. *Fly (Austin)*. **6**(2): 80–92. doi:10.4161/fly.19695.
- Coppe, A., Danieli, G.A., and Bortoluzzi, S. 2006. REEF: searching REgionally Enriched Features in genomes. *BMC Bioinformatics* **7**(1): 453. doi:10.1186/1471-2105-7-453.
- Destefanis, M., Nagy, I., Rigney, B., Bryan, G.J., McLean, K., Hein, I., Griffin, D., and Milbourne, D. 2015. A disease resistance locus on potato and tomato chromosome 4 exhibits a conserved multipartite structure displaying different rates of evolution in different lineages. *BMC Plant Biol.* **15**: 255. doi:10.1186/s12870-015-0645-8.
- Djian-Caporalino, C., Fazari, A., Arguel, M.J., Vernie, T., VandeCastele, C., Faure, I., Brunoud, G., Pijarowski, L., Palloix, A., Lefebvre, V., and Abad, P. 2007. Root-knot nematode (*Meloidogyne* spp.) Me resistance genes in pepper (*Capsicum annuum* L.) are clustered on the P9 chromosome. *Theor. Appl. Genet.* **114**(3): 473–486. doi:10.1007/s00122-006-0447-3.
- Ercolano, M.R., Sanseverino, W., Carli, P., Ferriello, F., and Frusciante, L. 2012. Genetic and genomic approaches for R-gene mediated disease resistance in tomato: Retrospects and prospects. *Plant Cell Rep.* **31**: 973–985. doi:10.1007/s00299-012-1234-z.
- Fernandez-Pozo, N., Menda, N., Edwards, J.D., Saha, S., Tecle, I.Y., Strickler, S.R., Bombarely, A., Fisher-York, T., Pujar, A., Foerster, H., Yan, A., and Mueller, L.A. 2015. The Sol Genomics Network (SGN)-from genotype to phenotype to breeding. *Nucleic Acids Res.* **43**(D1): D1036–D1041. doi:10.1093/nar/gku1195.
- Gasc, C., Peyretailade, E., and Peyret, P. 2016. Sequence capture by hybridization to explore modern and ancient genomic diversity in model and nonmodel organisms. *Nucleic Acids Res.* (8): gkw309-.

- doi:10.1093/nar/gkw309.
- González, V.M., Aventín, N., Centeno, E., and Puigdomènech, P. 2013. High presence/absence gene variability in defense-related gene clusters of *Cucumis melo*. *BMC Genomics* **14**: 782. doi:10.1186/1471-2164-14-782.
- Grover, C.E., Salmon, A., and Wendel, J.F. 2012. Targeted sequence capture as a powerful tool for evolutionary analysis. *Am. J. Bot.* **99**(2): 312–319. doi:10.3732/ajb.1100323.
- Grube, R.C., Radwanski, E.R., and Jahn, M. 2000. Comparative genetics of disease resistance within the solanaceae. *Genetics* **155**(2): 873–887.
- Hirakawa, H., Shirasawa, K., Miyatake, K., Nunome, T., Negoro, S., Ohyama, A., Yamaguchi, H., Sato, S., Isobe, S., Tabata, S., and Fukuoka, H. 2014. Draft Genome Sequence of Eggplant (*Solanum melongena* L.): the Representative *Solanum* Species Indigenous to the Old World. *DNA Res.*: 1–12. doi:10.1093/dnares/dsu027.
- Hoberman, R., and Durand, D. 2005. The Incompatible Desiderata of Gene Cluster Properties. *Proc. RECOMB 2005 Int. Work. Comp. Genomics, RCG 2005* **3678**: 73–87.
- Hoberman, R., Sankoff, D., and Durand, D. 2005. The statistical significance of max-gap clusters. *Comp. Genomics*: 55–71. Available from <http://www.springerlink.com/index/VQQN6C6ECN9T0NM0.pdf>.
- Holub, E.B. 2001. The arms race is ancient history in *Arabidopsis*, the wildflower. *Nat. Rev. Genet.* **2**(7): 516–527. doi:10.1038/35080508.
- Jia, Y., Yuan, Y., Zhang, Y., Yang, S., and Zhang, X. 2015. Extreme expansion of NBS-encoding genes in Rosaceae. *BMC Genet.* **16**: 48. doi:10.1186/s12863-015-0208-x.
- Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A.F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S.Y., Lopez, R., and Hunter, S. 2014. InterProScan 5: Genome-scale protein function classification. *Bioinformatics* **30**(9): 1236–1240. doi:10.1093/bioinformatics/btu031.
- Joshi, R.K., and Nayak, S. 2013. Perspectives of genomic diversification and molecular recombination towards R-gene evolution in plants. *Physiol. Mol. Biol. Plants* **19**(1): 1–9. doi:10.1007/s12298-012-0138-2.
- Katoh, K., and Standley, D.M. 2013. MAFFT multiple sequence alignment software version 7:

- 522 Improvements in performance and usability. *Mol. Biol. Evol.* **30**(4): 772–780.
 523 doi:10.1093/molbev/mst010.
- 524 Kawchuk, L.M., Hachey, J., Lynch, D.R., Kulcsar, F., van Rooijen, G., Waterer, D.R., Robertson, a, Kokko,
 525 E., Byers, R., Howard, R.J., Fischer, R., and Prufer, D. 2001. Tomato Ve disease resistance genes
 526 encode cell surface-like receptors. *Proc. Natl. Acad. Sci. U. S. A.* **98**(11): 6511–5.
 527 doi:10.1073/pnas.091114198.
- 528 Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A.,
 529 Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., and Drummond, A. 2012. Geneious
 530 Basic: An integrated and extendable desktop software platform for the organization and analysis of
 531 sequence data. *Bioinformatics* **28**(12): 1647–1649. doi:10.1093/bioinformatics/bts199.
- 532 Kim, S., Park, M., Yeom, S.-I., Kim, Y.-M., Lee, J.M., Lee, H.-A., Seo, E., Choi, J., Cheong, K., Kim, K.-T.,
 533 Jung, K., Lee, G.-W., Oh, S.-K., Bae, C., Kim, S.-B., Lee, H.-Y., Kim, S.-Y., Kim, M.-S., Kang, B.-C.,
 534 Jo, Y.D., Yang, H.-B., Jeong, H.-J., Kang, W.-H., Kwon, J.-K., Shin, C., Lim, J.Y., Park, J.H., Huh,
 535 J.H., Kim, J.-S., Kim, B.-D., Cohen, O., Paran, I., Suh, M.C., Lee, S.B., Kim, Y.-K., Shin, Y., Noh, S.-
 536 J., Park, J., Seo, Y.S., Kwon, S.-Y., Kim, H. a, Park, J.M., Kim, H.-J., Choi, S.-B., Bosland, P.W.,
 537 Reeves, G., Jo, S.-H., Lee, B.-W., Cho, H.-T., Choi, H.-S., Lee, M.-S., Yu, Y., Do Choi, Y., Park, B.-
 538 S., van Deynze, A., Ashrafi, H., Hill, T., Kim, W.T., Pai, H.-S., Ahn, H.K., Yeam, I., Giovannoni, J.J.,
 539 Rose, J.K.C., Sørensen, I., Lee, S.-J., Kim, R.W., Choi, I.-Y., Choi, B.-S., Lim, J.-S., Lee, Y.-H., and
 540 Choi, D. 2014. Genome sequence of the hot pepper provides insights into the evolution of pungency in
 541 *Capsicum* species. *Nat. Genet.* **46**(3): 270–8. doi:10.1038/ng.2877.
- 542 Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S.J., and Marra, M.A.
 543 2009. Circos: An information aesthetic for comparative genomics. *Genome Res.* **19**(9): 1639–1645.
 544 doi:10.1101/gr.092759.109.
- 545 Li, H., and Durbin, R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform.
 546 *Bioinformatics* **25**(14): 1754–1760. doi:10.1093/bioinformatics/btp324.
- 547 Li, J., Ding, J., Zhang, W., Zhang, Y., Tang, P., Chen, J.Q., Tian, D., and Yang, S. 2010. Unique
 548 evolutionary pattern of numbers of gramineous NBS-LRR genes. *Mol. Genet. Genomics* **283**(5): 427–
 549 438. doi:10.1007/s00438-010-0527-6.

- 550 Lu, Y., Huggins, P., and Bar-Joseph, Z. 2009. Cross species analysis of microarray expression data.
551 *Bioinformatics* **25**(12): 1476–1483. doi:10.1093/bioinformatics/btp247.
- 552 Luo, S., Zhang, Y., Hu, Q., Chen, J., Li, K., Lu, C., Liu, H., Wang, W., and Kuang, H. 2012. Dynamic
553 Nucleotide-Binding Site and Leucine-Rich Repeat-Encoding Genes in the Grass Family. *Plant Physiol.*
554 **159**(1): 197–210. doi:10.1104/pp.111.192062.
- 555 McHale, L.K., Haun, W.J., Xu, W.W., Bhaskar, P.B., Anderson, J.E., Hyten, D.L., Gerhardt, D.J., Jeddelloh,
556 J.A., and Stupar, R.M. 2012. Structural variants in the soybean genome localize to clusters of biotic
557 stress-response genes. *Plant Physiol.* **159**(4): 1295–308. doi:10.1104/pp.112.194605.
- 558 Meyers, B.C., Chin, D.B., Shen, K. a, Sivaramakrishnan, S., Lavelle, D.O., Zhang, Z., and Michelmore,
559 R.W. 1998. The major resistance gene cluster in lettuce is highly duplicated and spans several
560 megabases. *Plant Cell* **10**(11): 1817–1832. doi:http://www.plantcell.org/content/10/11/1817.short.
- 561 Meyers, B.C., Kozik, A., Griego, A., Kuang, H., and Michelmore, R.W. 2003. Genome-wide analysis of
562 NBS-LRR – encoding genes in Arabidopsis. *Plant Cell* **15**(April): 809–834.
563 doi:10.1105/tpc.009308.During.
- 564 Molinier, J., Ries, G., Zipfel, C., and Hohn, B. 2006. Transgeneration memory of stress in plants. *Nature*
565 **442**(7106): 1046–1049. doi:10.1038/nature05022.
- 566 Nandety, R.S., Caplan, J.L., Cavanaugh, K., Perroud, B., Wroblewski, T., Michelmore, R.W., and Meyers,
567 B.C. 2013. The role of TIR-NBS and TIR-X proteins in plant basal defense responses. *Plant Physiol.*
568 **162**(3): 1459–72. doi:10.1104/pp.113.219162.
- 569 Nazar, R.N., Chen, P., Dean, D., and Robb, J. 2010. DNA chip analysis in diverse organisms with
570 unsequenced genomes. *Mol. Biotechnol.* **44**(1): 8–13. doi:10.1007/s12033-009-9212-6.
- 571 Peele, H.M., Guan, N., Fogelqvist, J., and Dixelius, C. 2014. Loss and retention of resistance genes in five
572 species of the Brassicaceae family. *BMC Plant Biol.* **14**(Cc): 298. doi:10.1186/s12870-014-0298-z.
- 573 Qin, C., Yu, C., Shen, Y., Fang, X., Chen, L., Min, J., Cheng, J., Zhao, S., Xu, M., Luo, Y., Yang, Y., Wu,
574 Z., Mao, L., Wu, H., Ling-Hu, C., Zhou, H., Lin, H., González-Morales, S., Trejo-Saavedra, D.L., Tian,
575 H., Tang, X., Zhao, M., Huang, Z., Zhou, A., Yao, X., Cui, J., Li, W., Chen, Z., Feng, Y., Niu, Y., Bi,
576 S., Yang, X., Li, W., Cai, H., Luo, X., Montes-Hernández, S., Leyva-González, M. a, Xiong, Z., He,
577 X., Bai, L., Tan, S., Tang, X., Liu, D., Liu, J., Zhang, S., Chen, M., Zhang, L., Zhang, L., Zhang, Y.,

- 578 Liao, W., Zhang, Y., Wang, M., Lv, X., Wen, B., Liu, H., Luan, H., Zhang, Y., Yang, S., Wang, X.,
 579 Xu, J., Li, X., Li, S., Wang, J., Palloix, A., Bosland, P.W., Li, Y., Krogh, A., Rivera-Bustamante, R.F.,
 580 Herrera-Estrella, L., Yin, Y., Yu, J., Hu, K., and Zhang, Z. 2014. Whole-genome sequencing of
 581 cultivated and wild peppers provides insights into *Capsicum* domestication and specialization. *Proc.*
 582 *Natl. Acad. Sci. U. S. A.* **111**(14): 5135–40. doi:10.1073/pnas.1400975111.
- 583 Rehrig, W.Z., Ashrafi, H., Hill, T., Prince, J., and Van Deynze, A. 2014. *CaDMRI* Cosegregates with QTL
 584 *Pc5.1* for Resistance to in Pepper (*Capsicum annuum*). *Plant Genome* **7**(2): 1–12.
 585 doi:10.3835/plantgenome2014.03.0011.
- 586 Remm, M., Storm, C.E., and Sonnhammer, E.L. 2001. Automatic clustering of orthologs and in-paralogs
 587 from pairwise species comparisons. *J. Mol. Biol.* **314**(5): 1041–1052. doi:10.1006/jmbi.2000.5197.
- 588 Richly, E., Kurth, J., and Leister, D. 2002. Mode of amplification and reorganization of resistance genes
 589 during recent *Arabidopsis thaliana* evolution. *Mol. Biol. Evol.* **19**(1): 76–84.
- 590 Sanseverino, W., and Ercolano, M.R. 2012. In silico approach to predict candidate R proteins and to define
 591 their domain architecture. *BMC Res. Notes* **5**(1): 678. doi:10.1186/1756-0500-5-678.
- 592 Seo, E., Kim, S., Yeom, S.-I., and Choi, D. 2016. Genome-wide Comparative Analyses Reveal the Dynamic
 593 Evolution of Nucleotide-Binding Leucine-Rich Repeat Gene Family among Solanaceae Plants. *Front.*
 594 *Plant Sci.* **7**(August): 1205. doi:10.3389/FPLS.2016.01205.
- 595 Shao, Z.-Q., Xue, J.-Y., Wu, P., Zhang, Y.-M., Wu, Y., Hang, Y.-Y., Wang, B., and Chen, J.-Q. 2016.
 596 Large-Scale Analyses of Angiosperm Nucleotide-Binding Site-Leucine-Rich Repeat Genes Reveal
 597 Three Anciently Diverged Classes with Distinct Evolutionary Patterns. *Plant Physiol.* **170**(4): 2095–
 598 2109. doi:10.1104/pp.15.01487.
- 599 Shao, Z.-Q., Zhang, Y.-M., Hang, Y.-Y., Xue, J.-Y., Zhou, G.-C., Wu, P., Wu, X.-Y., Wu, X.-Z., Wang, Q.,
 600 Wang, B., and Chen, J. 2014. Long-Term Evolution of Nucleotide-Binding Site-Leucine-Rich Repeat
 601 (NBS-LRR) Genes: Understandings Gained From and Beyond the Legume Family. *Plant Physiol.*
 602 **166**(September): 217–234. doi:10.1104/pp.114.243626.
- 603 Spoel, S.H., and Dong, X. 2012. How do plants achieve immunity? Defence without specialized immune
 604 cells. *Nat. Rev. Immunol.* **12**(2): 89–100. Nature Publishing Group. doi:10.1038/nri3141.
- 605 Steuernagel, B., Periyannan, S.K., Hernández-Pinzón, I., Witek, K., Rouse, M.N., Yu, G., Hatta, A., Ayliffe,

- 606 M., Bariana, H., Jones, J.D.G., Lagudah, E.S., and Wulff, B.B.H. 2016. Rapid cloning of disease-
607 resistance genes in plants using mutagenesis and sequence capture. *Nat. Biotechnol.* (August 2015).
608 doi:10.1038/nbt.3543.
- 609 Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary
610 genetics analysis version 6.0. *Mol. Biol. Evol.* **30**(12): 2725–2729. doi:10.1093/molbev/mst197.
- 611 Tomato, T., and Consortium, G. 2012. The tomato genome sequence provides insights into fleshy fruit
612 evolution. *Nature* **485**(7400): 635–41. Available from
613 [http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3378239&tool=pmcentrez&rendertype=abs](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3378239&tool=pmcentrez&rendertype=abstract)
614 [tract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3378239&tool=pmcentrez&rendertype=abstract).
- 615 Vossen, J.H., Jo, K.R., and Vosman, B. 2014. Mining the genus solanum for increasing disease resistance. *In*
616 *Genomics of Plant Genetic Resources: Volume 2. Crop Productivity, Food Security and Nutritional*
617 *Quality*. Springer. pp. 27–46. doi:10.1007/978-94-007-7575-6_2.
- 618 Wang, G.-L., Ruan, D.-L., Song, W.-Y., Sideris, S., Chen, L., Pi, L.-Y., Zhang, S., Zhang, Z., Fauquet, C.,
619 Gaut, B.S., Whalen, M.C., and Ronald, P.C. 1998. Xa21D Encodes a Receptor-Like Molecule with a
620 Leucine-Rich Repeat Domain That Determines Race-Specific Recognition and Is Subject to Adaptive
621 Evolution. *Plant Cell* **10**(5): 765. doi:10.2307/3870663.
- 622 Wang, Y., Diehl, A., Wu, F., Vrebalov, J., Giovannoni, J., Siepel, A., and Tanksley, S.D. 2008. Sequencing
623 and comparative analysis of a conserved syntenic segment in the solanaceae. *Genetics* **180**(1): 391–408.
624 doi:10.1534/genetics.108.087981.
- 625 Wei, C., Chen, J., and Kuang, H. 2016. Dramatic Number Variation of R Genes in Solanaceae Species
626 Accounted for by a Few R Gene Subfamilies. *PLoS One* **11**(2): e0148708.
627 doi:10.1371/journal.pone.0148708.
- 628 Witek, K., Jupe, F., Witek, A.I., Baker, D., Clark, M.D., and Jones, J.D.G. 2016. Accelerated cloning of a
629 potato late blight–resistance gene using RenSeq and SMRT sequencing. *Nat. Biotechnol.* (August
630 2015): 1–8. doi:10.1038/nbt.3540.
- 631 Yeaman, S. 2013. Genomic rearrangements and the evolution of clusters of locally adaptive loci. *Proc. Natl.*
632 *Acad. Sci. U. S. A.* **110**(19): E1743–51. doi:10.1073/pnas.1219381110.
- 633 Zhang, R., Murat, F., Pont, C., Langin, T., and Salse, J. 2014. Paleo-evolutionary plasticity of plant disease

634 resistance genes. BMC Genomics **15**(1): 187. BMC Genomics. doi:10.1186/1471-2164-15-187.

635 Zhang, Y., Dorey, S., Swiderski, M., Jones, J.D.G., Centre, J.I., and Lane, C. 2004. Expression of RPS4 in

636 tobacco induces an AvrRps4-independent HR that requires EDS1, SGT1 and HSP90. Plant J. **40**(2):

637 213–24. doi:10.1111/j.1365-313X.2004.02201.x.

638

Draft

640 **Tables**

641

642

643

Table 1. Results of identification of PRG clusters using Sliding window (window size: 0.5 and 1 Mb) and Max Gap analysis.

	HEINZ 1706			ZUNLA-1		
	2 GENES	3 GENES	4 GENES	2 GENES	3 GENES	4 GENES
Sliding window analysis (0.5 M)						
average n. genes for cluster	5,6	6,1	6,9	4,3	5,3	9,3
n. of clusters	66	61	50	147	101	59
n. of genes in cluster	368	370	344	632	540	412
NB-ARC genes in cluster	159	147	153	211	178	126
RLP genes in cluster	83	80	67	162	134	112
RLK genes in cluster	79	94	87	154	140	110
average lenght of clusters (bp)	281917	314068	355599	255315	313651	393368
Sliding window analysis (1 Mb)						
average n. genes for cluster	8,1	8,1	8,1	4,6	6,5	8,1
n. of clusters	39	45	47	106	91	62
n. of genes in cluster	317	365	382	597	591	504
NB-ARC genes in cluster	140	160	151	193	189	161
RLP genes in cluster	56	80	84	148	151	128
RLK genes in cluster	81	93	103	154	139	138
average lenght of clusters (bp)	721002	829940	780434	510068	647393	818992
Max Gap analysis						
average n. genes for cluster	3,2	4,5	5,7	3,9	5,3	6,6
n. of clusters	146	72	40	201	114	72
n. of genes in cluster	471	323	227	778	604	478
NB-ARC genes in cluster	192	142	100	250	205	164
RLP genes in cluster	110	74	49	197	156	120
RLK genes in cluster	112	72	54	206	150	121
average lenght of clusters (bp)	67980	106576	120984	407856	510818	655767

644

Table 2. PRG orthologous clusters identified by merging of max-gap (MG) and sliding-window (SW) results.

	HEINZ 1706	ZUNLA-1
no. of clusters	73	103
no. of genes in cluster	415	643
average no. of genes per cluster	5.7	3.9
average length of clusters (bp)	298.565	733.549
NB-ARC genes in cluster	171	209
RLP genes in cluster	96	163
RLK genes in cluster	104	170

Table 3. Gene variant categories detected by targeted sequencing in pathogen recognition genes. Downstream and upstream variants located in coding sequences and their putative promoter regions (2Kb upstream the translation start site).

	S. lycopersicum var. Pyrella .	S. peruvianum 10543	S. melongena Cima Viola	C. annum 1014
Upstream	21	3121	25	101
Codon_change	0	18	0	1
Frame_shift	1	14	0	1
Intron	8	648	0	17
Non_synonymous_coding	5	1567	11	52
Splice_site_acceptor	0	2	0	0
Splice_site_donor	0	1	0	0
Splice_site_region	0	17	0	1
Stop_gained/lost	0	20	0	5
Synonymous_coding	2	980	19	13
Downstream	35	3080	25	52
Total	72	9468	80	243

Figure legends

Figure 1. Chromosome distribution of main pathogen recognition protein classes (RLP: receptor-like protein, RLK: receptor-like kinase and NLR: nucleotide-binding leucine rich repeat) of *S. melongena* Nakate-Shinkuro (NAK) and *C. annuum* Zunla-1 (ZUN). For comparative propose we reported the number of PRGs identified in *S. lycopersicum* Heinz 1706 (HEI) for each chromosome by Andolfo et al. 2013.

Figure 2. Bar chart of Solanaceae resistance orthologous groups (ROGs). Three species (pepper, tomato and eggplant) were used to generate the diagram. The shared ROGs between pepper-tomato-eggplant, tomato-pepper, tomato-eggplant and pepper-eggplant in cyan, green, yellow and red bars were shown, respectively. The number of pathogen recognition genes (PRGs) of common groups for each plant species (HEI: tomato; NAK: eggplant; ZUN: pepper) is reported below the horizontal line.

Figure 3. Comparative map of conserved PRG loci identified in this study. External rings (A) represent tomato chromosomes (red), pepper chromosomes (orange) and eggplant chromosome (purple) that are reported on a common scale. Eggplant chromosomes were designed using assembled sequencing blocks (Methods). The location of Solanaceae cloned resistance genes is indicated by gene id on tomato chromosomes. B) Genomic location of detected NLR genes (red bars). C, D) Genomic location of RLP and RLK detected genes (green and blue bars, respectively). E) Genomic location of detected PRG clusters (black arrows). F) Internal arcs connect the PRG clusters that share at least one orthologous gene among all genomes (red), between pepper and tomato (grey), between tomato and eggplant (purple), between eggplant and pepper (yellow).

Figure 4 Reconstruction of the gene duplication history of target genomic region 4. (a) Schematic representation of postulated gene duplication events occurring in the genomic region. Detected gene variants are reported as green (low impact on coding gene) or yellow (medium impact on coding gene) ticks. Phylogenetic analysis performed using the maximum likelihood method, based on the general time reversible model, for homologous sequences of cytoplasmic (NLR; panel b) and transmembrane (RLP and RLK; panel c) receptor proteins. Bootstrap values are indicated above branches. (d) Multiple alignment of pepper, tomato and eggplant genomic sequences. We reported gene locus (green), the exons (yellow) and the transposable element (brown). Pepper region includes Capana11g001502 (12), Capana11g001503 (13) genes and a transposable element in the intergenic region while tomato (7) and eggplant (1)

homologous genes lack any transposon insertion. Red rectangles display the most highly conserved sequences, indicated as Region 1 (Pairwise Identity 82.4%; Identical Sites 73,5%), Region 2 (Pairwise Identity 100%; Identical Sites 100 %) and Region 3 (Pairwise Identity 79.3 %; Identical Sites 70,9 %).

Figure 5. Reconstruction of the gene duplication history of target genomic region 4. region 10. (a) Representation of postulated duplication and of identified variants in green (low impact on coding gene) or in yellow (medium impact on coding gene). Phylogenetic analysis performed on homologous sequences of cytoplasmic (NLR; panel b) and transmembrane (RLP and RLK; panel c) receptor proteins. Bootstrap values are indicated above branches. (d) Multiple alignment of pepper (13,14), tomato (11) and eggplant (3) sequences and for each genes, we reports: locus (green), exons (yellow) and transposable element (brown). An insertion of transposable element on sequence of pepper gene 14 was evidenced. Red rectangles display highly conserved sequences, indicated as Region 1 (Pairwise Identity 78.3%;, Identical Sites 64.4%), Region 2 (Pairwise Identity 89.6%; Identical Sites 89.6%) and Region 3 (Pairwise Identity 75.8%; Identical Sites 85.6%).

Draft

Supplementary materials

Supplementary tables legends:

Table S1. Classification of *S. melongena* and *C. annuum* pathogen recognition genes that encode domains similar to plant R proteins.

Table S2. Annotated PRGs list and their relative genomic positions. *The chromosome position of eggplant PRGs were assigned on the base of results reported in table S17 by Hirakawa et al. (2014)

Table S3. Resistance ortholog groups (ROGs) list. In the table for each identified PRGs, we reported plant species, cluster ID, ortholog confidence score, protein class and short name of R-gene homologue. *For the partial proteins the class was not reported (-).

Table S4. List of PRG clusters obtained from data merging of cluster prediction in pepper and tomato.

Table S5. Syntenic genomic regions sequenced in tomato, eggplant and pepper genomes. The regions length and the number of mapped reads are related to three reference genomes (*S. lycopersicum*_ Heinz 1706; *S. melongena*_ Nakate-Shinkuro; *C. annuum* Zunla-1). *Target regions used for microsynteny analysis.

Table S6. Detected variants (SNPs and InDel) of selected genomic regions for target sequencing experiment.

Supplementary figures legends:

Figure S1. Workflow of the targeted sequencing and variant-calling experiment.

Figure S2. Proportion of homozygous and heterozygous SNPs assessed in four species. Zygosity classes are colour-coded as indicated. Accession IDs and the percentage of SNPs are indicated on the x and y axes, respectively.

Figure S3 Proportion of DNA variant types assessed in the four species. Variant types are colour-coded as indicated. Accession IDs and the percentage of SNPs are indicated on the x and y axes, respectively.

727

Draft

1402x623mm (72 x 72 DPI)

Draft

213x120mm (96 x 96 DPI)

116x117mm (600 x 600 DPI)

Figure 4

35x15mm (600 x 600 DPI)

Figure 5

35x15mm (600 x 600 DPI)

Draft