Chapter 1
Genomic Tools for Improving Tomato to Biotic Stress Resistance

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Abstract Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops. It also represents a model plant for studying genetic traits related to disease and pest resistance and molecular processes underlying plant-pathogen interactions mechanisms. Tomato crop can be endangered by stressful conditions, which can cause intensively yield lost in temperate areas. In the next years, it has been forecast that rising temperature and CO_2 levels, will affect agricultural production globally. The sequencing of tomato reference genome (*S. lycopersicum* Heinz 1706) allowed to improve our knowledge on important agronomic traits. In this species, important breeding achievements have been obtained thanks to extensive molecular mapping and molecular assisted selection (MAS) efforts. The advent of genomic-based technologies facilitated the identification of genes involved in tomato biotic stress and the design of more tailored varieties. Databases collected on tomato large-scale data were developed and are available to support the identification of genetic resources, markers, key genes, proteins and biochemical processes involved in biotic stress resistance. Different plant genetic engineering approaches were applied to promote more precise genome modification processes. Stable or transient plant transformations can be used to develop new resistant tomato lines able to adapt to the rapid climate

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changes and new diseases spreading. To date, laws about genetic modified (GM) tomatoes are quite stringent in many countries, but researchers made great progress using alternative biotechnological methodologies, based on DNA repair mechanisms such as genome editing technology, able to generate short insertion/deletion (InDel) in specific genomic locations leading to highly selective mutation. The current legal system on plant variety rights should be updated according to new biotechnological advances. The increasing knowledge on tomato overall response to biotic stress, including genome signature, gene identification, proteins and metabolite function combined to emerging biotechnological methodologies will unfold the full potential for accelerating tomato breeding for biotic stress resistance.

Keywords *Lycopesicon esculentum* · Disease resistance · Sequencing · Molecular markers · Database · Biotechnology · Plant-breeding rights

1.1 Introduction

1.1.1 Economic Importance of Tomato

Tomato (*Solanum lycopersicum* L.) is a species native of South America belonging to Solanaceae family that includes many other economically important vegetable crops such as potato (*Solanum tuberosum* L.), pepper (*Capsicum annuum* L.), and eggplant (*Solanum melongena* L.). Tomato production in 2019 reached a worldwide global value of 182 million tons with a cultivated area of 4.8 million hectares. More than 60% of total production is concentrated in Asia, followed by Europe, America, and Africa with 13.5%, 13.4%, 11.8% of total production, respectively (FAOSTAT 2019). A picture of the economic importance of tomato worldwide is given by its global market value. The six major countries playing a significant role in the tomato international market are USA, Spain, Portugal, Italy, China and India (Fig. 1.1), which in 2018 produced a total revenue of $190.4 billion with an average annual rate of increase of 3% in the previous 10 years.

The economic and nutritional importance of tomato, place it among the most widely studied crop, becoming a plant model to understand molecular process related to development, fruit metabolism, and plant pathogen interaction (Liu et al. 2018; Quinet et al. 2019). Tomato genome sequence released in 2012 represents an important resource for the improvement of agronomic traits, becoming in few years an essential tool for basic and applied research (Tomato Genome Consortium 2012; Sahu and Chattopadhyay 2017).

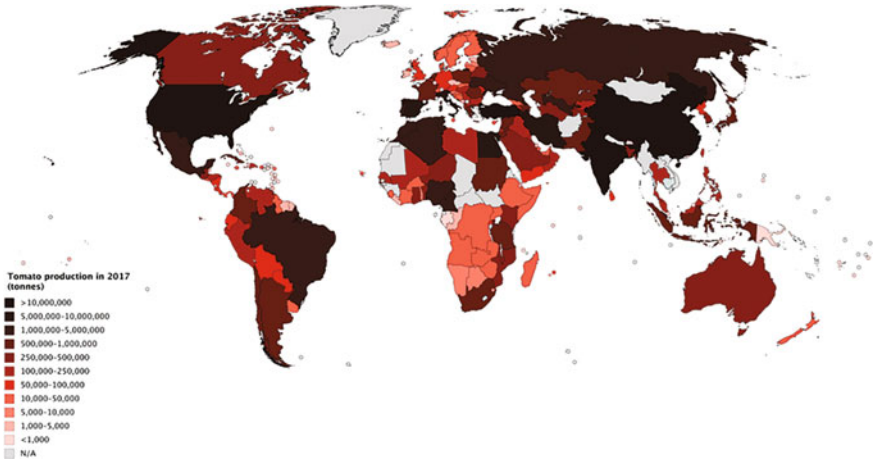

Fig. 1.1 Tomato production in tons, based on data from the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT 2017)

1.1.2 Reduction in Yield and Quality Due to Stress

Severe yield losses due to major pests and diseases can cause considerable yield and fruit quality reduction in tomato (Severin et al. 2001). Several diseases are caused by bacteria (*Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae* pv. *syringae*) fungi (*Alternaria porri* f. sp. *solani*, *Cladosporium fulvum*, *Phytophthora infestans*, *Verticillium dahliae* and *Fusarium oxysporum*) and virus such as *Tobacco Mosaic Virus* (TMV), *Tomato Spotted Wilt Virus* (TSWV), *Tomato Yellow Leaf Curl Virus* (TYLCV) and *Tomato Brown Rugose Fruit Virus* (ToBRFV) (Thompson and Tepfer 2010; Mândru et al. 2017). High atmospheric humidity and the presence of drops of water on the foliage can promote infection of *Phytophthora infestans, Xanthomonas campestris* pv. *Vesicatoria,* and *Pseudomonas syringae* pv. *syringae* (Costache et al. 2007; Tamir-Ariel 2007). *Cladosporium fulvum* in favorable conditions may cause premature defoliation, affecting the photosynthetic activity of affected plants and the consequent productions (Babadoost 2011). *Alternaria porri* f. sp. *solani* and other major tomato pathogens, can cause collar rot in the basal part, leaf and stem stains and rotting of fruits (Walker 1952). Sometimes biotic and abiotic stresses can act synergistically or additively causing stronger symptoms and serious damages (Cappetta et al. 2020a, b). Some studies showed that modulating the reactive oxygen species (ROS) response could be an important way to improve plant multi-stress tolerance (Sewelam et al. 2016). Depending on the plant stage and duration of the stress and interaction with other stresses yield loss can increase up to 70%. Taken together these data point out that if tomato stresses are not adequately treated it can lead to more than $133 billions of economic losses every year.

1.1.3 Impact of Climate Change

The major agricultural challenge is to provide food and nutritional security to the annually growing global population. Tomato world consumption is increasing from year to year. In 2018/2019 the estimated global consumption was 38.3 million mT (raw material equivalent) with an 8% increase against the previous year (35.5 million mT) and 4% increase compared to the average of the three previous years (Branthôme 2020).

Countries that typically showed the highest tomato consumption belong to the North American and Western European nations that to date remains the main commercial route for tomato products. However, it is important to highlight the increasing importance in the global market of emerging regions especially in the Middle East, South America, the Far East, and West Africa. Tus, the increasing tomato demand places these markets at the same level of the "classical" markets of America and Europe demand of which is in slightly decline; in total these two areas are accounted for approximately the 44% of world tomato consumption. It seems that on mentioned markets are growing fast from the beginning of the new millennium, and it is probable that in the next years they will reach a complete "maturity".

It is known that the climate is changing, average temperatures of our planet have risen about 1 grade Celsius over the last 200 years. In particular, the past 20 years have seen a rapid increase in global warming (Fig. 1.2). Every year there are new record temperatures with 2020 that has been registered as the warmest year ever.

Climate changes are in part consequential stages of our planet, but they are also driven and speed up by atmospheric greenhouse gases, land transformation and other human-made emissions into the atmosphere (Asseng et al. 2015). The "global warming" process is arousing an increasing interest in recent years, due to its high impact on human life, including the rivers and lake drying, animal species extinction and a substantial reduction of crop productivity (Wheeler and Von Braun 2013; Fahad et al. 2017). There is a real risk that climate changes that can affect the food security

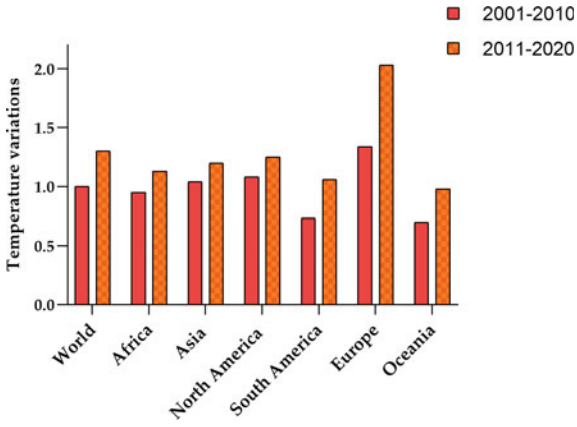

Fig. 1.2 Mean annual temperature measured globally and, in each continent in last two decades (FAOSTAT 2021)

worldwide. The global warming can reduce food availability or affect food quality. Climate change is mainly refleced in extreme weather events, and reductions in water availability, with huge impacts on agricultural productivity. For instance, in Italy, one of the major tomato producers worldwide, 2019 production season registered a reduction of tomato yield due to persistent rainfall and temperature variation from the seasonal average. Due to these climate effects, tomato plants showed a slow fruit ripening, because of winds and storms that damaged the fruits, and sudden heatwaves that reached 40 °C. Overall stressful conditions caused a 50% of total yield lost in temperate areas. Different published models show how in the next years rising temperature, and more elevated CO_2 levels will affect agricultural production all around the world (Kheir et al. 2019).

1.1.4 Limitations of Traditional Breeding and Rational of Genome Designing

Traditional plant breeding allowed breeders to obtain improved tomato varieties through techniques based on phenotypic selection. However, several years are required to develop a new and stable variety (in terms of phenotypical and genotypical traits), which may not meet the requirements related to the fast climate changing scenarios described above. Innovative technologies potentially can address many of these challenges. The design of more tailored varieties can take advantage of a more precise and complete understanding of plant functioning. A global vision of overall tomato response to biotic stress, including genome signature, gene identification, proteins and metabolite function can be obtained by combining different genomic methodologies. Integration of computational data showed to be effective in identifying key components of stress response (Cappetta et al. 2020a). The development of molecular marker techniques and their applications drastically changed the fate of plant breeding for biotic stress in tomato (Ercolano et al. 2012). However, marker assisted selection (MAS) for quantitative trait loci (QTLs) is promising and strategies able to predict the genomic potential can be more effective. In this regard, genomic selection (GS) provides new opportunities for selection using genome-wide marker data (Cappetta et al. 2020a, b). Transcriptomic analysis of plants exposed to biotic stresses allow identifying important targets involved in disease resistance process (Padmanabhan et al. 2019; Zhao et al. 2019). To date, different engineering approaches to obtain disease resistant varieties based on genetic transformation, RNA silencing strategies, and emerging gene editing techniques were developed. Overall, established and emerging technologies such as transcription activator-like effector (TALE) and clustered regularly-interspaced short palindromic repeats (CRISPR) associated Cas protein 9 (CRISPR/Cas9)-based technologies enlarged the range of opportunities for obtaining tomato resistant varieties (Andolfo et al. 2016). Genomic editing tools allow to modify DNA sequence in a thoroughly selective manner, resulting very promising breeding tools (Malzahn et al. 2017; Waltz 2018).

1.2 Molecular Mapping for Disease Resistance

1.2.1 A Brief History of Mapping Efforts

Since restriction fragment length polymorphism (RFLP) marker was first used for genetic mapping in 1980 (Botstein et al. 1980), a variety of DNA-based molecular markers have been developed that have been used in plant breeding to select the plants of interest from segregating populations without phenotype screening (Tanksley et al. 1989; Yang and Francis 2005; Foolad 2007; Foolad and Panthee 2012). The abundance of single nucleotide polymorphisms (SNP) and the advent of next-generation sequencing (NGS) makes it more feasible to simultaneously select thousands of markers, which allows cultivar development with significantly reduced phenotypic screening, hence shortening the breeding cycle. Although, single marker cost is low, the high total cost prevents many breeders from adapting GS in their breeding practice.

Different approaches have been adopted to map and fine-map the gene(s) and QTLs in tomato. Depending upon the purpose, various mapping populations have been used for mapping QTLs in tomatoes. An F_2 population derived from crossing two inbred lines has the advantage to reduce the time to generate it. Backcross populations (BC) including BC1 and BC2 are extremely useful while doing targeted mapping. Both F_2, as well as BC populations, are early generations. Recombinant inbred line (RIL) populations get a better estimation of additive effects of QTLs and trials can be replicated. However, it takes a long time to develop them. Several tools such as Map Maker, QTL Cartographer, Join Map, iCIMapping, QTL Mapper, MapChart, SolQTL, R/QTL, and Map/QTL can be employed to perform a mapping experiment, two major reviews report details to better exploit them (Cheema and Dicks 2009; Semagn et al. 2010).

1.2.2 Molecular Genetic Maps

Tomato genetic maps has been created by using the previously mentioned software. There are several genetic maps developed using mapping populations derived from *Solanum lycopersicum* by wild relatives (*S. pimpinellifolium, S. pennellii*, or *S. habrachaites*). Those populations used for mapping are F_2, backcross, or RILs. The first molecular linkage map in tomato was developed in 1992 using RFLP molecular markers consisting of 1,030 RFLP markers (Tanksley et al. 1992). This map was updated combining cleaved amplified polymorphic sequences (CAPS), RFLP and simple sequence repeat (SSR) marker information in Tomato EXPEN2000 (Fulton et al. 2002; Frary et al. 2005). A more comprehensively map was later obtained adding a few more CAPS, SNPs, and expressed sequence tag (EST) and SSR markers which is widely called the Tomato-EXPEN2000 map (Shirasawa et al. 2010). The total length of the chromosome was 1,503.1 cM resulting from a total of 2,116 molecular

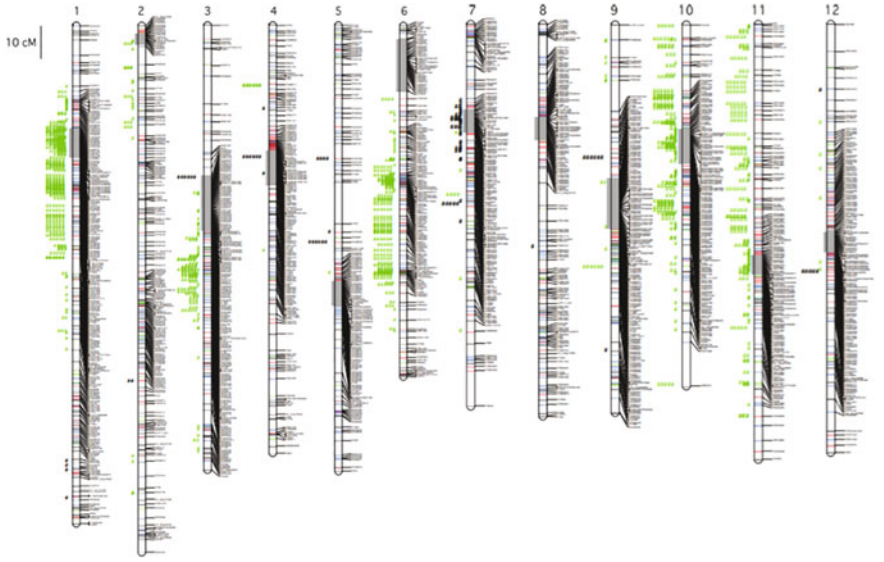

Fig. 1.3 Genetic linkage map of tomato genome derived from *S. lycopersicum* × *S. pennellii* using 2,116 molecular markers spanning 1,503.1 cM genetic distance (Shirasawa et al. 2010)

markers (Fig. 1.3; Shirasawa et al. 2010). A comprehensive list of mapping populations, markers types, number of markers, and publication information is provided by Labate et al. (2007).

1.2.3 Mapping Efforts for Identifying Resistance Traits to Major Tomato Fungal Diseases

Several bacterial, fungal, and virus diseases are common in tomatoes causing a significant yield loss throughout the world. There is a considerable research interest to investigate the genetic control of these diseases so that resistance genes or QTL can be introgressed.

Among the major diseases, late blight (LB), caused by *Phytophthora infestans* de Bary, is one of the most important diseases in the world in tomato. Three genes *Ph1, Ph2,* and *Ph3* have been identified to confer resistance to this disease. The dominant gene *Ph1* was identified in the wild relative *Solanum pimpinellifolium* and was mapped to the distal end of chromosome *7* (cited in: Foolad et al. 2008). However, this gene was not effective for a long time due to the emergence of new races of *P. infestans.* The *Ph2*, a partially dominant gene was found in the same wild relative *S. pimpinellifolium*, which was mapped to chromosome *10* (Moreau et al. 1998). The resistance conferred by this gene was also not found effective for a

long time. The *Ph3* was identified from LA3708 of *S. pimpinellifolium*, which was mapped to chromosome *9* (Chunwongse et al. 2002).

In addition, QTLs associated with late blight resistance were found on chromosome *4 7, 8* and *12* in *Solanum habrochaites* (Brouwer et al. 2004; Li et al. 2011).

Quantitative resistance to LB has also been reported from LA716 (*S. penelli*) (Smart et al. 2007). In addition, QTLs conferring resistance to LB were mapped on chromosome *5* (Haggard et al. 2013), and on chromosome *11* (Haggard et al. 2015). In order to make the resistance durable, Li et al. (2011) have suggested the pyramiding of resistance gene and/or QTLs from multiple species.

Subsequently, fine mapping of these QTLs made potential MAS for LB resistance. In another population derived from intraspecific crosses, the location of minor QTLs was found close to the R gene (Panthee et al. 2017). Such QTLs resulted consistent in all the environments tested, although the LOD score was slightly different (Fig. 1.4; Panthee et al. 2017).

Early blight (EB) resistance is a quantitative trait, which makes selection more difficult. Foolad et al. (2002) used a backcross population derived from NC84173 × PI126445 to map resistance QTLs for EB. They found ten resistance QTLs for EB in both BC_1 and BC_1S_1 populations, which were highly consistent across generations, and years explaining 8.4–25.9% of total phenotypic variation (Foolad et al. 2002). A selective genotyping approach detected seven QTLs for EB resistance, validating

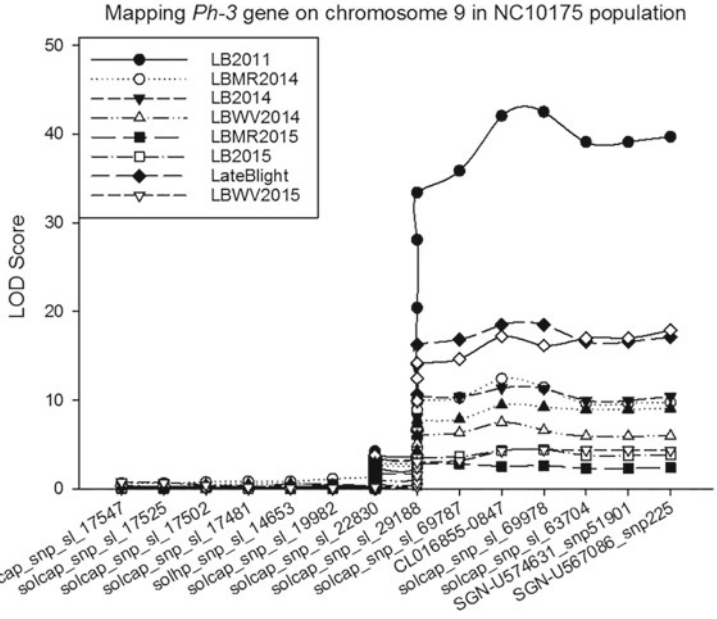

Fig. 1.4 Mapping *Ph-3* on chromosome *9* in segregating tomato population derived from an intraspecific cross (Panthee et al. 2017)

four of detected in a previous study using PI126445 of *S. habrochaites* (Zhang et al. 2003). A trait-based marker analysis for resistance to EB was performed in F_2 and F_3 populations derived from a cross between *S. lycopersicum* cv. Solentos (susceptible) and *Solanum peruvianum* LA2157 (resistant) (Chaerani et al. 2007). A total of six QTL regions were mapped to chromosomes *1, 2, 5, 6, 7*, and *9*, including three resistance QTLs to stem lesions in the field that explained 35% of the phenotypic variation. After extensive screening of 300 accessions of *S. pimpimellifolium*, an accession LA2093 with good EB resistance was selected for QTL mapping (Ashrafi and Foolad 2015a, b). Ten QTLs conferring EB resistance on chromosomes *2, 3, 4, 5, 6, 7, 9*, and *12* with individual effect of 7.6×13.4% and combined effect of 44% of total phenotypic variance were detected (Foolad et al. 2008). In another study, five major QTLs for EB resistance were identified on chromosomes *2, 5, 6*, and *9*, using RILs of the same cross (LA2093 × NCEBR-1) (Ashrafi and Foolad 2015a). QTLs on chromosomes *2* and *6* were from LA2093, whereas QTLs on chromosomes *5* and *9* were from NCEBR-1. Two stable QTLs on chromosomes *5* and *6* were used in EB resistance breeding. The detected QTLs were also co-localized with other resistant genes and candidate ESTs (Ashrafi and Foolad 2015a). A review on EB resistance including QTL mapping is provided by Adhikari et al. (2017).

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is a devastating disease of tomato (Agrios 2005). Three races, race-1, race-2, and race-3, of *Fol* have been reported to cause this disease. Corresponding to these races, three loci *I-1, I-2,* and *I-3*, have been identified which confer resistance in tomato (Sarfatti et al. 1989, 1991). The *I-2* was mapped between the RFLP markers TG105 and TG36, 0.4 cM from TG105 on chromosome *11* (Ori et al. 1994). The *I-3* gene from wild tomato *S. pennellii* accessions LA716 and PI414773 that confers resistance to *Fol* race 3 was mapped to chromosome *7* (Hemming et al. 2004).

In contrast to the fungal diseases discussed above, there is a lack of knowledge on QTL and molecular markers for Septoria leaf spot (SLS), Verticillium wilt (VW), Powdery mildew (PM), and other fungal diseases of tomatoes.

In summary, several disease resistance genes have been mapped onto the tomato genome. It has helped to advance the MAS in tomato breeding programs throughout the world.

1.3 Marker-Assisted Breeding for Disease Resistance

1.3.1 Germplasm Characterization and DUS

Germplasm characterization is one of the foundations for launching successful plant breeding. Phenotypic characterization was the basis for the identification of suitable germplasm to be used as parents in a breeding program. With the abundance of molecular markers and their association with several disease resistance traits, this information can be utilized for the selection of germplasm in a breeding program.

After selection, variety registration is an important step to provide the plant breeders right and to regulate the seed production process. For that, a variety to be eligible to be released as a unique variety, should meet the criteria of distinctness, uniformity, and stability (DUS). Some of the traits are difficult to measure phenotypically to provide the DUS certification. In this case, molecular testing might be useful. It has been optimized and employed for the testing of some of the diseases in tomatoes as explained by Arens et al. (2010). A similar approach can be adapted for other crops as well.

1.3.2 Marker-Assisted Gene Introgression

Molecular markers associated with disease resistance genes have been optimized and used extensively (Foolad and Panthee 2012). Molecular markers can be used when plants are very young, saving the field stage. The use of molecular markers at early generation also helps to discard the unwanted materials advancing the useful materials. The use of reliable molecular markers helps to even avoid phenotypic characterization. This is useful when inoculum pressure or screening facility is an issue for some of the diseases or evaluation of some of the diseases may be extremely difficult because of their safety concern. The MAS can be more effective than phenotypic selection under certain situations, including when there is a lack of selection environment such as enough inoculum pressure, trait expression is developmentally regulated, the trait is controlled by a recessive gene(s), or multiple trait selection is desired (Foolad and Panthee 2012).

1.3.3 Gene Pyramiding

Combining multiple sets of genes in a single genotype is the goal of a plant breeder. While they have been doing it by conventional breeding for a long time, it is very time-consuming. The MAS has been instrumental to combine the multiple genes in a single genotype. Gene pyramiding has been done to combine late blight (*Ph2* and *Ph3*), root-knot nematode (*Mi-1.2* gene), and *Tomato Yellow Leaf Curl Virus* (*Ty1*, *Ty2*, and *Ty3* genes) resistance genes in tomato (Kumar et al. 2019; Kim et al. 2020; Prabhandakavi et al. 2021). It would have taken at least ten years to combine all three genes in a single genotype by a conventional method. It took a single season by the use of molecular markers.

1.3.4 Limitations and Prospects of MAS

In most of the modern tomato breeding programs, the MAS is integral component and is being used on regular basis. These will be used even more frequently with the development of SNP markers. This approach has been helpful to advance the breeding programs. With the reduction of cost per sample analysis, tomato breeders may likely integrate the molecular approach even at wider level. They may expand the use in more traits. One of the limitations is that it may be challenging to keep up with the fast-changing technologies. Also, it may be challenging to handle the ever-increasing genotypic data since sequence-based SNPs are being generated in most cases.

1.4 Genomics-Aided Breeding for Resistance Traits

1.4.1 Structural and Functional Genomic Resources

Rapid advances in genomics technologies provide new opportunities to assess the biological function of important tomato loci, which, in turn, will greatly enhance our ability to utilize these genes in breeding programs. A high-density molecular map containing >2,000 markers (Rick and Yoder 1988; Sim et al. 2012) a large collection of well-characterized mutants, wild species and near-isogenic lines (NILs) are available for tomato (Eshed and Zamir 1995). In addition, tomato has a relatively small genome size (~950 Mb), (Martin et al. 1992; Bonnema et al. 1996), and a routine *Agrobacterium*-mediated transformation system (McCormick et al. 1986). The publicly available tomato large insert libraries (YAC: yeast artificial chromosome; BAC: bacterial artificial chromosome and TAC: transformation-competent artificial chromosome) were used as valuable research tools for the isolation of several agriculturally important genes by positional cloning (e.g., Martin et al. 1993; Geethanjali et al. 2010) to be directly transformed into the plant genome via *Agrobacterium*-mediated transformation (Hamilton et al. 1996; Li et al. 2000) or to be used as templates in shotgun sequencing (Boysen et al. 1997). More recently, the development of high-throughput genomics resources is improving our understanding the entire tomato genome organization and functioning.

1.4.2 Genome Sequencing

In 2004, an international consortium of 10 countries (Korea, China, the United Kingdom, India, the Netherlands, France, Japan, Spain, Italy, and the United States), as part of a larger initiative called 'International Solanaceae Genome Project' (SOL), launched the initiative to sequence the tomato genome. The first step of SOL project

was to generate a high-quality tomato euchromatic genome sequence. An ordered BAC approach was chosen to sequence the tomato genome and the libraries were constructed from the Heinz 1706 tomato line (Barone et al. 2009). The BAC-by-BAC strategy involves the anchoring of BACs or contigs of BACs to a reference genetic map. These anchored BACs are sequenced, and the sequence information is used to further extend the contigs. A total of 837 markers were used to anchor the contigs, mainly composed of euchromatic sequences, to the tomato genetic map. The tomato physical map was validated using fluorescent in situ hybridization (FISH) on pachytene complements with entire BAC clones as probes, and by genetic mapping of anchored BACs using panels of tomato introgression line populations. The genome of the inbred tomato cultivar 'Heinz 1706' has been released over nine years ago (TGC 2012). The tomato genome was sequenced and assembled using a combination of Sanger and 'next generation' technologies. The scaffolds were linked with two BAC-based physical maps and anchored/oriented using a high-density genetic map, introgression line mapping and BAC FISH. The predicted genome size is approximately 900 megabases (Mb), of which 760 Mb were assembled in *12* tomato chromosomes (TGC, 2012) . The latest tomato genome version (SL4.0) was assembled de novo from PacBio long reads and scaffolded using Hi-C contact maps and it is available at the Solanaceae Genomics Network Current (SGN; http://sgn.cornell.edu) (Hosmani et al. 2019).

1.4.3 Gene Annotation

A high-quality automated annotation of the genome was produced by the international tomato annotation group (iTAG) to rapidly allow the use of sequenced sequences to the tomato breeders community. The iTAG performed repeats annotation, and masking of pseudomolecules, mapping of different protein sequence sets, ESTs and full length cDNAs, as well as RNA-Seq reads from Illumina, 454 and SOLiD platforms. In addition, independent ab initio predictions were performed with GENEID (https://genome.crg.es/software/geneid/), AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/), and TWINSCAN (https://bio.tools/twinscan), all specifically trained for tomato. The above listed extrinsic data were integrated using the a priori informed gene prediction software. EuGene prediction, followed by manual expert curation, produced a consensus annotation of 34,727 protein encoding genes for the tomato (iTAG v2.3) nuclear genomes (TGC, 2012) . To date, the latest tomato gene annotation available at the Solanaceae Genomics Network is iTAG4.1 (SGN; http://sgn.cornell.edu) through BLAST database, Pathway database (SolCyc: https://solgenomics.net/pages/solcyc/) and Apollo (Dunn et al. 2019). About 5,000 novel genes were identified and most of the updated genes have extensions in the 5′ and 3′ UTRs (Hosmani et al. 2019). The release of the tomato genome annotation provided an excellent opportunity to steer the studies of gene characterization. Scientists and breeders around the world actively use the tomato genome sequence for breeding and research activities. Indeed, a full annotation of pathogen recognition genes was

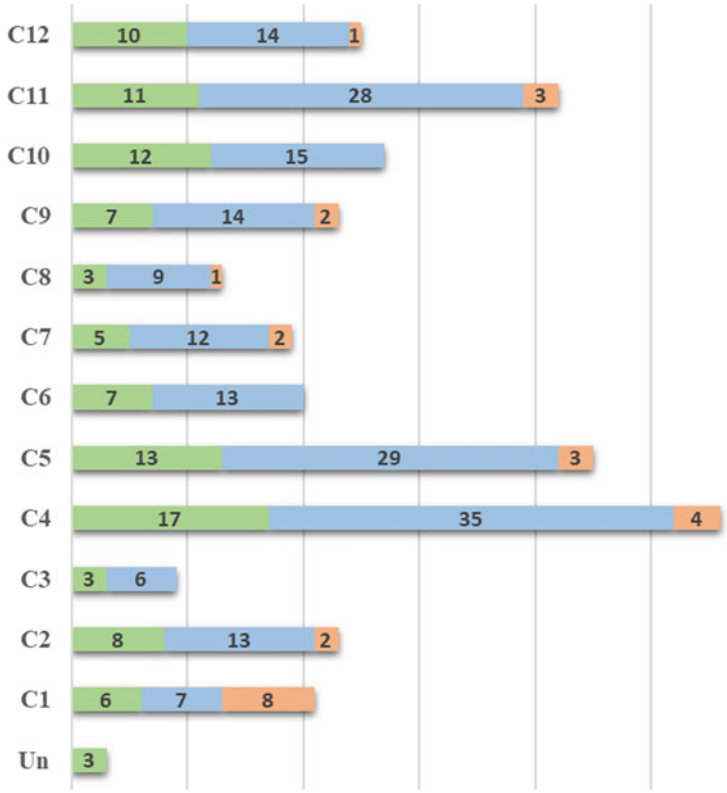

Fig. 1.5 R-gene family identified and annotated in *S. lycopersicum* Heinz 1706 v2.4 genome. The tomato defense arsenal was displayed with respect to chromosomal position (C1–C12 and unassembled region (Un)) and R protein domain structure (CNL in blue; TNL in orange and partial genes in green). The total number of CNLs, TNLs, and partial genes was shown

released immediately after the publication of tomato genome sequence (Andolfo et al. 2013). Over 770 genes, belonging to nucleotide binding domain and leucine-rich repeat (NLR), receptor-like protein (RLP) and receptor-like kinase (RLK) protein classes, were finely annotated and characterized in tomato genome (Andolfo et al. 2013) providing a useful tool, for breeders and scientists, to identify novel disease resistance traits to introduce in tomato cultivars (Andolfo et al. 2014) (Fig. 1.5).

1.4.4 Impact on Germplasm Characterization and Gene Discovery

The sequencing of tomato genome has totally revolutionized the accuracy of germplasm characterization and the pace of gene discovery (Andolfo et al. 2021). The

development of the *S. lycopersicum* Heinz 1706 reference genome made possible to study the genetic variation of tomato accessions and wild relatives. Considering the overwhelming interspecies genetic variability, tomato germplasm collections represent a gene pool with unprecedented possibilities to address new breeding demands imposed by climate change, world population increase, and consumer needs. During the domestication the tomato genome went through a genetic bottleneck, reducing its genetic diversity to less than 5% (Sim et al. 2010). Moreover, several disease resistance traits have been disregarded as a result of human selection for yield and quality related traits. Consequently, tomato cultivars have become more susceptible to various pathogens (Foolad 2007). Introgression of traits from wild-species into domesticated species is a widely used practice for increasing diversity in crop plants. Indeed, numerous disease resistance genes have been introgressed in tomatoes from wild species such as *Solanum chilense, S. peruvianum, S. habrochaites, S. pennellii*, and *S. pimpinellifolium* (Catanzariti et al. 2017; Yamaguchi et al. 2018; Andolfo et al. 2021). The selection process can be accompanied by linkage-drag, which require many rounds of backcrossing and fine-mapping to eliminate (Labate and Robertson 2012). Thus, the ability to define the borders and contents of wild-species introgressions can contribute significantly to speed up the selection process and can help to identify the putative resistance gene loci (Andolfo et al. 2021). The whole-genome sequencing approach provides detailed information on genic content and the origins of the introgressed regions through comparison of wild species genomes with genomic background of breeding lines obtained (Labate and Robertson 2012).

The increasing of accessions resequencing allowed to explore extant genetic variation in tomato, providing a major boost to identification of valuable alleles (Aflitos et al. 2014; Ercolano et al. 2014; Gupta et al. 2020). The millions of informative markers (SNPs/InDels) and structural variations identified through comparison of genome sequences of domesticated and wild tomatoes will promote investigations into the genetic and molecular basis of the disease resistance process. This will not only help identify useful SNPs from the wild accessions but also rare SNPs within domesticated varieties (Ercolano et al. 2014; Tranchida-Lombardo et al. 2018). Tomato breeders can identify gene variants in the wild species associated with desirable traits such as disease or pest resistance and introduce them into cultivars to exploit the diversity of tomato germplasm. The tomato genome sequence facilitates QTL identification, mapping and cloning of underlying genes, and provide new SNP markers for marker-assisted breeding (Arafa et al. 2017; Gonda et al. 2019). Availability of the tomato genome sequence will speed up the understanding of gene function in plant disease resistance by mapping relevant wild tomato traits. The advent of NGS and available genome sequences should make characterization of large collections of tomato accessions even more rapid and robust.

1.4.5 Application of Structural and Functional Genomics in Tomato Breeding

The growing body of tomato genomic data is accelerating the transfer of beneficial traits into new tomato varieties (Andolfo et al. 2021). The use of the reference genome for genetic analysis has become increasingly beneficial to enhance tomato breeding efforts. Genetic mapping of resistance traits speeds up breeding for plant disease resistance. Markers available for tomato have been widely used to locate and tag genes or QTLs for disease resistance (Arafa et al. 2017; Panthee et al. 2017). Indeed, mapping of resistance genes to different viruses, bacterial, nematode, and fungal diseases provided important information for tomato genomics aided breeding. The success of this strategy depends on the availability of technological platforms based on automated large-scale screening. To date, several technologies for automatic large-scale small-variants detection have been set up, increasing markers specificity levels. The completed genome sequence of *S. pennellii, S. pimpinellifolium* and *S. chilense* (Bolger et al. 2014; Stam et al. 2019; Wang et al. 2020) and several transcriptomic data for wild tomatoes are available. Therefore, the polymorphism between resistant and susceptible genotypes could be more easily explored in order to identify SNPs or InDels useful as gene markers in dissecting complex resistance traits (Pachner et al. 2015). The increasing availability of information on resistance genes deriving from the sequencing of the wild tomato genomes (Seong et al. 2020), will facilitate large-scale annotation for gene-assisted selection (Andolfo et al. 2014). Several tomato wild relatives are used to broaden the genetic diversity of tomato through the introgression of required alleles (Jablonska et al. 2007; Zang et al. 2014; Catanzariti et al. 2017). The identification and transfer of new resistance alleles assisted by genomic data provide more reliable and precise methods for tomato breeding. In many cases, one or few polymorphic amino acids are sufficient to determine resistance in the plant host (Ashikawa et al. 2012; Stirnweis et al. 2014; Giannakopoulou et al. 2015).

GS is a predictive approach that has emerged as a valuable method for improving complex traits that are controlled by many genes with small effects (Cappetta et al. 2020a, b). This promising breeding framework has already been shown to be feasible superior genotypes during breeding programs (Liabeuf et al. 2018). Genome editing technologies can improve the development of varieties with desirable wild genes/alleles (Wang et al. 2019).

1.5 Genetic Engineering for Resistance

1.5.1 Transgenic Technologies

Since 1983 with the first transgenic tobacco plant, the genetic engineering science have undergone great improvements, reaching impressing accuracy levels (Lemaux 2008). To date, plant genomes can be modified in a highly selective manner and in

near future, it is expected that engineered plants (free from the transgenic backbone and selectable marker genes) may take an important role in agricultural productions. The genetic engineers are working hard to promptly enhancing desired tomato traits by genome modification processes. In this context, transgenic approach of genetically modified (GM) tomatoes represent an important weapon. To date, laws about GM tomatoes are quite stringent, but researchers made great progress using transgenic technologies. Genes isolated in sexually compatible species (cisgenes) can be introduced through genetic engineering. Cisgenic science should be considered similar to traditional breeding, because the final result is the same of a crossing between two compatible species. Cisgenic tomato plants resistant to *Phytophthora infestans* were obtained by Faino et al. (2010). More recently cisgenic tomato lines resistant to bacterial wilt disease (*Ralstonia solanacearum*) were obtained by Morais et al. (2019) through the identification of *PPC20*, an alpha-helical (AH) peptide derived from plant protein sequences, and *SlP14a* (a pathogenesis-related protein). Cisgenic methods have been also used in other Solanaceae such as potato, introducing two R genes conferring resistance to *Phytophthora infestans*: *Rpi-sto1* and *Rpi-vnt1.1* in three potato commercial varieties, from the crossable species *Solanum stoloniferum* and *Solanum venturi;* they obtained resistant marker-free potatoes plants (Jo et al. 2014). A more efficient homologous recombination system, with a subsequently highly precise transgene insertion can be obtained with plastid DNA transformation. Foreign proteins can be expressed to extremely high levels with the absence of epigenetic effects (Oey et al. 2009). More genes can be introduced simultaneously stacking them in operon systems (Boehm and Bock 2018). Furthermore, plastid engineered does not allow the transmission of transgenic genes to the progeny. The genetic sequence of the tomato chloroplasts (plastome) has also been determined by Kahlau et al. (2006) facilitating tomato plastid experiments (transplastomic tomato).

1.5.2 Gene Silencing

In order to discover new gene functions, scientists can downregulate gene expression by several gene-silencing approaches. A method to downregulate gene expression was originally developed by Hiatt et al. (1989), using the expression of an antisense RNA strand which then caused base pairing with the sense RNA strand originally synthetized by plant, reducing the availability of targeted RNA and subsequently the protein accumulation. More efficient silencing technologies were further developed after discovering of RNA interference (RNAi) and virus-induced gene silencing (VIGS), two post-transcriptional gene silencing techniques. Through RNAi approach, a gene portion is expressed in double-strand flanking a linker DNA region. At this point a dicer protein cuts the double-stranded RNA into smaller pieces of approximately 22 nucleotides long, producing small interfering RNA (siRNA). These siRNA form the RNA-induced silencing complex (RISC) with the target gene, blocking the translation. Gene downregulation can also be achieved using

microRNA (miRNA) which binding to the 3′ untranslated regions of target mRNAs represses its expression. In the last decades these methods became quite popular among researchers worldwide (Eulalio et al. 2008; Galvez et al. 2014; Tiwari et al. 2014). VIGS involves the use of engineered viral vectors that contain a sequence of a gene of interest to silence. The recombinant virus can be introduced into plant cells through *Agrobacterium tumefaciens* infections. In many studies, it has been demonstrated that the use of gene silencing in tomato provides resistance against biotic and abiotic stress. Singh et al. (2020a, b) targeting a key polyamine (PA) biosynthesis gene *ornithine decarboxylase* (*ODC*) of the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* using RNAi, obtained transgenic lines with moderate and high resistance to *Fusarium oxysporum.* Singh et al. (2014) obtained transgenic tobacco and tomato plants, using small interfering RNA, targeting two RNAi suppressor proteins (*AC2* and *AC4*) of *Tomato Leaf Curl New Delhi Virus* (ToLCNDV); showing that after virus inoculation, most of the plants displayed no disease symptoms. Other experiments carried out in tomato, showed that silencing of *miR482b* (involved in *Phytophthora infestans* infections) using short tandem target mimic (STTM) resulted in enhancement of tomato resistance (Jiang et al. 2018). *SLNLC1* gene silencing using VIGS technology provides resistance against the pathogen *Stemphylium lycopersici* in tomato (Cui et al. 2018).

1.5.3 Gene Editing

Plant genetic editing (GE) involves technologies that could be applied to modify valuable plant traits for increasing resistance to herbicides, insects, and diseases. Gene editing technologies enable scientists to make DNA modifications, leading to changes in phenotypic traits. To date, widespread genome editing technologies allow scientists to alter, add, or remove a specific locus. Gene editing requires engineered enzymes (endonucleases) able to bind a specific DNA sequence to achieve the desired genetic changes. Once reached the nucleus, they can introduce cuts into the double-strand of DNA, leading to an non-homologous end joining (NHEJ) that subsequently results in a random mutation or in presence of a DNA donor, to an homology directed repair (HDR) useful to introduce determined DNA fragments. There are different types of nucleases: the zinc finger nucleases (ZFNs) and transcriptional activator-like effector nucleases (TALENs) operate through the fusion of sequence-specific DNA binding domains (DBDs) and nucleases. Following the recognition of the target sequence by the DBDs, nucleases provide double-strand breaks (DSBs) leading to NHEJ and to InDel causing gene mutation and a consequently loss-of-function (Chandrasegaran and Carroll 2016). More recently, the CRISPR/Cas9 system is already being explored for a wide number of applications in agriculture fields. This technology consists of a nuclease driven to the DNA target sequence by a specifically designed guide RNA (gRNA). To date, several tomato genes involved in biotic or abiotic stress pathways have been well characterized through this technique. The CRISPR/Cas9 system it

was extensively used in the scientific community because it requires only a protospacer adjacent motif (PAM), usually NGG, and a complementary 17–22 bp guide RNA to match the target gene (Ran et al. 2013). However, the genome editing technique mentioned above requires a sequenced plant genome to selectively identify the genome targets.

1.5.4 Nanotechnology

Agricultural engineered crops are a promising solution to meet the increasing food demand worldwide also in the face of a growing population and climate changes. In the last few years, new strategies in plant genetic engineering have been developed, including the use of nanoparticles (Fig. 1.6). Nanomaterials (NMs) offer new solutions for incorporating agrochemicals and biochemical molecules into plants (Kole et al. 2013; Khan et al. 2017). To date, systems used to transfer biomolecules into plant cells such as a DNA fragment are mainly based on biological delivery systems such as *Agrobacterium*-mediated transformation. However, not all plant species can be transformed by *Agrobacterium*. Another commonly used tool for plant transformation is a biolistic particle delivery (gene gun) in which microparticles of

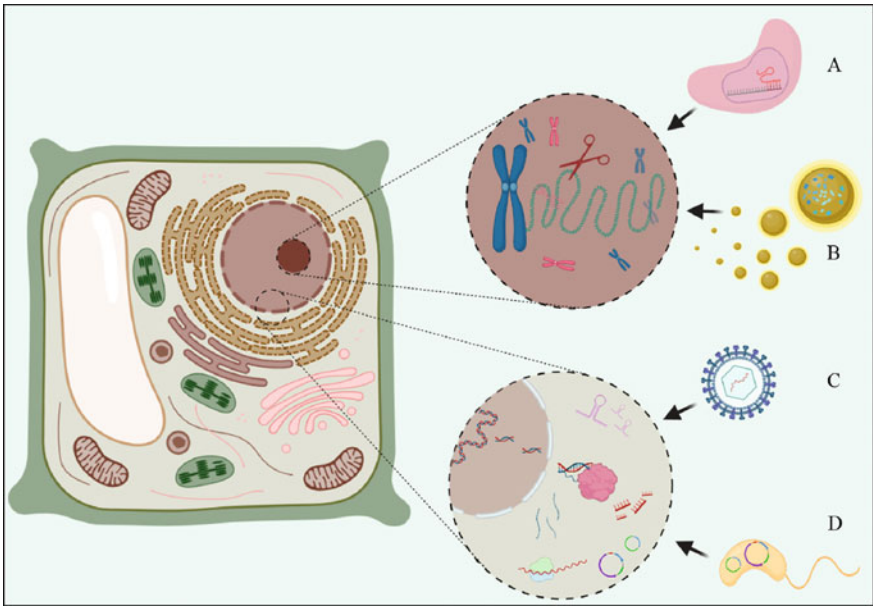

Fig. 1.6 Schematic representation of different biotechnological techniques for gene modifications: (A) Genome editing; (B) Biolistic approach; (C) Virus delivering ssRNA; (D) Agrobacterium-mediated Virus Inducing Gene Silencing. Created with BioRender.com

gold are introduced in plant tissues through a high-pressure gene gun. Recently, interesting results have been obtained with the use of nanoparticles with size of less than 100 nm able to penetrate the plant cells main barriers: (1) the hydrophilic cell walls able to exclude molecules bigger than 5–20 nm; (2) internal double-layer lipid membrane which can exclude molecules of more than 500 nm. Multi-walled carbon nanotubes (CNTs) and carbon dots allowed efficient DNA delivery into both nuclear and chloroplast genomes achieving gene silencing (Demirer et al. 2019, 2020; Kwak et al. 2019). Graphene, fullerenes, and polymeric nanoparticles (NPs) including polyethyleneimine-coated NPs have promising efficiency for DNA, RNA, or protein delivery into plant cells (Cunningham et al. 2018). Mesoporous silica nanoparticles (MSNs) were employed in Arabidopsis plants (Chang et al. 2013) and double-layered hydroxide clay nanosheets in *Nicotiana tabacum* (Mitter et al. 2017). More recently Zhang et al. (2019) using a system of DNA origami nanostructures delivered RNAi molecules in *Nicotiana benthamiana.* Nevertheless, further studies are needed to improve NMs' physic-chemical properties and to optimize nanoparticles characteristics for different cellular destinations and plant tissue or organ explant.

1.5.5 Target Traits for Biotic Stress Resistance

Genome editing techniques are generally applied in the perspective of producing genetically improved crop varieties. Target traits might be chosen to improve plant resistance to a specific biotic stress, or an established plant pathogen. Specific application, targeting multiple genes, can lead to wild species domestication. Several plant species have been genetically modified using genome editing tools, especially CRISPR/Cas9 technology and RNAi or VIGS. Tomato represents one of the most well-studied crops, probably because of its economic importance and the availability of a whole-sequenced genome. CRISPR/Cas9 mediated genome editing allowed enhancing tomato resistance to biotic stress (Nekrasov et al. 2017; Tashkandi et al. 2018). Moreover, using RNAi targeting *HyPRP1* gene (to inhibit gene translation) scientists obtained tomatoes with improved characteristics of resistance against both biotic and abiotic stresses (Li et al. 2016). In Table 1.1 are shown CRISPR/Cas9 studies related to tomato biotic stress resistance, conducted in last three years.

1.6 Bioinformatics Repositories

1.6.1 Gene and Genome Databases

In last years, a large amount of tomato genome and gene sequences was generated and stored in public repository, affecting the research approaches for carrying out

Table 1.1 List of CRISPR/Cas9 experiments conducted to improve tomato resistance to biotic stresses

Organism	CRISPR/Cas9 system	Target	Effects	References
S. Lycopersicum cultivar Moneymaker	CRISPR/Cas9 double guide RNAs	Jas domain of *SlJAZ2* gene	*Sljaz2Δjas* mutants are resistant to *Pseudomonas syringae* (PtoDC3000)	Ortigosa et al. (2019)
S. Lycopersicum cultivar Moneymaker	CRISPR/Cas9 four single-guide RNAs	*Powdery Mildew Resistance 4* (*PMR4*)	Enhanced resistance against *Oidium neolycopersici*	Martínez et al. (2020)
S. Lycopersicum *cultivar BN-86*	CRISPR/Cas9 four guide RNAs and two guide RNAs respectively	eRF1_1 domain in the *SlPelo* gene and exon 11 of the *SlMlo1* gene	complete resistance to powdery mildew fungus and reduced accumulation of TYLCV virus	Pramanik et al. (2021)
S. Lycopersicum cultivar Micro-Tom	CRISPR/Cas9 double guide RNAs	*SlPDS* and *SlMYC2* genes	reduced the plant growth and fruit resistance to *B. cinerea*	Shu et al. (2020)
S. Lycopersicum cultivar Zaofen No. 2	CRISPR/Cas9 multiplexing—three guides RNA targeting the stem-loop structure	MicroRNAs miR482b and miR482c	Mutants showed a reduced disease symptom against *Phytophthora infestants*	Hong et al. (2020)
S. Lycopersicum cultivar Ailsa Craig	CRISPR/Cas9 Not specified	*SlMAPK3*	reduced resistance to *B. cinerea* and enhanced the content of *ROS*	Zhang et al. (2018)
S. Lycopersicum cultivar Moneymaker	CRISPR/Cas9 duble guide RNAs	*SlMlo1*	Improved resistance against *Oidium neolycopersici*	Nekrasov et al. (2017)
S. Lycopersicum cultivar Moneymaker	CRISPR/Cas9 single guide RNA	TYLCV genome at coat protein (CP) site	Improved resistance against TYLC virus	Tashkandi et al. (2018)

genetic investigations and expanding the opportunity to get a response to a scientific question. The availability of an high-quality reference genome, the resequencing of hundreds of genomes (Aflitos et al. 2014; Lin et al. 2014; Ercolano et al 2014) and the release of large RNA-seq experiment data (Du et al. 2015; Yang et al. 2017; Shi and Panthee 2020) provided new insight into biological knowledge of *Solanum* species. Several databases collect tomato data and allow cross analysis of metadata coming from various entries. The Sol Genomics Network (SGN; http://solgenomics.net), a clade-oriented genomics platform for Solanaceae species, hold

several features and tools able to deal with tomato genome variation and gene family structural and functional investigation. Other large data access portals such as Ensembl Plants, PlantGDB Phytozome, and PLAZA, collect sequenced genomes, providing powerful tools to analyze annotated gene family datasets. The proper utilization of the existing large scale tomato data is challenging and many collection databases have been developed, including: KaTomicsDB, (http://www.kazusa.or.jp/tomato), TOMATOMICS (http://bioinf.mind.meiji.ac.jp/tomatomics), and Tag-SNP, an online Solanaceae genome Browser for capturing information on SNPs (Jeong et al. 2020). Tomato large scale RNA-seq data are available at the Tomato Functional Genomics Database (TFGD) (Fei et al. 2011), (TFGD, http://ted.bti.cornell.edu), TomExpress (http://gbf.toulouse.inra.fr/tomexpress/www/welcomeTomExpress.php), Kazusa Tomato Genomics Database Plant Expression Database (PLEXdb, http://www.plexdb.org/index.php).

Tomato Genetics Resource Center database (TGRC, http://tgrc.ucdavis.edu) can be interrogated for genetic resources and information on microRNA identified in expressed sequence tags (ESTs) can be obtained by miSolRNA (Bazzini et al. 2010) and in SolmiRNA (Kim et al. 2011). In addition, several mutant resources derived by Ethyl methanesulfonate (EMS), gamma-rays, fast neutron mutagenesis are publicly available and can be exploited by tools such as Mutmap, and MutChromeSeq, to accelerate the mutation breeding in tomato (Chaudhary et al. 2019).

To retrieve information related to tomato R genes and other Solanaceous species we can easily browse through the plant resistance gene database (PRGdb: http://prgdb.org/prgdb/). This web resource collects manually curated reference R-genes as well as plant putative R-genes. The PRGdb database is organized in four sections: plants, genes, pathogens, and disease. A set of pre-defined queries can be cross explored to identify putative R-proteins thanks to the distinctive structural domains of resistance genes such NB-LRR and TIR present into NB-LRR proteins and receptor kinase domains belonging to RLK and RLP proteins (Sanseverino et al. 2010). In addition, a BLAST search tool and a DRAGO pipeline allows to annotate resistance genes (Osuna-Cruz et al. 2018). A new section reporting plant-pathogen transcriptome experiments in model species, was added in the last database updated. (PRGdb 4.0). From the home page (Fig. 1.7A) is possible to select the species for visualizing data related to reference and predicted genes (Fig. 1.7B) or to explore the results of different expression studies. Differential gene expression analysis (DEG) lists to conduct further analyses are also provided (Fig. 1.7C).

1.6.2 Comparative Genome Databases

The evolution selection pressure acting on resistant loci significantly can affect species variation. The reconstruction of evolutionary trajectories that shaped tomato gene repertories can be improved using orthologs analysis. Comparison among plant species showed to be a valuable strategy to facilitate proper classification of genes and for exchanging information related to putative protein functions across species,

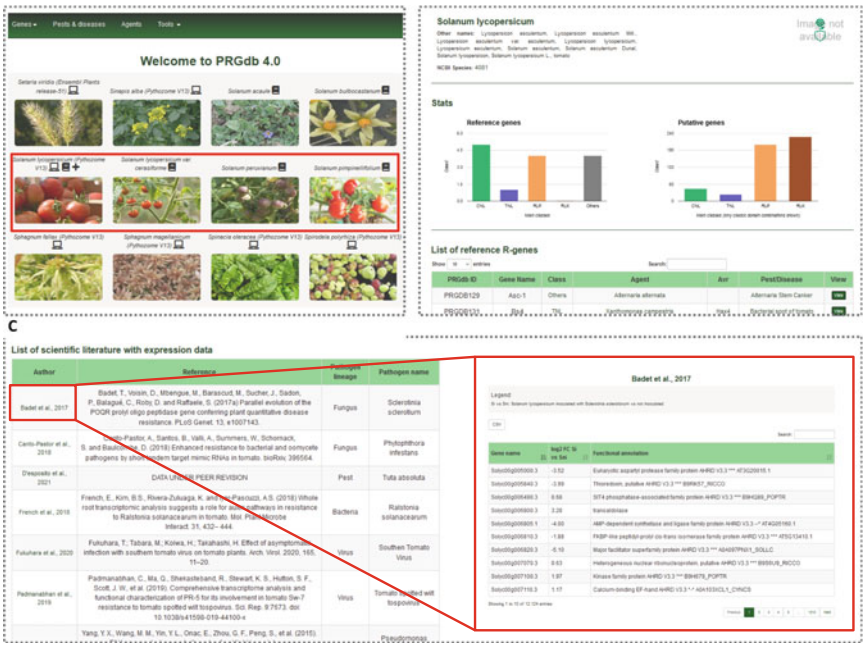

Fig. 1.7 Overview of PRGdb 4.0 main sections

raising important questions related to genome organization (Andolfo et al. 2021). Tools able to identify putative orthologous genes from different plant species are available through several websites such: Ensembl Plants, PlantGDB, Phytozome and PLAZA. A phylogenetic analysis can help to identify the likely orthologs of resistance genes for species of interest (Andolfo et al. 2013). It represents a good starting point to identify putative tomato orthologs of a given gene involved in a resistance process. Translational and/or comparative genomics methodologies can be integrated to detect homology sequences and block of synteny for trait-associated genes discovering (Di Donato et al. 2018).

1.6.3 Gene Expression Databases

Numerous tomato RNA-Seq datasets have been generated and published. Although the raw data are publicly available (e.g., via the NCBI sequence read archive, https://www.ncbi.nlm.nih.gov/sra), they are not curated and their use in direct comparisons can be tedious due to the diversity of genetic sources, pathogen treatments and sequencing methodologies. Expression browsers aim to collect and reanalyzing public datasets, normalizing parameters used to count expressed reads, and ideally

allowing retrieval of expression information in a list of genes under different conditions. Three main tomato expression browsers are currently available: The Tomato Expression Atlas that provides tissue-specific expression data based on single cell laser dissection (Fernandez-Pozo et al. 2017). The TomExpress platform developed to support tomato research community of a public RNA-Sequencing browser with integrated web tools, including data mining graphic outputs, such as expression bar plots, heatmaps of hierarchically clustered expression data and co-expressed genes networks (Zouine et al. 2017) and Co-expressed Pathways DataBase on Tomatoma platform (http://cox-path-db.kazusa.or.jp/tomato) developed by Narise et al. (2017). All these resources provide a powerful way for generating hypothesis using tomato-specific data. The web-based resources can be explored to get useful information for specific experimental aims. However, comparisons with gene expression profiles in response to various treatments could be more useful to gain new insights in specific tomato stress interactions. A dedicated platform to plant-pathogen interaction transcriptomic experiments is definitely needed.

1.6.4 Protein or Metabolome Databases

TomatoCyc (https://plantcyc.org/content/tomatocyc-5.0) is a large-scale computational prediction platform for pathways and their catalytic enzymes, compounds, and genes. Most of pathway pictures were extracted from literature. Kegg (https://www.genome.jp/kegg/pathway.html) can be widely used to check reference proteins as well as Biocyc (https://biocyc.org/web-services.shtml) that allows to retrieve pathways, reactions, compounds, genes, proteins, and RNA or transcription-unit resembling the underlying pathway tools schema. The Co-expressed Pathways DataBase for Tomato (http://cox-path-db.kazusa.or.jp/tomato) allow to predict pathways that are relevant to a query gene, which would help to infer gene functions. Predicted tomato interactome resource (PTIR) (http://bdg.hfut.edu.cn/ptir/index.html), covering approximately the 30% of the entire tomato proteome, is based on experimentally determined orthologous interactions in six model organisms, evaluated by shared gene ontology (GO) terms, co-evolution, co-expression, co-localization and available domain-domain interactions (DDIs) (Yue et al. 2016). Reconstructing protein interaction networks may be a powerful method for deciphering molecular mechanisms and potential gene function.

1.6.5 Integration of Different Genomic Data

Various web resources-based tomato omics information and bioinformatics tools have been developed. In addition, repositories collecting genetic valuable material including natural and artificial mutants are available. To enhance the efficiency of

acquiring tomato biology information coming from different sources we must integrate knowledges. Large-scale sequencing projects continue to be launched and it is important to combining them with validated data on genes function and interaction. SGN (Fernandez-Pozo et al. 2015) and TOMATOMICS (Kudo et al. 2017) provide large-scale omics information with gene structures, expression profiles and functional annotations, full-length mRNA through search functions and the genome browser. However, a more comprehensive effort for integrating genomic tools and datasets can facilitate gene characterizations. Translational strategies showed to be feasible to investigate plant defense responses. Multi-layered omics data can be combined to better explore network of interactions and biological behavior in a synthetic manner (Choi 2019). A broader vision will provide deeper insights in studied process accelerating the discovery of new traits. Knowing the location of given R-gene locus can be of great advantage for mining its nucleotide sequences using both genetic recombination analysis and protein prediction data. Once a resistance source has been phenotypically characterized, sequencing, genetic and functional analysis can be employed to link predicted sequence to gene function. Identification of syntenic regions among related genomes or collocation of a predicted gene with similar function in a related species can help to select candidate genes for the given trait. Analysis of chromosome recombination rate data and putative R-gene prediction resulted useful to select promising candidate genes (Andolfo et al. 2014).

1.7 Plant Protection and Patent Regulatory Issues

In many countries the regulation for the protection of plant varieties is based on a traditional approach set up prior the development of genetic engineering and genomics methodologies (Official Journal of the European Union n° L 227 of 01/09/1994 pp. 0001–0030) here in after "ROV". The UPOV Convention establishes a specific title for the protection of plant varieties, different from the patent, excluding from patentability, in its first drafts, all plant varieties. This prohibition is also included in article 53(b) of the ROV, relating to the community protection of plant varieties. On the other hand, Directive 98/44/EC of the European Parliament and of the Council of July 6, 1998 on the legal protection of biotechnological inventions (Official Journal of the European Union n° L 213 of 30/07/1998 pp. 0013–0031), allows the patentability of inventions consisting of plants or plant material, provided that no whether they are new plant varieties, or their application is not limited to a specific plant variety (Garcia-Vidal 2017). Effects and intensity of the protection are different from those of patent law, since they touch, in principle, the variety's reproduction material and, only when it has not been possible to exercise actions against the production and commercialization of this vegetal material, cascading actions can be exercised against the fruits and the products obtained by said fruits. Hence, despite the prohibition of the patentability of plant varieties, there have been several attempts to achieve their patentability. TOMATE II case was successful in this regard since the High Chamber of Resources of the Office European Patent, interpreting that

article 53 b) of the European Patent Convention did not exclude the patentability of plants as products (Torralba-Simon 2019). The search for the patentability of plant varieties shows the interest of the tomato industry in greater protection for their biotechnological inventions, so that they can recover and obtain greater profitability from the investment made. This forces to consider the fundamentals of the Law of plant varieties and consider whether the protection granted is currently sufficient, taking into account the development of biotechnological research. The holder of the plant variety rights has the right to exclusively carry out certain operations with the plant material, requiring any third party of their authorization for its execution (Arts. 13.1 and 2 ROV, 12 LOV and 14.1 UPOV Convention). These operations, which are exhaustively listed, are production or reproduction (multiplication), packaging for propagation, putting up for sale, sale or other commercialization, export, import and storage with a view to perform any of the above operations (Petit-Lavall 2017).

The extension of the scope of protection of the breeder's rights to the product of the harvest and to the products directly obtained from the plant material is nuanced by the cascade configuration of said protection, which already places the plant variety right at a clear disadvantage with respect to the right of patent. In this way, the harvested material is only protected if the following two conditions apply: it has been obtained through the unauthorized use of components of the protected plant variety and the owner has not had a reasonable opportunity to exercise his rights over said components of variety (Arts. 13.3 Regulation ROV, 13.1 LOV related to art. 7 ROV and art. 14.1 CUPOV). For the holder of the right to benefit from the extension of the protection on the crop product, he must have previously carried out the necessary actions to exercise said right in the multiplication or reproduction phase and, only in the case of proving these actions are not possible, he may try to exercise his rights over the harvest product.

It could well underlined a limitation of the protection of the breeder's rights to protect farmers and traditional breeders interests. It is necessary to reflect on the interests that base the plant variety right and the adequacy of the current legal system for its protection, since there is no doubt that any weakening of the breeder's rights must cause a flight to other protection systems such as know-how or patent law, as has been seen, is occurring despite the express prohibition of patentability of plant varieties, through recourse to product claims obtained by a certain procedure. The pressure on the patent system to protect plant varieties, which as has been advanced has been successful on several occasions but has been stopped by the Enlarged Board of Appeal of the European Patent Office issued Opinion G 3/19 (Pepper) on May the 14th, 2020. As with other industrial property rights (art. 59 LP and art. 38 LM, in Europe see art. 67 CPE and 9.3 RMC), the applicant for a plant variety has the right to demand reasonable compensation appropriate to the circumstances of whoever performs acts of exploitation that, granted the plant variety to be protected, would constitute acts of infringement, during the period started with the publication of the application and ended with the concession (Arts. 95 ROV; in art.18.2 LOV and art. 13 UPOV). The actions for violation of the right do not extend to this period of provisional protection, in which the protection of the owner of the rights is limited to compensation for the negative effects caused by the exploitation of the plant

variety by third parties. Obviously, whatever the criteria used to fix the amount of reasonable compensation (Espinosa-Calabuig 2016), only the negative consequences of the exploitation of the variety during the period of provisional protection would have to be take into account.

Biotechnological advances, which require an investment in plant innovation fully comparable to that made in other technology matters, together with a possible consolidation of the interpretation of the courts that is very restrictive of the scope of the protection of breeder's rights, translate into pressure on patent offices to achieve the patentability of plant varieties, by considering them products obtained through the use of microbiological procedures. Undoubtedly, the cascade protection of plant variety rights and their extension only to essentially derived varieties, and not to all derived varieties or dependent varieties, is a transcript of a traditional or "natural" conception of plant variety law that must probably outperform.

The attaching of regional and national regulation in the UPOV Convention places the international community before a huge challenge, such as the debate and reform of the Law of plant varieties, attending to all interests in presence, the public interest in food safety and the sustainability of agriculture, and the interest of farmers and rights holders, ceasing to oppose said interests and seeking a balance between them, but taking into account the current reality of the state of science, such as new publishing techniques genetics that are being developed, and the need to promote the advancement of technology.

1.8 Future Perspectives

Genomic information extracted in different stages of resistant plant design process can be used to define target genes, to select target trait to begin studies, to extract information relevant for identifying a gene or obtaining desired varieties. The genetic advance achieved through genomic scanning depends on the ability of capturing superior alleles. Modern breeding is a dynamic, and evolving research discipline for minimizing efforts. Traditional breeding has been integrated with molecular aided selection, but many traits are very complex to dissect and variation in gene expression level may cause difference in resistance response variability. In such complex situation, it is important to offer the possibility to screen for allelic differences at the expression level (Torti et al. 2021) and to discriminate superior allelic forms with high throughput and sensitive detection methods (Singh et al. 2020a, b). After generating and analyzing new data, the comparison with information stored in large-scale repositories is essential to understand and interpret the resulting data and to draw conclusions. A wide range of technologies that might be used to genetically engineering plant's genome are also available or are under development. Several countries (Argentina, Australia Japan Canada and US) acknowledge the potential of gene editing to improve plant traits without introducing foreign DNA. In other countries, the debate is still ongoing (EU, UK, Russia, India, China and South Africa). A

more comprehensive effort for making use of genomic tools and datasets can enlarge the availability of new tomato resistance traits to biotic stress in the next future.

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