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CICLO: XXXV

CRISPR/CAS9 TARGETED MUTAGENESIS OF SUSCEPTIBILITY GENES FOR RESISTANCE IN TOMATO

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The experimental works described in this thesis are part of scientific papers published or submitted to international journals.

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Li R, Maioli A, Yan Z, Bai Y, Valentino D, Milani AM, Pompili V, Comino C, Lanteri S, Moglia A, Acquadro A. CRISPR/Cas9-Based Knock-Out of the PMR4 Gene Reduces Susceptibility to Late Blight in Two Tomato Cultivars. International Journal of Molecular Sciences. 2022; 23(23):14542. https://doi.org/10.3390/ijms232314542

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Li R, Cui L, Bai Y Martina M, Moglia A, Acquadro A (202-) Less is More: CRISPR/Cas9-based Mutations in DND1 Gene Enhance Tomato Resistance to Powdery Mildew with Low Fitness Costs. *Submitted*.

CHAPTER 4:

Li R, Maioli A, Lanteri S, Moglia A, Bai Y, Acquadro A (202-) Genomic Analysis Reveals Defective Susceptibility Genes in Tomato Germplasm. *Submitted*

Chapter 1 - Introduction

1.1 Focus

Diseases like late blight and powdery mildew, are major concerns for tomato growers and breeders, causing yield reduction and ecomomic losses. Researchers have explored different approaches to enhance the resistance of tomato plants, including CRISPR-Cas9 gene editing to disable susceptibility genes (S-genes), and identified defective alleles as a potential source for breeding.

This thesis is related to the possibility to produce **new resistant tomato varieties** with the genome editing approach, **using** the **CRISPR-Cas9 technology**. The **Chapter 1** will briefly introduce the importance of breeding resistant tomato cultivars, the target genes we selected, and the new breeding techniques we used.

The **Chapter 2** and **3** discussed the reliable use of the CRISPR-Cas9 gene-editing technology to disable two susceptibility genes (*PMR4* and *DND1*) in tomato plants to increase their resistance to various diseases (late blight and powdery mildew, respectively), showing promising results in terms of resistant materials generated.

The **Chapter 4** focuses on a genome-wide analysis of 360 tomato genotypes with the goal to identify existing defective S-gene alleles in germplasm for potential applications in breeding for resistance. These results provide a valuable resource for plant genetics, with potential

applications in genomic-assisted breeding programs. However, we have also provided new insights on single-guide RNA (sgRNA) design for the application of a gene editing in targeted S-genes, as a powerful alternative for the obtainment of tomato elite genotypes resistant to biotic stresses.

1.2 Tomato, a species of economic relevance

Tomato (*Solanum lycopersicum* L.) is the most cultivated and consumed vegetable worldwide, with more than 189.1 million tons harvested in 2021. Its gross production value rose from approximately 32.9 billion US$ in 2000 to 92.8 billion US$ in 2021 (FAOSTAT, https://www.fao.org/faostat). The global demand for tomato has considerably increased in recent years because of its various uses as a raw, cooked, and processed food, as well as its substantial contribution to the human diet, as it is rich in lycopene, vitamins, and minerals. Tomato also represents a model plant for biological research because of its short life cycle, chromosomal ploidy (2n = 2x), genome sequence availability, and amenability to transformation methodologies (*Chaudhary, Alisha et al. 2019, Xia, Cheng et al. 2021*).

Until the 1950s, tomato breeders developed multipurpose cultivars to meet agricultural and market demands. Subsequently, breeding focused on developing cultivars that were specifically suitable for industrial transformation (*Ronga, Francia et al. 2019*) or fresh markets (*Schouten, Tikunov et al. 2019*). Aside from raw tomato and tomato added to other foods, a number of processed products such as pastes, whole peeled tomato, diced products, and other kinds of juice, sauces, and soups have gained popularity (*Foolad, Merk et al. 2008*). The differentiation between fresh and processed accessions reflects all breeding efforts to provide welcomed, commercial tomato cultivars (*Bergougnoux 2014*).

1.3 Pathogens

Tomato can be cultivated in a range of climates, in open fields or greenhouses, and the fruit can be harvested manually or automatically. The fruits are both fresh market and food-processing industry end-products. During cultivation or post-harvest storage, it is susceptible to more than 200 diseases caused by an array of pathogenic fungi, nematodes, bacteria, and viruses, which diminish yield and alter product quality (*Singh, Singh et al. 2017, Lu, Ehsani et al. 2018, Panno, Davino et al. 2021*). The main route for disease control is fungicide application, which is an onerous practice with a high environmental impact and contributes to the selection of resistant isolates (*Laurindo, Laurindo et al. 2018*). The development of cultivars with reduced susceptibility to pathogens represents an important alternative for pathogen control and environmental sustainability (*Brouwer and St. Clair 2004*).

The study of tomato as a plant-pathogen system aids in the discovery and understanding of the molecular mechanisms underlying disease resistance, as well as the possibility of increasing the productivity and quality of its edible products. The application of functional genomics has contributed to this goal by allowing the identification of plant critical functional genes in susceptible and resistant responses (*Campos, Félix et al. 2021*). Understanding the mechanisms behind tomato-pathogen interactions and developing resistant cultivars are thus significant research objectives for attaining sustainable agriculture (*Ercolano, Sanseverino et al. 2012*).

1.3.1 Late blight

Late blight (LB), caused by the etiological agent *Phytophthora infestans*, is a devastating disease and a serious concern for plant productivity (*FRY 2008*), as it can destroy an entire unprotected tomato crop within 7-10 days of infection (*Nowicki, Foolad et al. 2012*). *Phytophthora* has around 120 recognized species, all of which are plant diseases, they colonise different host tissues, such as roots, tubers, herbaceous stems, woody trunks, foliage, and fruit, causing leaf and stem necrosis, fruit rot and eventual plant death (*Martin, Blair et al. 2014, Whisson, Boevink et al. 2016*), even infecting tomato seed (*Rubin and Cohen 2004*). Late blight lesions emerge first at the leaflet margins. Water-soaked lesions that are purple, dark brown, or black, frequently have a pale yellowish-green border that merges into healthy tissue. Lesions on the leaves may appear elsewhere as the disease progresses. In wet weather, fluffy, white sporangia may form on the lower (abaxial) leaflet surface. As the illness proceeds, the leaflets shrivel and die, and the disease spreads to the remainder of the leaves, resulting in widespread defoliation (*Foolad, Merk et al. 2008*).

Infection involves two phases: a biotrophic phase up to 36 h post inoculation (hpi) in which *P. infestans* forms haustoria and requires living plant tissue, and an ensuing necrotrophic phase in which infected host tissue becomes necrotic (*Whisson, Boevink et al. 2007*). *P. infestans* generates unique cellular stages during their infection cycle, from one

stage to the next (**Figure 1**) (*Hardham 2007, Whisson, Boevink et al. 2016*).

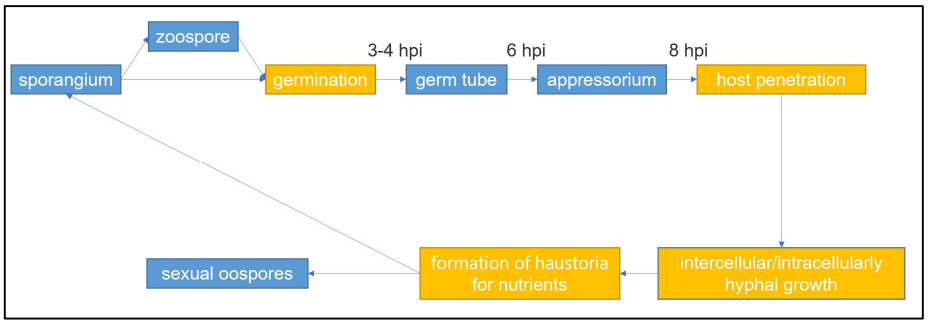


Figure 1. Diagram of the *P. infestans* infection and life cycle.

The beginning steps of dispersal consist of multinucleate sporangia and uninucleate motile zoospores. The multinucleate sporangium either germinates immediately or releases zoospores. Sporangia are multinucleate cells that can be blown or splattered to new hosts, allowing them to either germinate directly (at temperatures above 21°C, optimally at 25°C) or release uninucleate zoospores (below 21°C) to commence infection; zoospores shed their flagella and build a cell wall, resulting in the formation of a cyst. Once the zoospores have reached the host, within around 2 hours, they differentiate into walled cysts, which subsequently germinate. The germ tube develops into an appressorium from which a penetration peg emerges that enters through the leaf cuticle, or less frequently, the stomata. A spherical initial infection vesicle is generated upon host cell penetration, from which hyphae emerge to ramify through plant tissue. The hyphae of *P. infestans* grow intercellularly, projecting digit-like haustoria into host cells. Haustoria are structures that form an

intimate interaction with host cells, removing the plant cell wall but leaving the membrane intact to facilitate molecular exchange between the pathogen and a living plant cell. Successful colonization of the host in suitable relationships often results in sporulation, with the formation of asexual sporangia on the plant surface or sexual oospores within the host tissues. Oospores are huge, thick-walled spores that allow the fungus to persist outside of the living host plant in plant debris or soil, can overwinter in the field and survive well under adverse conditions, and may serve as an inoculum source for the following year's crop (*Gavino, Smart et al. 2000, Perfect and Green 2001, Latijnhouwers, Ligterink et al. 2004, Hardham 2007, Avrova, Boevink et al. 2008, Foolad, Merk et al. 2008, Fry, Birch et al. 2015*).

Knowing the life cycle of *P. infestans*, we can better understand how LB has been identified as one of the most devastating plant diseases of tomato and potato. Each LB lesion can produce up to 300,000 sporangia each day, leading to the disease's rapid spread. The asexual disease cycle, which includes pathogen penetration, colonization, sporulation, and dissemination, can take as little as five days (*Fry and Goodwin 1997*). Low levels of *P. infestans*, on the other hand, are difficult to detect in the field, and by the time the illness is discovered, it is sometimes too late to preserve the crop by fungicide application (*Foolad, Merk et al. 2008*).

1.3.2 Powdery mildew

Powdery mildew (PM), caused by genuine ascomycete fungus that belongs to the Erysiphales order and has just one family, the *Erysiphaceae*, infects the aerial regions of higher plants and may reduce yield by up to 30% (*Braun, Cook et al. 2002, Hückelhoven 2005*). PM is a group of termed obligate biotrophs, which only grow and reproduce on living plants and do not directly cause plant cell death, as they need living cells to obtain nutrients and complete their life cycle (*Spanu, Abbott et al. 2010*). There are roughly 700 PM species that can colonize nearly 10,000 plant species; three of these species, *Oidium neolycopersici*, *Oidium lycopersici*, and *Leveillula taurica* can live on tomato (*Seifi, Gao et al. 2014*). Among them, *Oidium neolycopersici* is a highly polyphagous powdery mildew fungus that infects all tomato cultivars (*Seifi, Gao et al. 2014*) and causes powdery white lesions on the adaxial tomato leaf surface, abaxial surfaces, petioles, and the calyx; its severe infection leave behind leaf chlorosis, premature senescence and a marked reduction in fruit size and quality (*Whipps, Budge et al. 1998*), thus it currently poses a significant threat to tomato (*Jones, Whipps et al. 2001*).

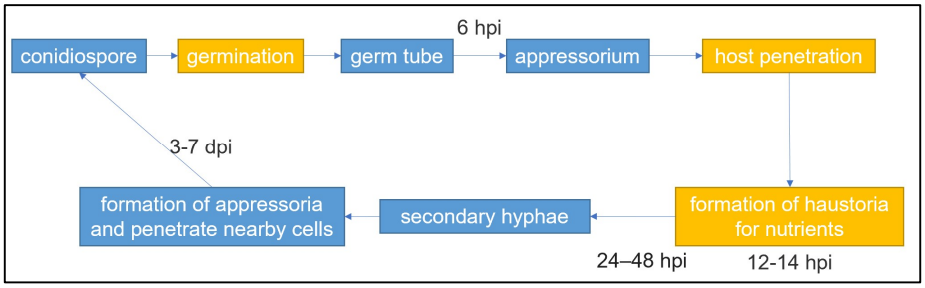


Figure 2 . Diagram of the powdery mildery fungi infection and life cycle. Hpi: hours post inoculation; dpi: day post inoculation.

PM fungi are biotrophic parasites invading only epidermal cells (*Perfect and Green 2001*). After a conidial spore land on the host surface, the PM fungus needs to attach to the surface and to penetrate the host cuticle and cell wall. The fungi build one or two germ tubes, depending on the genera (*Yarwood 1957*), which surroundedred by an invagination of the epidermal plasma membrane. Six hours after inoculation, the appressorium, a thickened infection structure, forms at the tip of this hypha. The haustorium, a specialized hyphal feeding structure with protrusions for surface enlargement, is produced in 12-14 hpi following successful cell wall penetration by the fungus, which is supposed to acquire the nutrients required for its epiphytic growth. The PM colony's secondary hyphae become evident as a result (24–48 hpi onwards). The secondary hyphae also create new appressoria and invade neighboring cells. Conidiophores are specialized hyphae that produce new conidiospores at the end of the cycle (3-7 dpi) (*Kuhn, Kwaaitaal et al. 2016*). To sum up, this process is assumed to ensure nutrient uptake which in turn enables the formation of aerial mycelium and asexual spores (conidia) (**Figure 2**) (*Hippe-Sanwald, Hermanns et al. 1992, Shirasu, Nielsen et al. 1999*).

Although cultural and biological techniques may minimize the risk of powdery mildew infection, they do not provide adequate protection. As a result, chemical control, including the use of fungicides from several

chemical groups, is the most effective method for managing powdery mildew in practice. Unfortunately, because standard spray programs entail numerous applications per season, the danger of resistance development is significant. Furthermore, some of the most economically damaging powdery mildew fungi are considered high-risk infections capable of developing resistance to a variety of chemical classes (*Vielba-Fernández, Polonio et al. 2020*). Therefore breeding varieties with resistance to powdery mildew is needed.

1.4 R-genes and S-genes

Plants cannot move to escape environmental challenges, in turn, they have evolved sophisticated mechanisms to perceive such attacks, and to translate that perception into an adaptive response. Plant-pathogen interactions, particularly those involving biotrophic parasites, are governed by specific interactions between pathogen *Avr* (avirulence) gene loci and alleles of the corresponding plant disease resistance (R) locus. These R-genes mostly encode NB-LRR proteins which are named after their characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains (*Dangl and Jones 2001*).

In plants, there is a two-layered defense system against pathogens. In the first layer, pattern recognition receptors on the cell surface perceive conserved microbial elicitors called ‘pathogen-associated molecular patterns’ (PAMPs), leading to PAMP-triggered immunity (PTI). Adapted pathogens can overcome PTI by deploying effector proteins, leading to

effector-triggered susceptibility (ETS). In the second layer, if one effector is recognized by a corresponding NB-LRR protein coded by an R-gene, effector-triggered immunity (ETI) starts. The recognized effector is termed an Avr protein. ETI is a faster and stronger version of PTI that often culminates in hypersensitive response (HR) (*Jones and Dangl 2006, Koseoglou, van der Wolf et al. 2022*).

Each dominant R-gene in the host corresponded with a dominant *Avr* gene in the pathogen, resistance is only conferred if both the R-gene and the corresponding *Avr* gene are present in the same interaction (*Flor 1971, Zachary Nimchuk, Thomas Eulgem et al. 2003, Balint-Kurti 2019*). The earliest events following R engagement are calcium influx, alkalinization of the extracellular space, protein kinase activation, production of reactive oxygen intermediates (ROIs) and nitric oxide (NO), and transcriptional reprogramming. NO, and ROI could also contribute to rapid transcriptional activation of a battery of 'defence genes' in and surrounding the infected cell (*Piedras, Hammond-Kosack et al. 1998*). Functions of these defence genes include biosynthesis of salicylic acid (SA), induction of ethylene biosynthesis, cell-wall strengthening, lignification, production of various antimicrobial compounds, and a form of rapid cells death where adjacent to the pathogen termed the HR. HR contributes to disease resistance by fostering release of antimicrobial enzymes and metabolites, by physically isolating the pathogen within defined lesions, and/or by enhancing local and systemic signaling signalling defenses in non-infected cells (*Scheel 1998, Clarke, Volko et al. 2000, Clough, Fengler et al. 2000, Balint-Kurti*

2019). In short, R-mediated recognition in most cases leads to HR (*Zachary Nimchuk, Thomas Eulgem et al. 2003*).

To establish a compatible interaction, pathogens use host factors encoded by plant susceptibility genes (S-genes) (*Eckardt 2002, Schie and Takken 2014, Zaidi, Mukhtar et al. 2018*). Any plant gene that allows compatible contact with the pathogen can be classified as an S-gene; thus, S-genes belong to a wide range of gene families, provide a variety of activities, and many of them are essential in plant physiological processes (*Schie and Takken 2014*). Pathogen effectors target plant factors encoded by S-genes for suppression of defenses, nutrient acquisition, and transport of bacterial proteins in the host cell to promote disease (*Koseoglou, van der Wolf et al. 2022*).

Researchers and breeders have long been working to increase plant resistance by focusing their efforts on R-genes, which mediate recognition of race or isolate-specific effector proteins of the pathogen, with the subsequent activation of plant defense responses (ETI). However, introgression of resistance genes in elite genotypes is time-consuming and often short-lived, because the widespread deployment of R-genes selects for pathogen strains capable of overcoming plant resistance (*FRY 2008, Michalska, Sobkowiak et al. 2016*); thus, continuing the discovery and introgression of new R-genes is of prime importance (*Wastie 1991, Huibers, Loonen et al. 2013*), it can be particularly difficult due to new

pathogen races overcoming resistance and global agricultural markets introducing new diseases (*Ercolano, Sanseverino et al. 2012*).

Evidence shows that mutation of S-genes can lead to durable, recessively inherited, and potentially broad-spectrum resistance in plants (*Pavan, Jacobsen et al. 2009*), to overcome the disadvantage of altering the narrow-spectrum R-genes (*Schie and Takken 2014*). In my work, *PMR4* and *DND1*, as well as the remaining 8 S-genes (*PMR5*, *PMR6*, *MLO1*, *BIK1*, *DMR6*, *DMR1*, *CPR5*, *SR1*), were specially examined:

1.4.1 PMR4

Many mutants in S-genes are already known in plants and there are some encouraging results in the literature related to their switch-off or down-regulation, like the powdery mildew-resistant (*pmr*) mutants (*Vogel and Somerville 2000*). PMR5 and PMR6 are pectin acetyltransferase and a pectate lyase-like genes respectively involved in resistance to powdery mildew, as well as cell wall composition in *Arabidopsis*; mutations in those genes have been shown to confer resistance to the pathogen and alter the host cell wall (*Vogel, Raab et al. 2002, Vogel, Raab et al. 2004*), highlighting the connection between cell wall composition and fungal disease resistance (*Chiniquy, Underwood et al. 2019*). Biotrophic diseases like PM must overcome host defenses, as a result, these pathogens must strike a delicate balance between collecting enough nutrients from the plant to complete their life cycle while not kill the host (*Vogel and Somerville 2000*). For this reason, pathogens deploit genes to

export nutrient from plant cells and creating site as a metabolic sink, therefore these genes became S-genes.

PMR4, coding a wound and pathogen-associated callose synthase (*Nishimura, Stein et al. 2003*), was discovered and characterized as a glucan callose synthase responsible for the generation of pathogen-inducible callose (*Østergaard, Petersen et al. 2002, Jacobs, Lipka et al. 2003, Nishimura, Stein et al. 2003*). *Pmr4* resistance was dependent on a functional SA signal transduction pathway (*Nishimura, Stein et al. 2003*). Callose is a high-molecular-weight amorphous-1,3-glucan, deposited in papillae produced beneath infection sites after infection by oomycetes or fungi, it works as a physical barrier or as a matrix that concentrates antimicrobial chemicals at potential fungal penetration sites (*An, Hückelhoven et al. 2006*). Surprisingly, changes in this gene provide resistance rather than vulnerability to various powdery mildew species (*Jacobs, Lipka et al. 2003, Nishimura, Stein et al. 2003*). This resistance is based on an increased SA response, implying a negative cross-talk between the callose response and signalling (*Nishimura, Stein et al. 2003*). It was anticipated that callose deposition during the early stages of fungal infection could hinder late SA-dependent defense mechanisms that could be damaging to the plant.

PMR4 has been identified as an S-gene that no longer supports the normal growth of the powdery mildew pathogen in *Arabidopsis* mutants (*Vogel and Somerville 2000*) and has been proven to be a potential

candidate gene in disease resistance breeding because its silencing in tomato and potato did not affect crop growth (*Huibers, Loonen et al. 2013, Sun, Wolters et al. 2016*). It has been the target of multiple studies investigating its role in plant defense against various pathogens, including powdery mildew and late blight in tomato (*Huibers, Loonen et al. 2013*), in potato it has been reported that knocking down *PMR4* leads to high tolerance against several diseases, including late blight (*Sun, Wolters et al. 2016*). The use of CRISPR/Cas9-based gene editing has sbeen hown to reduce susceptibility to these pathogens in tomato (*Santillán Martínez, Bracuto et al. 2020, Li, Maioli et al. 2022*).

1.4.2 DND1

The *dnd* (defense, no death) class of mutants, including *dnd1*, *dnd2*, and *Y15*, were identified by their reduced ability to produce the hypersensitive response (HR) in response to avirulent *Pseudomonas syringae* pv. *glycinea* (Psg) and were isolated in a screen designed to discover additional components of the *AvrRpt2* (an effector present in all *Pseudomonas syringae pv. tomato* race 1 strains)-*RPS2* (resistant to *p. syringae2*, the nucleotide binding-leucine rich repeat resistance protein) disease resistance pathway in Arabidopsis (*Clough, Fengler et al. 2000*). The Arabidopsis *dnd1* mutant is a rare autoimmune mutant that was identified by its reduced ability to produce a cell death response, known as the HR, a central feature of gene-for-gene plant disease resistance, and their mutants exhibit enhanced resistance against a broad spectrum

of virulent fungal, bacterial, and viral pathogens (*Yu, Parker et al. 1998, Clough, Fengler et al. 2000*). In details, SA is required for local and systemic acquired resistance (SAR) to elevate disease resistance (*Zachary Nimchuk, Thomas Eulgem et al. 2003*). But *dnd1* mutants suppressed HR, exhibited high levels of SA, while resistance increased (*Clough, Fengler et al. 2000*), made *DND1* recognized as an S-gene that act as negative regulators of defences (*Schie and Takken 2014*).

The DND1 protein is a Cyclic Nucleotide-Gated ion Channel (CNGC) that plays a role in plant defense and senses changes in intracellular cyclic nucleotide monophosphate (cNMP) levels to regulate numerous cellular responses, including calcium (Ca^{2+}) fluxes, indicating its importance for the HR in plants against pathogens (*Wilkins, Matthus et al. 2016, Ren, Zhao et al. 2021*). Dysregulated Ca^{2+} signalling may prevent the induction of PCD, which is a process that plants use to eliminate old, damaged, or unwanted cells in response to biotic and abiotic stresses (*Zheng, Zhan et al. 2020, Ren, Zhao et al. 2021*). *Dnd1* mutant exhibits a broad-spectrum resistance in absence of hypersensitive response (HR) to several biotrophic and necrotrophic pathogens, since HR is one of the most effective ways to impede growth of biotrophic pathogens, however it is considered to facilitate the growth of necrotrophic pathogens like *Botrytis cinerea* (*Govrin and Levine 2000, Sun, van Tuinen et al. 2017*).

In previous study, it was reported that silencing of potato orthologs to *DND1* resulted in resistance to *Phytophthora infestans* (*Sun, Wolters et*

al. 2016, Sun, Schipper et al. 2022), and reduced susceptibility to *Botrytis cinerea* in both tomato and potato (*Sun, van Tuinen et al. 2017*), as well as to two powdery mildew species, *Oidium neolycopersici* and *Golovinomyces orontii* (*Sun, Wolters et al. 2016*). In particular, the number of *Botrytis cinerea* conidia attached to the leaf surface of *DND1* silenced potato and tomato plants was significantly reduced, as was germling hyphal growth, implying that *DND1* silencing reduced susceptibility through impaired conidial germination and attachment, as well as hyphal growth (*Sun, van Tuinen et al. 2017*). However, in these studies, *DND1* may be an interesting gene in breeding resistant crops with negtive side effects (*Clough, Fengler et al. 2000*), especially in tomato, whose silencing caused a severe dwarf phenotype, autonecrosis and decreased male fertility (*Sun, Wolters et al. 2016, Sun, van Tuinen et al. 2017*). To examine the possibility of *DND1* orthologs being useful in tomato breeding, it is vital to investigate a method to reduce susceptibility as well as limiting at the same time the fitness costs.

1.4.3 Other S-genes

Elite barley lines carrying introgressed homozygous mutated alleles of an S-gene (*Mildew Locus O, mlo*) have been successfully used in European agriculture for approximately three decades because of the exceptional efficacy and longevity of powdery mildew resistance (*Büschges, Hollricher et al. 1997, Consonni, Humphry et al. 2006*). The *mlo*-based resistance has also been described in several other monocotyledonous and

dicotyledonous plant species (*Acevedo-Garcia, Gruner et al. 2017*). The existence of *mlo* mutants in different plant species of *Solanaceae* (e.g.: tomato), *Rosaceae* (apple, peach, and strawberry), *Cucurbitaceae* (melon, watermelon and zucchini) is widely documented and has been used to confer resistance to powdery mildews (*Acevedo-Garcia, Gruner et al. 2017*); the role of *MLO* in resistance to PM has been demonstrated through various mutagenesis approaches, including chemical mutagenesis, RNAi, and CRISPR-Cas9 (*Pessina, Pavan et al. 2014, Appiano, Pavan et al. 2015, Yan, Appiano et al. 2021, Sunarti, Kissoudis et al. 2022*). Recently, homoeoalleles in hexaploid bread wheat have been modified using transcription activator-like effector nuclease (TALEN) and CRISPR-Cas9 to confer heritable resistance to powdery mildew (*Wang, Cheng et al. 2014*). The first line of defence against invading pathogens in plants is triggered by the recognition of microbe-associated molecular patterns (MAMPs) through pattern recognition receptors (PRRs) located on the plasma membrane. Receptor-like cytoplasmic kinases (RLCKs) act as convergent regulators that work with multiple PRRs. However, the process of activating PM-tethered RLCKs is not yet fully understood (*Ma, Claus et al. 2020*).

BIK1 (*Botrytis*-induced kinase1) is an RLCK from subfamily VII (46 members in Arabidopsis) that plays a role in plant defence against pathogens and insects in *Arabidopsis thaliana*, acting specifically or redundantly in immune signalling (*Ma, Claus et al. 2020*). The *bik1* mutant

of Arabidopsis was found to exhibit strong resistance to the obligate biotrophic protist *Plasmodiophora brassicae* (*Chen, Bi et al. 2016*). Conversely, loss of BIK1 function in Arabidopsis increased susceptibility to green peach aphids (*Lei, A. Finlayson et al. 2014*). BIK1 also plays distinct roles in resistance to necrotrophic and biotrophic pathogens in Arabidopsis (Veronese et al. 2006).

One of the most intriguing S-gene is downy mildew resistance 6 (*DMR6*), encoding for a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase, which has a salicylic acid (SA) 5-hydroxylase activity and thus reduces the active SA pool (*Zhang, Zhao et al. 2017*). Inactivation of DMR6 results in increased SA level (*Zeilmaker, Ludwig et al. 2015, Thomazella, Seong et al. 2021*). Tomato *DMR6-1* mutants (characterized by high accumulation of SA) showed enhanced resistance against evolutionarily distinct classes of pathogens: bacteria, oomycetes, and fungi (*Thomazella, Seong et al. 2021*). Besides, the mutation of the *Arabidopsis* homoserine kinase gene *DMR1* confers enhanced resistance to *Fusarium graminearum* and *F. culmorum*, which cause *Fusarium* ear blight disease in small grain cereals (*Brewer, Hawkins et al. 2014*). The study showed that common Arabidopsis DMR1-mediated susceptibility mechanisms occur in both vegetative and reproductive plant tissues during infection by both obligate biotrophic oomycete and hemi-biotrophic fungal pathogens. A highly efficient CRISPR/Cas9-mediated gene editing system was established for targeted mutagenesis in sweet basil (*Ocimum basilicum*) (*Navet and Tian 2020*). In Arabidopsis, the resistance to the *Cauliflower*

mosaic virus (CaMV) is regulated by SA and jasmonic acid (JA)/ethylene (ET) signalling pathways. Mutations in these pathways, such as *cpr1-1* (constitutive expressor of pathogenesis-related genes) and *cpr5-2*, can result in constitutive activation of SA-dependent signalling and increased resistance to systemic infection with CaMV (*Love, Laval et al. 2007*).

In addition, the *cpr* (constitutive expressor of PR genes) mutants, including *cpr5*, exhibit both enhanced disease susceptibility (*EDS1*)-dependent and independent components of plant disease resistance (*Clarke, Aarts et al. 2001*). Additionally, mutations in the antiviral RNAi defense pathway can affect the brome mosaic virus (BMV) RNA recombinant profiles (*Dzianott, Sztuba-Solińska et al. 2012*). *SR1* (signal responsive 1) is a calmodulin-binding transcription factor, it modulates plant defense. A gain-of-function mutation in *SR1* using CRISPR in Arabidopsis enhances disease resistance to powdery mildew and regulates ET-induced senescence by directly regulating *NDR1* (non-race-specific disease resistance 1) and *EIN3* (ethylene insensitive 3) (*Nie, Zhao et al. 2012*). Similarly, in tobacco, *SR1* mutants (activation-tagged EPC-resistant, *ATER1*, to *ATER7*) generated via T-DNA activation tagging are less susceptible to tobacco mosaic virus (TMV) due to reduced microtubule dynamics (*Ouko, Sambade et al. 2010*).

1.5 CRISPR-Cas9

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system is a powerful and

multiplexable genome editing tool that allows researchers to precisely manipulate specific genomic elements for editing cells, tissues and whole organisms, with widespread uses in experimental and applied systems (*Jinek, Chylinski et al. 2012, Barrangou and Doudna 2016*). CRISPR is a prokaryotic adaptive immune system that offers DNA-encoded, RNA-mediated, and sequence-specific virus defense. Bacteria and archaea incorporate short fragments of foreign nucleic acid into the host chromosome at one end of the repetitive element (*Barrangou, Fremaux et al. 2007, Andersson and Banfield 2008, Jinek, Chylinski et al. 2012, Wiedenheft, Sternberg et al. 2012, Barrangou and Doudna 2016*).

The CRISPR–Cas systems have been classified into two Classes (Class 1 and Class 2) and six types (Type I–VI) based on the different arrangements of *Cas* genes and the subunits of effector complexes (*Makarova, Wolf et al. 2015, Koonin, Makarova et al. 2017, Hille, Richter et al. 2018, Yan, Hunnewell et al. 2019, Chen, Liu et al. 2020*), Class 1 CRISPR-Cas systems (types I, III, and IV) employ multi-Cas protein complexes for interference, whereas in class 2 systems (types II, V, and VI), interference is accomplished by a single effector protein: type II (Cas9), type V (Cas12), and type VI (Cas13) (*Makarova, Haft et al. 2011, Makarova, Wolf et al. 2015, Chen, Liu et al. 2020*). The type I and III systems, from class 1, share some overarching features: Pre-crRNAs are processed by specialized Cas endonucleases, and once complete, each crRNA assembles into a large multi-Cas protein complex capable of

recognizing and cleaving nucleic acids complementary to the crRNA (*Hille, Richter et al. 2018*).

Type II systems, however, process pre-crRNAs through a different mechanism in which a trans-activating crRNA (tracrRNA) complementary to the pre-crRNA repeat sequences triggers processing by the double-stranded (ds) RNA-specific ribonuclease RNase III in the presence of the Cas9 protein (*Deltcheva, Chylinski et al. 2011, Gottesman 2011, Jinek, Chylinski et al. 2012*). The Cas9 endonuclease family can be programmed to cleave specific DNA sites using single RNA molecules to generate dsDNA breaks for genome targeting and editing (*Jinek, Chylinski et al. 2012*). Therefore the biotechnological development of molecular components of type II CRISPR-Cas system, especially CRISPR-Cas9, has been exploited into a powerful new generation of genome editing and engineering tools (*Mali, Esvelt et al. 2013*).

The effector modules of Class 2 are each comprised of a single large, multidomain protein, resulting in a much more straightforward and uniform organization of the corresponding CRISPR-Cas loci than those of Class 1 (*Koonin, Makarova et al. 2017*). In the type II effector system, tracrRNAs first hybridize to repetitive regions of the pre-crRNA (*Deltcheva, Chylinski et al. 2011*). Second, endogenous RNase III cleaves the hybridized crRNA-tracrRNAs, and a second event eliminates the 5′ end of each spacer, resulting in mature crRNAs that are still linked to the tracrRNA and Cas9 (*Deltcheva, Chylinski et al. 2011*). Three Cas proteins

(*Deltcheva, Chylinski et al. 2011*), Cas6e (Cse3 or CasE) (*Brouns, Jore et al. 2008*), Cas6 (*Carte, Wang et al. 2008*) and Csy4 (*Haurwitz, Jinek et al. 2010*), have also been identified as endoribonucleases that cleave within the repeat sequences of pre-crRNA to generate the mature crRNAs. Third, each mature complex locates and cuts both strands of a specific dsDNA sequence (*Mali, Esvelt et al. 2013*). Target recognition and subsequent cleavage by the crRNA-tracrRNA-Cas9 requires both complementary sequence between the spacer and the target 'protospacer' sequence as well as the presence of an appropriate protospacer-adjacent motif (PAM) sequence at the 3′ end of the protospacer sequence (*Gasiunas, Barrangou et al. 2012*). RNA-guided DNA recognition and cleavage strictly require the presence of a protospacer adjacent motif (PAM) in the target DNA (*Jinek, Chylinski et al. 2012, Anders, Niewoehner et al. 2014*), composed of a nonspecific Cas9 nuclease and a set of programmable sequence-specific CRISPR RNA (crRNA) (*Brouns, Jore et al. 2008, Deltcheva, Chylinski et al. 2011*), which can guide Cas9 to cleave DNA and create double-strand breaks at target locations (*Gasiunas, Barrangou et al. 2012*). Subsequent cellular DNA repair process leads to desired insertions, deletions or substitutions at target sites (*Zhang, Wen et al. 2014*).

The application of CRISPR technologies enables the RNA-guided Cas9 endonuclease to first recognize and bind with PAM (*Anders, Niewoehner et al. 2014*), and then Cas9 interrogates the flanking DNA sequences for base-pairing complementarity to its guide RNA, looking for

complementarity between their first 12 base pairs (*Sternberg, Haurwitz et al. 2012, Anderson, Haupt et al. 2015*). If they match, Cas9's precise cleavage of each DNA strand results in a blunt double-strand DNA (dsDNA) break (DSB) three base pairs upstream of the protospacer sequence's 3′ edge, measured from the PAM (*Gasiunas, Barrangou et al. 2012, Barrangou and Doudna 2016*). The programmable DNA cleavage of CRISPR-Cas9 provides for efficient, site-specific genome engineering in single cells and whole organisms. Controlling transcription, altering epigenomes, running genome-wide screens, and visualizing chromosomes are just a few medical study applications that have made use of CRISPR-enabled genome editing (*Barrangou and Doudna 2016*). For obtaining desired mutagenesis, the CRISPR-Cas9 genome editing technology has recently emerged as a revolutionary tool and has been applied in tomato since 2014 (*Brooks, Nekrasov et al. 2014*), becoming the primary genome editing tool applied in this species to characterize gene function in precision plant breeding (*Wang, Zhang et al. 2019*). Recently, the finding of the CRISPR/Cas9 genetic scissors, which have revolutionized genome editing, by Emmanuelle Charpentier of the Max Planck Unit for the Science of Pathogens and Jennifer Doudna of the University of California, Berkeley, earned them the 2020 Nobel Prize in Chemistry.

1.6 Whole genome sequencing

High-throughput sequencing and computational tools have opened up a new era of genomics. The application of such technologies will make it

easier to undertake comparative and functional analyses, and perform in silico breeding to improve the models for genome selection, direct the choice of parents in crosses, or select a novel set of CRISPR-Cas constructs for genome engineering (*Furbank, Jimenez-Berni et al. 2019*). The whole genome sequencing technique exploits the presence of conserved resistance gene homologues in diverse plant genomes that can be isolated by using polymerase chain reaction (PCR) or identified by more modern sequencing techniques such as NGS (next generation sequencing) (*Ercolano, Sanseverino et al. 2012*).

NGS technologies, as opposed to the traditional Sanger capillary electrophoresis sequencing technique (*Maxam and Gilbert 1977, Sanger, Nicklen et al. 1977*), which is considered a first-generation sequencing technology, provide greater throughput data at a lower cost and allow population-scale genome research (*van Dijk, Auger et al. 2014, Park and Kim 2016*). The first most complete collection of an individual's genetic variation was provided by whole genome sequencing (WGS) (*Ng and Kirkness 2010*). The development of NGS instantly transformed genomics study by bringing the sequencing of entire genomes within the reach of many small laboratories. Besides the well-known human genetic variation study, the 1000 Genomes Project (*Consortium 2010*), WGS is becoming more popular in translational study fields such as clinical diagnostics, and agricultural genomics, like barley and wheat (*Poland, Brown et al. 2012*), rice (*Project 2005*), and grape (*Velasco, Zharkikh et al. 2007*).

Using these promising technologies, a sequencing-based strategy could lead to the identification of thousands of putative gene sequences in a diverse range of organisms. Tomato is one of the most studied model plants for investigating defense response mechanisms. The International SOL consortium recently released the tomato genome sequence utilizing a Whole Genome Shotgun method, which included 350,000 BAC and fosmid end-sequence pairs. The SOL Genomics Network, SGN (http://solgenomics.net/) hosts the draft versions. The tomato reference genome is available and several tomato genotypes have begun to be sequenced:

SOL100 initiative; http://solgenomics.net/organism/sol100/view).

A similar approach (ftp://ftp.solgenomics.net/genomes/tomato_360) based on the genome sequences of 360 accessions, was used in my study (*Lin, Zhu et al. 2014*).

However, the unprecedented level of sensitivity and the large amount of available data produced by NGS platforms provide clear advantages as well as new challenges and issues. Discovering the methods for arranging resistance loci will be critical for developing innovative or diverse pathogen identification capacities in order to face new disease problems. With the advent of second-generation sequencing, enormous amounts of genomic sequence data may now be produced at a reasonable cost. This technology will make comparative genomics and gene finding much easier (*Ercolano, Sanseverino et al. 2012*).

1.7 SNPs

There are several classifications for NGS applications. Researchers can use DNA-sequencing, RNA-sequencing, and epigenome sequencing to quantify genetic variation in an organism with an existing reference genome. In the instance of DNA sequencing, NGS technologies enable whole genome, whole exome (for eukaryotes), and targeted sequencing. Researchers can see genetic variation such as structural variations, copy number variations, and other variations, including single nucleotide polymorphisms (SNPs), by comparing sequencing results to reference genomes using different software programs (*Park and Kim 2016*). SNPs are the most common genetic variations, as well as significant markers with virtually infinite numbers due to single nucleotide variations among individuals (*Kim and Misra 2007*), which are rapidly becoming the marker system of choice in many plants (*Ganal, Altmann et al. 2009*). Every SNP in single copy DNA is a potentially useful marker.

SNP are infinitely frequent variations of individual nucleotides, and have gained much interest in the scientific and breeding community (*Rafalski 2002*). In tomato, direct genome sequencing of several SNP or indel sites in Micro-Tom and *L. esculentum* E6203 revealed that more than 69% of the candidate sites were genuinely polymorphic, indicating that they could be used to create DNA markers (*Yamamoto, Tsugane et al. 2005*). The

knowledge of existing alleles not only provides a resource for tomato genetics but could aid genomic-assisted breeding programs as well as tailored gene editing approaches for resistance to biotic stresses, in particular to guide for a proportionate risk assessment step.

1.8 History of safe use (HoSU)

In the last decade, EFSA (European Food Safety Authority), at the request of the European Commission, has released scientific opinions (*Organisms, Mullins et al. 2022*a) on plants obtained through new genomic techniques (NGTs; i.e.: targeted mutagenesis, cisgenesis and intragenesis) and elaborated criteria to be considered for the risk assessment of plants produced by NGTs. In particular, EFSA has proposed six main criteria to assist the risk assessment of these plants (*Organisms, Mullins et al. 2022* b). History of safe use (HoSU) is one of the six criteria and it is related to the function and structure associated with the new allele. In particular, when HoSU and/or familiarity can be demonstrated, for a history of use as food and feed, and/or familiarity with the environment, and/or presence in a consumed variety, the donor plant and/or gene/allele and the associated trait can be subjected to a reduced risk assessment activity (*Organisms, Mullins et al. 2022* a). In other words, the risk assessment will consider both the probability for such an allele to be obtained by conventional breeding or that the allele is already in place in the breeders' gene pool. For these reasons, a genomic survey on the genetic diversity already present in a germplasm group can assist this step.

Chapter 2 - CRISPR/Cas9-based knock-out of the *PMR4* gene reduces susceptibility to late blight in two tomato cultivars

2.1 Abstract

Phytophthora infestans, the causal agent of late blight (LB) in tomato (*Solanum lycopersicum* L.), is a devastating disease and a serious concern for plant productivity. The presence of susceptibility (S) genes in plants facilitates pathogen proliferation; thus, disabling these genes may help provide a broad-spectrum and durable type of tolerance/resistance. Previous studies on *Arabidopsis* and tomato have highlighted that knockout mutants of the *PMR4* susceptibility gene are tolerant to powdery mildew. Moreover, *PMR4* knock-down in potato has been shown to confer tolerance to LB. To verify the same effect in tomato in the present study, a CRISPR-Cas9 vector containing four single guide RNAs (sgRNAs; sgRNA1, sgRNA6, sgRNA7, and sgRNA8), targeting as many *SlPMR4* regions, was introduced via *Agrobacterium tumefaciens*-mediated transformation into two widely grown Italian tomato cultivars: 'San Marzano' (SM) and 'Oxheart' (OX). Thirty-five plants (26 SM and 9 OX) were selected and screened to identify the CRISPR/Cas9-induced mutations. The different sgRNAs caused mutation frequencies ranging from 22.1 to 100% and alternatively precise insertions (sgRNA6) or deletions (sgRNA7, sgRNA1, and sgRNA8). Notably, sgRNA7 induced in seven SM genotypes a -7 bp deletion in the homozygous status, whereas sgRNA8 led to the production of 15 SM genotypes with a bi-allelic

mutation (-7 bp and -2 bp). Selected edited lines were inoculated with *P. infestans*, and four of them, fully knocked out at the *PMR4* locus, showed reduced disease symptoms (reduction in susceptibility from 55 to 80%) compared to control plants. The four SM lines were sequenced using Illumina whole-genome sequencing for deeper characterization of on-/off-target effects.

2.2 Materials and methods

2.2.1 Plant material and transformation vector

Seeds of SM and OX cultivars used in this study were provided by Agrion (www.agrion.it) and maintained in the Germplasm Bank of the Department of Agricultural, Forest and Food Science (University of Torino, Italy). The vector used for plant transformation was previously reported by Santillán Martínez et al (*2020*). It carries the *NptII* resistance gene, *Cas9* gene, and four sgRNA sequences (sgRNA1: GTTAAAGCAGTCCCATACTCG, sgRNA6: GTACTGCCCCACACTCTGCG, sgRNA7: GCCAAGGTT-GCCAGTGGCAA, and sgRNA8: GGATATCAGAGAAGGATCAG), designed to target several regions of *SlPMR4* (Solyc07g053980.3.1, ITAG4.1). sgRNA6 targeted the FKS1dom1 domain, while the other three sgRNAs targeted the glucan synthase domain. The four sgRNAs were used to increase the editing efficacy and promote the emergence of deletions between sgRNAs. The transformation vector was cloned into *Escherichia coli* strain DH5α and then into *Agrobacterium tumefaciens* strain LBA4404. The transformed *A. tumefaciens* was conserved as a stock at -80°C and subsequently used for plant transformation, as described below.

2.2.2 Plant transformation, regeneration, and acclimation to soil

Tomato seeds of both SM and OX cultivars were sterilized with 75% EtOH for 30 s and 1% sodium hypochlorite for 20 min. Sterilized seeds were washed in sterile water for 5 min and then sown on a germination medium

(2.2 g/l MS basal salts, 10 g/l sucrose, and 8 g/l Daishin agar; pH 5.8). Seeds were incubated for approximately 3 days at 4°C and then transferred at 24°C for approximately 10 days. The expanded cotyledons were used for transformation with *A. tumefaciens*. A single colony of *A. tumefaciens* was streaked in 2 ml Luria–Bertani (LB) medium with antibiotics (50 mg/l rifampicin and 50 mg/l kanamycin) and grown at 28°C for two days, under continuous shaking. The culture was then refreshed in 10 ml LB with the same antibiotics and shaken overnight. The following day, the culture was centrifuged for 15 min at 2,000 g, and the pellet was resuspended in liquid induction medium containing acetosyringone (200 mM). The optical density (OD) of the culture was measured at 600 nm (OD600) and adjusted to a final OD of approximately 0.125. After 1 h, the culture was used for transformation. With occasional swirling, the explants were incubated in culture for 15 min and then incubated at 25°C in the dark for 48 h. Briefly, cotyledons were cut into four pieces and placed on top of the induction medium (4.3 g/l MS basal salts, 108.73 mg/l vitamins Nitsch, 30 g/l sucrose, 8 g/l micro agar, 1.5 mg/l zeatin riboside, 0.2 mg/l IAA, and 1 ml/l acetosyringone; pH 5.8), with two pieces of filter paper soaked with liquid induction medium (4.3 g/l MS basal salts, 0.4 mg/l thiamine, 100 mg/l Myo-inositol, and 30 g/l sucrose; pH 5.8) supplemented with acetosyringone (200 µM). The explants were then placed in an incubator at 25°C. Control groups were explants from the wild-type (WT) of each variety subjected to the same treatments using a mock solution (liquid induction medium without *A. tumefaciens*). After two

days of incubation in the dark, the explants were transferred to a solid induction medium with timentin (300 mg/l) and kanamycin (100 mg/l) and incubated at 25°C with a 16/8-h light/dark cycle. The medium was renewed every two weeks until shoot proliferation. Two weeks later, the regeneration process was visible, and calli with regenerated shoots were transferred to a shooting medium (3% sucrose, MS + vitamins Nitsch, pH 5.8) supplemented with zeatin riboside (1.5 mg/l), IAA (0.2 mg/l), timentin (300 mg/l), and kanamycin (100 mg/l). Explants from the control group were transferred to the same medium without antibiotics. Once shoots reached 1–2 cm in length, they were transferred to a rooting medium (3% sucrose, MS + vitamins B5, pH 5.8) with or without kanamycin (100 mg/l) and maintained under growth chamber conditions (*McCormick, Niedermeyer et al. 1986, Santillán Martínez, Bracuto et al. 2020*).

2.2.3 Identification of transformed plants and detection of the *SlPMR4* editing

Genomic DNA was isolated from the leaves of *in vitro* regenerated plants using the Plant DNA Kit (Omega Bio-tek, USA), analyzed using 0.8% agarose gel electrophoresis, quantified on a Qubit fluorometer (Thermo Fisher, USA), and then subjected to PCR screening aimed at amplifying the *Cas9* gene as proof of transgene integration. A positive control (Cas9) was used to amplify a vector containing the *Cas9* gene, whereas a negative control (CTRL) was used on a tomato plant generated *in vitro* without transformation. PCR was conducted on 5 ng of genomic DNA

using the KAPA HiFi HotStart ReadyMix (Kapa Biosystems, USA), according to the manufacturer's instructions, and Cas9 primers listed in **Suppl. Table 1**. Amplification of the *Cas9* gene was performed using a real-time PCR (RT-qPCR) assay to quantify the number of T-DNA integration events. Reactions were carried out on the same genomic DNA in triplicate, using the Power SYBR® Green Master Mix (Thermo Fisher) and the StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The following PCR protocol was used: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Data were quantified using the $2^{-\Delta\Delta Ct}$ method based on the Ct values of *SlActin* (Solyc11g005330.1, ITAG_eugene; primers listed in **Suppl. Table 1**) as the housekeeping gene. The number of *Cas9* integrations was expressed as the relative DNA abundance with respect to single-gene amplification (**Suppl. Table 2**).

To detect the deletions between the sgRNAs, *Cas9* positive transformants were analyzed by PCR amplification (as reported above) using different primer combinations (**Suppl. Table 1**), flanking the four predicted target gene regions in the *SlPMR4* gene, and compared with control amplicons to detect deletions. Sanger sequencing was performed (BMR Genomics Service, Italy) on PCR-amplified gene fragments, as described above, using the primers listed in **Suppl. Table 1**. Amplicons of transformants smaller than those detected in control plants, suggesting the occurrence of large deletions between sgRNA targets, were cloned into pGEM®-T Easy Vector Systems (Promega, USA) according to the manufacturer's

instructions, and eight colonies of each transformant were picked and subjected to Sanger sequencing.

Transformants without deletions were PCR amplified using other primers (**Suppl. Table 1**) and then subjected to Sanger sequencing and analyzed using Tracking of Indels by DEcomposition (TIDE, https://tide.nki.nl) assay (*Brinkman, Kousholt et al. 2017*) to quantify the editing efficacy and identify the predominant types of insertions and deletions (indels). TIDE calculates a goodness of fit value (R^2) as a measure of the reliability of the estimated alleles and the overall efficiency of each TIDE assay as the estimated total fraction of DNA with mutations around the break site.

2.2.4 Detached Leaf Assay with *Phytophthora infestans*

P. infestans (Westerdijk Fungal Biodiversity Institute strain CBS 120920) was grown on rye sucrose agar medium (*Caten and Jinks 1968*) in the dark at 15°C. The sporangia were harvested from a 10–14-day old cultured plate by flooding the plate with 5 ml ice-cold water (4°C) and mixing it properly with a spreader. The plate was maintained at 4°C for 2–4 h to release zoospores. Zoospores were harvested by filtering the liquid from each plate through two layers of cheesecloth. Motile zoospores were counted using a hemocytometer under a microscope, and the concentration was adjusted to 2.5×10^4 spores/ml (*Karki and Halterman 2021*).

Six healthy, fully grown leaves from each soil-acclimated line (SM and OX) were used in a detached leaf assay (DLA) according to the procedure

described by Foolad (*Foolad, Sullenberger et al. 2015*). The six leaves of each mutant were randomly placed bottom side up into plastic trays (six replicates) containing water agar (20 g/l). Each plastic tray was divided into 18 areas, and two groups of mutant leaves and one group of control leaves were randomly placed in each area of the tray. All leaves were infected with the *P. infestans* isolate by dropping a suspension of zoospores at a concentration of 2.5×10^4 spores/ml (12 µl/leaf). After infection, the trays were covered with lids, sealed with Parafilm, and placed in a growth chamber at 20°C in the dark with a relative humidity of 60%. The trays were examined on a daily basis. Eight days post-inoculation (dpi), images were captured and analyzed using ImageJ software (version 1.52a; LOCI, University of Wisconsin) for the percentage of leaf area damaged (LAD%). A scale of 0–5 was used to score LAD%; a score of 0 indicated the absence of any foliar infection (LAD = 0%), and a score of 5 indicated complete destruction due to LB infection (LAD = 100%; *Foolad, Sullenberger et al. 2014, Foolad, Sullenberger et al. 2015*). The control leaves were scored and compared to the mutants present in the same tray. The ratio of mutant/control scores was calculated and used to select less-susceptible plants. Statistical differences between mutants/controls were analyzed using a two-tailed t-test (* $p < 0.05$). Multiple comparisons were performed using a two-tailed Student's t-test with post-hoc Bonferroni correction.

2.2.5 Whole Genome Sequencing

One microgram of DNA was used to construct short-insert (length 350 bp) genomic libraries (Novogene, Hong Kong), which were sequenced using an Illumina sequencer (Illumina Inc., San Diego, CA, USA) with paired-end chemistry (2×150 bp). Raw reads were cleaned with Scythe (v0.991, https://github.com/vsbuffalo/scythe) to remove contaminant residual adapters and Sickle (v1.33, https://github.com/najoshi/sickle), which allows the removal of reads with poor quality ends (Q < 30).

A *de novo* genome assembly was performed using the MegaHit assembler (v1.2.9, https://github.com/voutcn/megahit), utilizing specific assembly parameters (k-min = 27, k-max = 141, k-step = 10, cleaning-rounds = 1, and disconnect-ratio = 0). Metrics for assessing the quality of a genome assembly (e.g., N50, contig/scaffold number/size/length, and genome length) were obtained using the Perl script Assemblathon_stats.pl (https://github.com/ucdavis-bioinformatics/assemblathon2-analysis). BLAST analysis was conducted on the assembled genomic sequences (mutants and WTs) to identify any possible insertions using the T-DNA sequence (*Santillán Martínez, Bracuto et al. 2020*) as a query. As preferential choice criteria, the e-value (e-value < $1 \times e^{-10}$), percentage similarity, and query coverage were considered. T-DNA coverage analysis using bedtools (https://bedtools.readthedocs.io) was conducted to infer the number of *Cas9* integrations within each edited plant by aligning cleaned reads on the T-DNA sequence available as a reference. The *Cas9* copy number

was inferred by comparing with the coverage recorded for a single-copy tomato gene (Solyc10g009390.3.1, CYP702).

2.2.6 On- and off-target analyses and SNP statistics

In edited plants, the emergence of genomic variants and allele frequencies in *SlPMR4* locus was highlighted using CRISPResso2 (http://crispresso2.pinellolab.org) and SNP/indel analysis. Clean reads derived from the edited plants were mapped to the tomato reference genome (SL4.0, https://solgenomics.net) using the Burrows-Wheeler Aligner (v0.7.17, https://sourceforge.net/projects/bio-bwa/files) program and 'mem' command with the default parameters. BAM files were processed and used for SNP calling using Samtools (v1.9-166-g74718c2) mpileup with default parameters, except for minimum mapping quality (Q = 20) and filtering out multimapping events (-q > 1). A variant call format (vcf) file was produced. The vcf file was inspected in the 100 bp window surrounding each sgRNA to highlight SNP/indels through bedtools intersect (https://bedtools.readthedocs.io).

WT and mutated PMR4 proteins were reconstructed using the 'getorf' tool (http://emboss.sourceforge.net) and proteins were multi-aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). Homology models for the WT and mutated PMR4 proteins were built using the Swiss-Model tool (https://swissmodel.expasy.org), utilising the AlphaFold-predicted crystal structure of Arabidopsis thaliana PMR4 (PDB ID AF-

Q9ZT82-F1), as a template. Pairwise juxtaposition of models were carried out with UCSF Chimera (v1.16, https://cgl.ucsf.edu/chimera).

For off-target analysis, the CasOT script (https://github.com/audy/mirror-casot.pl) was used to identify any off-target regions in the tomato genome. All designed sgRNAs were considered as bait in an sgRNA mode, with default PAM type (NGG = A) and specific numbers of permitted mismatches in the seed (2), non-seed (2) regions allowed. All the candidate off-target genomic region coordinates were intersected with the vcf file through bedtools for edited as well as for the control plants to filter-out monomorphic regions among the latter. The results were analyzed using custom bash scripts.

2.3 Results

2.3.1 CRISPR/Cas9-based mutagenesis of *SlPMR4*

A CRISPR-Cas9 vector containing the *NPTII* resistance gene and four sgRNAs (**Figure 1A, 1B**) targeting many regions of the *SlPMR4* gene was introduced via *A. tumefaciens*-mediated transformation into two commercially available tomato cultivars, SM and OX. Both the cultivars were susceptible to *P. infestans*. A total of 132 SM and 136 OX explants were transformed. Moreover, 87 tomato regenerants (T_0 generation; 70 in SM and 17 in OX) were obtained. The observed regeneration efficiencies were 65.9% and 12.5% for SM and OX, respectively. From this initial screening, the more robust T_0 plantlets (26 SM and 9 OX) were recovered from in vitro cultivation, and all of them were positive for *Cas9* PCR amplification (**Figure 1C, 1D**).

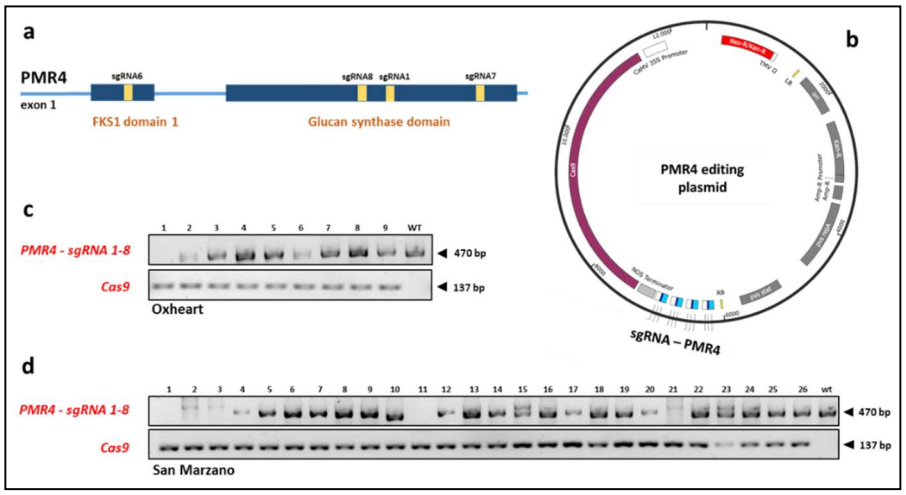


Figure 1. a) Gene structure of the tomato *PMR4* gene with domains and sgRNAs. b) Vector containing features for *PMR4* editing through four sgRNAs. Gel

electrophoresis of the *Cas9* PCR products in c) Oxheart and d) San Marzano tomato mutants.

Sanger sequencing revealed different editing outcomes for each of the four sgRNAs (**Table 1**, **Suppl. Table 3**). sgRNA7 was the most efficient, as seven SM (4, 5, 6, 13, 17, 19, and 22) and five OX (2, 3, 4, 9, and 11) genotypes were characterized by more than 99% editing effects, estimated using TIDE. In all cases, the *PMR4* gene was fully knocked out due to the introduction of a deletion (-7 bp) in the homozygous state. sgRNA8 also led to the production of 15 SM (4, 6, 7, 8, 9, 12, 13, 14, 17, 18, 19, 22, 24, 25, 26) genotypes with a bi-allelic mutation (-2 bp, -7 bp) and one plant with a 1 bp insertion present in homozygous status (16). sgRNA8 generated three OX genotypes, with a 2 bp deletion in the homozygous state (2, 4, 11), one with a 1 bp insertion in the homozygous state (3), and one (9) with a bi-allelic mutation (-2 bp, +1 bp). The outcome of editing at the sgRNA7 and 8 was the introduction of premature stop codons, which produced shorter truncated proteins. sgRNA1 and 6 showed less efficient results (**Suppl. Table 3**), with the persistence of reference alleles at medium-high frequency and the emergence of few indels in the heterozygous or chimeric state (**Suppl. Table 3**).

Overall, the most frequent indels were small deletions (from 1 to 10 bp, with a predominance of -7 bp and -2 bp) and small insertions (from 1 to 4 bp, with a predominance of +1 bp). For one target (sgRNA7), a 7-bp deletion was observed as the predominant mutation (**Suppl. Table 3**) for both the cultivars. Following Sanger sequencing, we also identified two

SM mutants containing large deletions, one of which (SM2) contained a 3200-bp deletion between sgRNA6 and 7 (**Suppl. Figure 1**), and the other one (SM5) contained a 146-bp deletion at sgRNA1, although not in the homozygous state (data not shown).

Table 1. Mutational status of the edited genotypes, as revealed by the TIDE analysis of the Sanger sequences. Four regions surrounding the adopted sgRNA were analyzed.

Mutant	Mutational status (%) - TIDE			
	sgRNA6	sgRNA8	sgRNA1	sgRNA7
SM1	1.2	-	-	-
SM2	2.5	-	-	-
SM3	8.7	-	-	-
SM4	38.5	89.4	-	98
SM5	24.2	-	-	97.6
SM6	40.2	95.1	4.1	98.9
SM7	13.1	94.5	58.1	-
SM8	34.6	95.5	33.6	-
SM9	-	95.4	35.3	-
SM12	-	93.3	9.1	-
SM13	93.4	90.3	48.1	99
SM14	-	92.8	3.4	-
SM16	-	97.8	7.6	-
SM17	64.6	94.2	76.2	97.9
SM18	-	92.9	80.1	-
SM19	1.5	93.1	31.3	99.3
SM20	1.8	1.1	3.7	2.2
SM22	69.3	94.2	9.7	98.3
SM24	-	93.6	34.2	-
SM25	-	94.2	5.2	-
SM26	-	93.9	4.4	-
OX1	55.3	-	-	-
OX2	17.7	98.6	6.6	99
OX3	26.8	98.2	56.7	99.3
OX4	14.2	99	9.9	99
OX9	13.5	95.9	4.7	99.3
OX11	48.6	94.4	32.5	99.3

2.3.2 Reduced susceptibility to Late Blight (LB)

A DLA was conducted as previously reported (*Foolad, Sullenberger et al. 2015*). The assay was performed in two independent experiments on 35 edited T_0 lines (26 SM and 9 OX; **Suppl. Table 3**). In both experiments, the plants with reduced susceptibility showed smaller chlorotic and necrotic foliar lesions than the control plants (**Figure 2**). In the first experiment, 12 SM and 2 OX mutants showed reduced disease symptoms (**Suppl. Table 4**), whereas in the second experiment, only 8 SM mutants showed a significant reduction in pathogen infection (**Suppl. Table 4**). Four SM mutants (6, 13, 17, and 19) showed the highest reduction in symptoms in two independent experiments (**Figure 2**, **Suppl. Table 4**). These lines showed good TIDE outcomes, which predicted the presence of truncated SlPMR4 proteins in the homozygous state (at the sgRNA7 level), as well as the presence of bi-allelic deleterious deletions (-2/-7 bp at the sgRNA8 level), generating even shorter truncated proteins (**Suppl. Figure 3**). For these reasons, those SM mutants were subjected to WGS for a deeper characterization of the on-/off-target regions. Two OX mutants (1, 4) showed good performance in terms of symptom reduction, but only in one of the independent experiments and thus were not selected for WGS. One T_1 line (SM17-1.2) did not highlight any chimerism (sgRNA7: -7/7; sgRNA1: -2/0; sgRNA8: -7/-2; sgRNA6: +1/0) and, like the T_0 plant, showed a reduced susceptibility (**Suppl. Figure 2**).

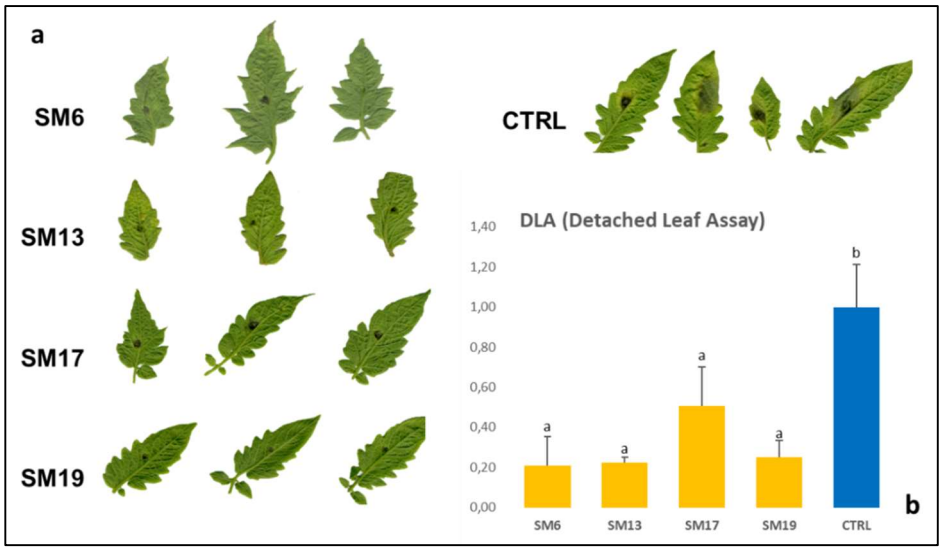


Figure 2. a) Detached-leaves assay with *Phytophthora infestans* performed on four *pmr4* San Marzano mutants (SM6, 13, 17, and 19) and a wild type plant as a control group at 8 days post-inoculation (dpi). b) In the histogram, normalized LAD% values are reported for each genotype. The y-axis shows the mean ratio of the score of the mutant/control group; bars represent standard deviation (sd). Statistical differences among mutant/control were analyzed with a two-tailed t test ($P < 0.05$). Multiple comparisons were performed using two-tailed Student's t test with post-hoc Bonferroni's correction.

2.3.3 Genomics of selected *pmr4* mutants

Four candidate edited lines of the cultivar SM (6, 13, 17, and 19) and one in vitro control plant were subjected to Illumina WGS. Two additional SM WT plants obtained from seedlings germinated in soil were also sequenced. Genome sequencing yielded 1,678 billion raw paired-end reads (252 Gb), with an average length of 150 bp (**Suppl. Table 5**). The latter was reduced to 1,577 billion (94%) after filtering and trimming high-quality reads. The sequence depth of coverage ranged from 38.1X (SM13) to 52X (SM6), being 46X on average (**Suppl. Table 5**). Sequence data

were deposited in the NCBI Short Read Archive with specific submission identifiers (PRJNA846963).

A *de novo* genome assembly of each mutant was produced (**Suppl. Table 2**), and blast analysis was used to scan the scaffolds for the presence of T-DNA integration. All four edited plants showed *Cas9* positive scaffolds, and a sequence coverage analysis was used to infer the number of *Cas9* integrations (**Suppl. Table 2**). This analysis highlighted two independent copies in the hemizygous state in each edited plant. The latter was confirmed by qPCR analysis (**Suppl. Table 2**). Within these four selected mutants, all candidate gene regions (sgRNA-7, 1, 8, and 6) within *SlPMR4* were scanned using Crispresso2 utilizing WGS data (**Suppl. Table 5**). In all cases, clear evidence of editing was observed (**Figure 3**, **Table 2**). Scanning of the *PMR4* in sgRNA7 region revealed a 100% editing effect and no reference alleles, confirming the TIDE analysis.

In general, the *PMR4* gene was knocked out due to the introduction of a deletion (-7 bp) present in the homozygous state (position 62.314.165-62.314.171 bp in chromosome 7). This mutation can result in a shorter protein lacking 431 amino acids due to the presence of a premature stop codon at position 1337 (instead of 1769 in the WT, **Suppl. Figure 3**), thereby affecting the general protein functionality. Scanning of *PMR4* in the sgRNA8 region revealed a 100% editing effect with bi-allelic mutations (a 2 bp deletion at position 62.315.066-62.315.067 in chromosome 7 and a 7 bp deletion at position 62.315.066-62.315.072 in chromosome 7) and

no reference alleles, confirming the TIDE analysis. Such mutations can result in shorter proteins lacking 753/751 amino acids, in the presence of premature stop codons at position 1015/1019 (**Suppl. Figure 3**), thereby affecting the general protein functionality. When both loci (sgRNA7 and sgRNA8) were affected, the resulting protein originated from a mutation in sgRNA8, which precedes sgRNA7 in the gene (**Suppl. Figure 3**). The analysis of *PMR4* at sgRNA1 and sgRNA6 regions showed less efficient editing effects, according to the TIDE analyses (**Table 2**), with some persistence of the reference alleles and a few indels in heterozygous or chimeric status (**Suppl. Table 3**).

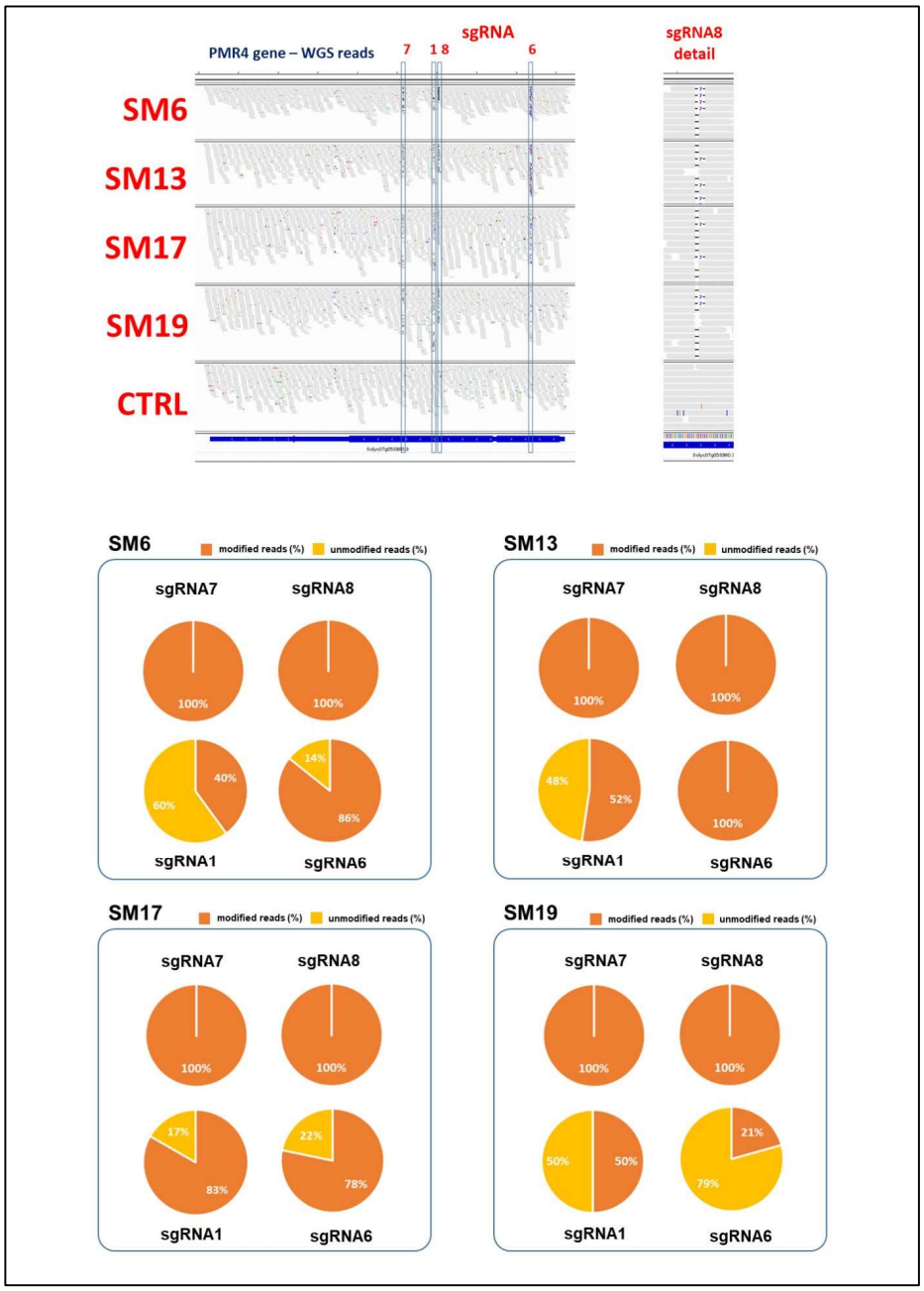


Figure 3. a) Sequence alignment view of the edited *PMR4* gene at the level of the four sgRNAs in the four mutants and the control plant. A focus on sgRNA8 is shown on the right-hand side. b) Mutational status (%) for each sgRNA region and in all the assayed genotypes as revealed by the Crispresso2 analysis.

Table 2. Editing effects and observed allelic forms (%) in each of the four sgRNA regions, in four selected genotypes and the San Marzano control. Data were retrieved through: a) Illumina sequencing analysis, analysed with Crispresso2 and b) TIDE analysis of Sanger sequences; the overall efficiency of each TIDE analysis is calculated as R^2.

Mutant		Alleles in sgRNA7 Region (%)												
		R^2	−18	−10	−8	−7	−6	−5	−3	−2	−1	1	ref	Allelic State
SM6	WGS	-	-	-	-	100.0	-	-	-	-	-	-	-	homozygous
	Tide	0.99	-	-	-	98.9	-	-	-	-	-	-	-	
SM13	WGS	-	-	-	-	100.0	-	-	-	-	-	-	-	homozygous
	Tide	0.98	-	-	-	98.5	-	-	-	-	-	-	-	
SM17	WGS	-	-	-	-	100.0	-	-	-	-	-	-	-	homozygous
	Tide	0.98	-	-	-	97.8	-	-	-	-	-	-	-	
SM19	WGS	-	-	-	-	100.0	-	-	-	-	-	-	-	homozygous
	Tide	0.99	-	-	-	97.2	-	-	-	-	-	-	-	
SM-CTRL	WGS	-	-	-	-	-	-	-	-	-	-	-	100.0	wild type
Mutant		Alleles in sgRNA1 Region (%)												
		R^2	−18	−10	−8	−7	−6	−5	−3	−2	−1	1	ref	allelic state
SM6	WGS	-	-	10.0	-	-	-	-	-	-	23.3		69.0	chimeric
	Tide	0.96	-	-	-	-	-	-	-	-	-	-	92.2	
SM13	WGS	-	-	-	-	-	52.4	-	-	-	-	-	47.6	heterozygous
	Tide	0.92	-	-	-	-	45.7	-	-	-	-	-	43.9	
SM17	WGS	-	-	-	-	-	-	-	-	62.5	8.3	8.3	20.8	chimeric
	Tide	0.94	-	-	-	-	-	-	-	54.7	15.0	3.2	18.3	
SM19	WGS	-	-	-	43.7	-	-	-	-	-	-	-	56.3	heterozygous
	Tide	0.91	-	-	27.6	-	-	-	-	-	-	-	60.0	
SM-CTRL	WGS	-	-	-	-	-	-	-	-	-	-	-	100.0	wild type
Mutant		Alleles in sgRNA8 Region (%)												
		R^2	−18	−10	−8	−7	−6	−5	−3	−2	−1	1	ref	allelic state
SM6	WGS	-	-	-	-	33.3	-	-	-	66.7	-	-	-	biallelic
	Tide	0.95	-	-	-	46.4	-	-	-	48.3	-	-	-	
SM13	WGS	-	-	-	-	66.7	-	-	-	33.3	-	-	-	biallelic
	Tide	0.9	-	-	-	43.4	-	-	-	45.8	-	-	-	
SM17	WGS	-	-	-	-	20.0	-	-	-	80.0	-	-	-	biallelic
	Tide	0.94	-	-	-	43.2	-	-	-	50.1	-	-	-	
SM19	WGS	-	-	-	-	50.0	-	-	-	50.0	-	-	-	biallelic
	Tide	0.93	-	-	-	45.1	-	-	-	47.7	-	-	-	
SM-CTRL	WGS	-	-	-	-	-	-	-	-	-	-	-	100.0	wild type
Mutant		Alleles in sgRNA6 Region (%)												
		R^2	−18	−10	−8	−7	−6	−5	−3	−2	−1	1	ref	allelic state
SM6	WGS	-	-	-	-	5.7	-	14.3	-	17.1	-	45.7	17.1	chimeric
	Tide	0.95	-	-	-	11.4	-	12.0	-	16.8	-	-	54.8	
SM13	WGS	-	25.0	-	-	-	-	3.1	21.9	-	3.1	47	-	chimeric
	Tide	0.93	27.5	-	-	-	-	12.0	8.1	-	-	38.8	-	
SM17	WGS	-	-	-	-	-	56.5	-	-	-	-	21.7	21.7	chimeric
	Tide	0.93	-	-	-	-	31.7	-	-	-	-	31.7	28.2	
SM19	WGS	-	-	-	-	-	-	-	-	-	-	17.2	82.8	heterozygous
	Tide	0.98	-	-	-	-	-	-	-	-	-	-	97.0	
SM-CTRL	WGS	-	-	-	-	-	-	-	-	-	-	-	100.0	wild type

2.3.4 Off-target events and SNP analysis

Based on the resequencing data, we evaluated the extent of off-target (OT) mutations caused by CRISPR/Cas9 editing in the four selected pmr4 mutants (SM6, SM13, SM17, and SM19). First, we generated a list of potential OTs (25 loci; **Suppl. Table 6**) for the four sgRNAs that were used to target *SlPMR4*. All 25 candidate OT regions showed sequence similarity with at least 2 bp mismatches with respect to sgRNAs (**Table 3**), of which seven fell in coding regions and 18 in non-coding regions (**Table 3**). For sgRNA6, the number of mismatches increased to five, because no other candidate OTs were observed. The analysis was conducted by mapping the Illumina reads of one control plant, two WT plants, and four mutants to the tomato Heinz 1706 reference genome. All 25 putative OT regions were fully covered by Illumina reads in the control, WT, and *pmr4* mutants (**Suppl. Table 6**), indicating the possibility that large deletions occurred in these plants. A side-by-side comparison of DNA alignments in the control, WTs, and mutants revealed that no SNPs/indels or large deletions were present in candidate OT regions. Indeed, some SNPs/indels were present in the surrounding regions, but they did not indicate any OT effect, being always: i) conserved nucleotides already in place in SM, but polymorphic with respect to the Heinz 1706 genome; and ii) outside of the 20 bp window related to the sgRNA-like sequence (putative OT region). In conclusion, we did not find any evidence of mutations in the potential OT regions within the genome of the selected SM *pmr4* mutants.

Table 3. Statistics on *PMR4* off-target regions analyzed in all the assayed genotypes and in the San Marzano unedited plants.

sgRNAs	off-target all	coding	Non-coding	Obs. SNP/indels
7	4	1	3	0
1	5	2	3	0
8	6	0	6	0
6	10	4	6	0
total	25	7	18	0

Polymorphisms in SM were searched and identified over seven genotypes, using the Heinz tomato genome as a reference: four selected *PMR4*-edited lines, one control, and two WT materials (**Table 4**). In total, 595,701 SNPs/indels were observed, with 153,977 cultivars specific for the SM genome with respect to the genome sequence of the Heinz variety. Genotype-specific SNP/indels were identified in all edited lines, as well as in the three unedited plants, to discriminate the emergence of spontaneous mutations (SNP/indels) from mutations induced by *in vitro* culture or genetic transformation/gene-editing processes. The average SNP number across edited (9.04 SNPs per Mb) and not edited plants (8.95 SNPs per Mb) was comparable, as was the average mutation rate (0.00113% for edited plants, 0.00110% for unedited plants), with no statistically significant differences among them (t-test, $p=0.78$, $\alpha=0.05$).

Table 4. SNP statistics for each Illumina sequenced genotype.

		SNP						
Genotype	**Plant type**	**Genot. specific**	**Homoz.**	**Heteroz.**	**in**	**(%)**	**per Mbp**	**per Mbp (avg)**
SM6	edited	8,141	3,318	4,823	240	0.0010%	9.6	8.95
SM13	edited	9,841	3,554	6,287	348	0.0013%	8.0	-
SM17	edited	8,589	3,491	5,098	286	0.0011%	9.1	-
SM19	edited	8,587	2,945	5,642	249	0.0011%	9.1	-
CTRL	*in vitro*	7,960	2,969	4,991	236	0.0010%	9.8	9.04
WT-1	from seed	8,784	2,925	5,859	269	0.0011%	8.9	-
WT-2	from seed	9,322	3,236	6,086	279	0.0012%	8.4	-

2.4 Discussion

We aimed at investigating whether full KO of an S-gene (*SlPMR4*) through CRISPR/Cas9 editing in two widely cultivated Italian tomato cultivars, SM and OX, may reduce susceptibility to LB, a devastating disease caused by *P. infestans*. We selected four SM *pmr4* mutants and, following their whole genome resequencing, assessed the overall editing efficiency, types of induced mutations, as well as the emergence of any unintended OT effects.

2.4.1 Reduced susceptibility to LB in tomato cultivars knocked-out in the *SlPMR4* gene

The S-gene *PMR4* was originally identified because of the powdery mildew resistance phenotype of a *pmr4* mutant in *Arabidopsis* (*Vogel and Somerville 2000*). *PMR4* appears to be the main biosynthetic enzyme that coordinates callose response to biotic, abiotic, and chemical stresses. Even if the callose response is widely recognized as an early response of host plants to microbial attack, callose may protect the fungus during pathogenesis (*Jacobs, Lipka et al. 2003, Nishimura, Stein et al. 2003*). Loss of function of PMR4 results in depletion of callose at fungal penetration sites (*Vogel and Somerville 2000, Jacobs, Lipka et al. 2003*) and resistance related to enhanced activation of the salicylic acid signal transduction pathway or constitutive expression of the pathogenesis-related protein 1 (PR-1) (*Nishimura, Stein et al. 2003, Flors, Ton et al. 2008*).

In previous studies, *PMR4* mutations have been shown to provide broad-spectrum resistance to powdery mildew (*Jacobs, Lipka et al. 2003, Flors, Ton et al. 2008, Santillán Martínez, Bracuto et al. 2020*). In this study, we generated loss-of-function mutants in the *SlPMR4* gene in two susceptible tomato cultivars, SM and OX, by applying CRISPR/Cas9 technology. The three main mutations (sgRNA7-indel-7 bp, sgRNA8-indel-7 bp, and sgRNA8-indel-2 bp) generated trunked copies of the *PMR4* protein, leading to the loss of a large part of the glucane synthase domain (amminoacidic range: 877-1677, **Suppl. Figure 3**) and likely depleting the *PMR4* callose deposition function. The degree of reduced susceptibility to LB was evaluated in the edited mutants using the DLA assay. Compared with the controls, 15 T_0 (14 SM and 1 OX) showed reduced susceptibility to *P. infestans* in one experiment, and six of them (SM4, 6, 13, 17, and 19 and OX4) showed a reduced susceptibility level in two independent experiments (**Suppl. Table 4**). One edited T_1 line (SM17-1.2) confirmed a reduction in LB susceptibility analogous to the one detected in T_0 plants (**Suppl. Figure 2**). Our results towards the LB confirmed the role of knocked-out *PMR4* in providing broad-spectrum protection against pathogens in two tomato cultivars. In our study, it was also proved that the KO of *PMR4* gene produced plants with normal growth and with the same habit as that of the WT plants. This was in agreement with the results of previous studies based on observations of *pmr4* mutants (*Vogel and Somerville 2000, Huibers, Loonen et al. 2013*).

2.4.2 Genome editing outcomes in tomato cultivars

Tomato is a model plant species, and its amenability to transformation methodologies via *A. tumefaciens*, biolistic, or direct protoplast DNA uptake makes it a suitable platform for the application of gene-editing technologies (*Pan, Ye et al. 2016, Hashimoto, Ueta et al. 2018, Jaganathan, Ramasamy et al. 2018*). Tomato is also a food crop, and the improvement of traits achieved by applying gene editing techniques to widely cultivated varieties has the potential for direct use in the field.

In our experiment, we applied CRISPR/Cas9 editing based on the use of 20 nt sgRNAs, which, with respect to longer or shorter sgRNAs, have been reported to be the most efficient in DNA cleavage efficiency. We applied four sgRNAs targeting as many regions of the *SlPMR4* gene, with the goal of increasing the frequency of random insertions or deletions by NHEJ editing (*Liu, Yang et al. 2022*). The most frequently induced mutations reported for CRISPR-based experiments are 1 bp and 3 bp deletions, followed by 1 bp insertions (*Pan, Ye et al. 2016*). Within the produced mutants, all candidate regions (sgRNA-7, 1, 8, and 6) within *SlPMR4* were scanned using TIDE and Crispresso2 utilizing WGS data (**Suppl. Table 5**). In all cases, clear evidence of editing was observed (**Suppl. Table 3**, **Figure 3**, and **Table 2**), and both TIDE and WGS approaches, showing a high concordance among each other (**Table 2**), highlighted that small indels at the target sites of sgRNAs were mainly 1 bp insertions (sgRNA6) and 2 bp or 7 bp deletions (sgRNA7, sgRNA1, and sgRNA8; **Suppl. Table 3**). However, as reported in previous studies

(*Santillán Martínez, Bracuto et al. 2020*), in some (rare) cases (for example SM2, **Suppl. Figure 1**), we also detected the induction of a large deletion (3,200 bp). Although it seems difficult to justify the presence of a common repair pattern (e.g., -7 bp), a recent characterization of indel patterns at multiple genomic locations revealed that individual targets show reproducible repair outcomes, with distinct preferences for the class (insertion or deletion) and size of indels (*van Overbeek, Capurso et al. 2016*). More recently, the role of genetic and epigenetic factors influencing CRISPR-Cas-mediated DNA editing has been clarified by performing large-scale genomic characterization of indel patterns over 1,000 sites in the human genome (*Chakrabarti, Henser-Brownhill et al. 2019*). Therefore, it is now clear that double-strand breaks (DSBs) can be repaired in both predictable and unpredictable manners and that the pattern relies on the target site. Positions -4 and -5 from the PAM seem to roughly predict the likely repair outcome (insertion or deletion). Based on these predictive criteria, we confirmed the tendency of sgRNA6 to introduce an insertion (5 nt preceding PAM=CTGCG-NGG), similar to sgRNA8 (5 nt preceding PAM=ATCAG-NGG) (**Suppl. Figure 4**). In contrast, sgRNA7 (5 nt preceding PAM=GGCAA-NGG) and sgRNA1 (5 nt preceding PAM=ACTGG-NGG) showed a tendency to introduce deletions. However, these trends cannot be easily explained if specific patterns of repair in plants are not postulated. Moreover, by analyzing the performance of the sgRNAs and the editing outcome in the two varieties in the study, sgRNA7 resulted in the introduction of a precise deletion and the same type of

deletion (-7 bp) in both SM and OX cultivars. These results suggest that by fine-tuning sgRNA at the design stage, it is possible to predict the outcome of gene editing in different plant genotypes. Although the analysis of thousands of repair patterns in plant-specific contexts is needed to provide more details of the CRISPR genome editing outcome and strengthen our prediction power.

Considerable variability in sgRNA efficiency has been detected, which does not appear to change with the expression system or *CAS9* delivery method (*Liu, Homma et al. 2016, Soyars, Peterson et al. 2018*), and it is often difficult to predict the specificity and stability of sgRNA sequences (*Moreno-Mateos, Vejnar et al. 2015*). In a previous study(*Santillán Martínez, Bracuto et al. 2020*), no mutations were found close to the sgRNA6 target site, suggesting that this sgRNA was not efficient in guiding CAS9 protein to induce DSBs. However, in our study, we found that the average efficiency of sgRNA6 was comparable to that of sgRNA1 and lower than those of sgRNA7 and sgRNA8, where no residual reference alleles were highlighted (**Figure 3**). sgRNA6 was also able to drive the emergence of DSBs in SM2 and generate a large deletion event (**Suppl. Figure 1**) of 3,200 bp between sgRNA6 and sgRNA7.

2.4.3 Off-target absence and emerging SNPs in *pmr4* mutants

One of the associated concerns in the application of CRISPR/Cas9 editing is that the endonuclease may act on non-selective and non-specific regions of genomic DNA, commonly known as OT sites (*Wada, Ueta et*

al. 2020). It is believed that the seed regions (8-12 nt most proximal to the PAM) in sgRNA govern the identification of targets, in which a high degree of homology can result in OT binding (*Semenova, Jore et al. 2011, Cong, Ran et al. 2013, Nishimasu, Ran et al. 2014*). Despite WGS being widely used in plant genomics, studies investigating the occurrence of OTs upon gene editing remain scarce (*Hahn and Nekrasov 2019, Manghwar, Li et al. 2020*), and the current studies have primarily highlighted the scarce presence of OT mutations resulting from the *CAS9* activity. In our study, no OT mutations were found (**Table 3**) in both the coding and non-coding regions in the four SM mutants investigated using Illumina sequencing. Our results confirm that CRISPR/Cas9 can be a highly precise genome editing tool in tomato, which is consistent with the results of previous reports (*Pan, Ye et al. 2016, Peterson, Bogomolov et al. 2016, Nekrasov, Wang et al. 2017, Maioli, Gianoglio et al. 2020*).

The ratio of spontaneous mutations is highly variable and unique to every organism (*Sung, Ackerman et al. 2016*). Base substitution mutations can often be explained as the result of two main processes: deamination of methylated cytosines and ultraviolet light-induced mutagenesis (*Ossowski, Schneeberger et al. 2010*). Somaclonal variation can further emerge in the conditions of *in vitro* cultivation, mainly as a consequence of hormone supplementation. In our study, the sequenced mutants (SM6, 13, 17, and 19) were obtained through genetic transformation starting from the WT 'SM' (cultivated *in vitro*). The four mutants showed similar genetic similarity (**Table 4**), with an average SNP mutation rate of

0.00113%, which was not statistically different from that of the WTs (0.00110%), indicating that the set of SNPs detected in the edited plants did not undergo a statistically significant increase due to editing side effects, as well as somaclonal variation, but arose from spontaneous mutations. Interestingly, the observed spontaneous mutation rate in control tomato (0.00110%) was 5.7-fold higher than that reported in rice (*Miyao, Nakagome et al. 2012*) and far higher than that reported in *Arabidopsis* (*Ossowski, Schneeberger et al. 2010*).

2.5 Supplementary materials

Suppl. Table 1. Primers used in PCR and RT-qPCR

Objective	Primer name	Region screened	Forward primer (5' - 3')	Reverse primer (5' - 3')	Amplicon size (bp)
Cas9 amplification	Cas9F;Cas9R	Cas9	CTATCCTCAGGCGGCAAGAG	AGTCATCCACGCGAATCTGG	137
Actin amplification	ActinF; ActinR	Actin	TCCGCGACATGAAGGAAAAGC	GCAACGGAACCTCTCAGCAC	146
Big deletion detecting	sg8F; sg7R	sgRNA8 + sgRNA1 + sgRNA7	GCGAATGCGTAGAGAAGGAA	CCCCACTAAGTGCCAGGTAA	1246
Big deletion detecting	sg6F; sg7R	sgRNA6 + sgRNA8 + sgRNA1 + sgRNA7	GCTTTTCTGAATCGGATCGTA	CCCCACTAAGTGCCAGGTAA	3564
TIDE	tide_sg6F; tide_sg6R	sgRNA6	GCTTTTCTGAATCGGATCGTA	ATTCCTGCATCAAGTAACGAC	440
TIDE	tide_sg8F; tide_sg1R	sgRNA8 + sgRNA1	AAATTTCTTGCAGCGAATGCGTAG	GGCCTTTAAACAACATACTCAC	470
TIDE	tide_sg7F; sg7R	sgRNA7	GTTCCGTGTCATCCCTTGCT	CCCCACTAAGTGCCAGGTAA	492

Suppl. Table 2. Assembly statistics of selected edited and control plants of San Marzano genotype

	SM6	SM13	SM17	SM19	CTRL
N° scaffold	390,956	408,513	403,795	391,614	415,830
assembled genome (Mb)	830.9	833.7	832.8	831.5	843.1
Mean scaffold size (bp)	2,125	2,041	2,062	2,123	2,027
N50 scaffold length (bp)	28,863	28,271	27,917	31,529	30,452
L50 scaffold count	7,286	7,416	7,556	6,621	6,913
Read coverage over Solyc10g009390.3 (single copy gene)	45.1	33.3	32.8	36.6	36.9
Read coverage over Cas9 gene	45.5	30.2	36.1	36.5	-
Inferred N° copies *Cas9* (hemyzigous)	2.0	1.8	2.2	2.0	-
N° copies *Cas9* (qPCR)	2.2	2.0	1.8	1.8	-

Suppl. Table 3. Details of the indels produced by the 4 sgRNAs in each edited line

Mutant	Alleles in sgRNA7 region (%)		R^2
	-7	0	
SM4	95	-	0,98
SM5	91,9	-	0,98
SM6	97,5	-	0,99
SM13	96	-	0,99
SM17	97,8	-	0,98
SM19	97,9	-	0,99
SM22	96,6	-	0,98
OX2	95,6	-	0,99
OX3	97,7	-	0,99
OX4	98,5	-	0,99
OX9	98,6	-	0,99
OX11	98	-	0,99

Mutant	Alleles in sgRNA8 region (%)				R^2
	-7	-2	0	1	
SM4	54.2	25.5	-	34.6	0,9
SM6	46,7	48,4	-	-	0,95
SM7	46,1	48,4	-	-	0,94
SM8	43,6	51,6	-	-	0,96
SM9	44,8	50,6	-	-	0,95
SM12	45	48,3	-	-	0,93
SM13	43,4	45,8	-	-	0,9
SM14	41,9	50,9	-	-	0,93
SM16	-	-	-	96,1	0,96
SM17	43,2	50,1	-	-	0,94
SM18	44,5	48	-	-	0,93
SM19	45,1	47,7	-	-	0,93
SM22	45,4	48,4	-	-	0,94
SM24	44,9	48,7	-	-	0,94
SM25	45,1	49,1	-	-	0,93
SM26	44,5	49,4	-	-	0,94
OX2	-	96.5	-	-	0,99
OX3	-	-	-	95	0,98
OX4	-	96,2	-	-	0,99
OX9	-	69.5	-	26,4	0,96
OX11	-	91,3	-	-	0,95

Mutant	Alleles in sgRNA6 region (%)										R^2
	-18	-8	-7	-5	-4	-3	-2	-1	0	1	
SM1	-	-	-	-	-	-	-	-	-	-	0,01
SM2	-	-	-	-	-	1,6	-	-	96,8	-	0,99
SM3	-	-	-	-	-	7.2	0.8	-	90.5	-	0,99
SM4	-	-	-	-	-	2.9	-	-	57.6	34.6	0,96
SM5	-	-	-	-	-	-	-	-	71.7	24.2	0,96
SM6	-	-	11.4	12	-	-	16.8	-	54.8	-	0,95
SM7	-	-	-	-	8.6	-	-	-	85.6	1.4	0,99
SM8	-	-	-	-	-	-	-	10.9	61.8	21.1	0,96
SM13	27,5	-	-	12,2	-	8,1	-	-	-	38,8	0,93
SM17	-	-	31,7	-	-	-	-	-	28,2	31,7	0,93
SM19	-	-	-	-	-	-	-	-	97	-	0,98
SM22	-	-	-	-	4	6,3	-	5,7	28,6	51,4	0,96
OX1	-	-	-	-	15	-	-	-	41.8	39.7	0,97
OX2	-	-	-	-	8.4	-	-	-	80.8	9.1	0,99
OX3	-	-	-	-	-	-	26.8	-	69.6	-	0,96
OX4	-	6.6	-	-	-	-	-	-	84.7	6.6	0,99
OX9	-	-	-	-	-	6,2	-	-	84,4	5,9	0,99
OX11	-	-	-	-	-	-	-	48.5	46,1	-	0,95

Mutant	Alleles in sgRNA1 region (%)										R
	-10	-8	-7	-6	-5	-3	-2	-1	0	1	
SM6									92,2		0,96
SM7							52,6		35,8		0,94
SM8	8,4				10,6			14,3	59,3		0,93
SM9	-	2	11.4	-	-	-	7	6.9	9.3	3.4	0,45
SM13				45,7					43,9		0,92
SM17							54,7	15	18,3	3,2	0,94
SM18		45,3		17,8					8,4	12,2	0,88
SM19		27,6							60		0,91
SM24				17,6					57,4	9,8	0,92
OX3		31,8							-		0,57
OX11		10,3	-	-		16,7			60	-	0,93

Suppl. Table 4. Detached-leaves assay with *P. infestans* performed on 26 PMR4 San Marzano mutants, 9 Oxheart mutants and control plants. Two independent experiments were separately conducted. LAD% values here reported were normalized using LAD% derived from the controls. Statistical differences among mutant/control were analyzed with a two-tailed t test (*, $p < 0.05$).

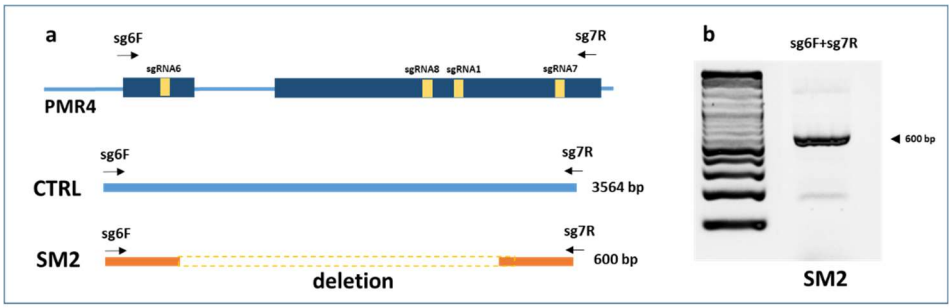
	1° experiment				2° experiment			
Genotype	mean LAD%	st. deviation	p-value	sig.	mean LAD%	st. deviation	p-value	sig.
SM1	0.57	0.14	0.021	*	1.13	0.47	0.687	
SM2	0.57	0.14	0.021	*	-	-	-	
SM3	0.28	0.14	0.004	*	1.43	0.47	0.254	
SM4	0.14	0.07	0.003	*	0.20	0.09	0.000	*
SM5	1.20	0.20	0.059		-	-	-	
SM6	0.60	0.20	0.049	*	0.20	0.09	0.000	*
SM7	0.80	0.04	0.192		0.82	0.18	0.207	
SM8	1.00	0.20	1.000		0.82	0.18	0.207	
SM9	0.66	0.46	0.343		0.19	0.19	0.011	*
SM10	0.60	0.53	0.325		0.56	0.50	0.267	
SM11	0.26	0.12	0.001	*	0.75	0.00	0.225	
SM12	1.26	0.95	0.674		0.06	0.11	0.009	*
SM13	0.26	0.00	0.007	*	0.29	0.25	0.019	*
SM14	0.44	0.31	0.097		0.69	0.30	0.224	
SM15	0.44	0.31	0.097		1.29	0.74	0.577	
SM16	0.71	0.15	0.054		1.43	0.49	0.279	
SM17	0.34	0.06	0.002	*	0.35	0.00	0.031	*
SM18	0.44	0.11	0.003	*	1.00	0.27	0.185	
SM19	0.19	0.00	0.003	*	0.06	0.07	0.003	*
SM20	-	-	-		0.32	0.29	0.059	
SM22	0.28	0.00	0.007	*	1.77	0.74	0.221	
SM23	1.04	0.72	0.921		0.62	0.13	0.036	*
SM24	0.47	0.16	0.014	*	0.92	0.40	0.787	
SM25	1.04	0.16	0.725		0.69	0.00	0.087	
SM26	1.42	0.29	0.103		1.70	0.62	0.187	
CTRL-SM	1.00	0.13	-		1.00	0.12	-	
OX1	0.30	0.00	0.019	*	0.67	0.13	0.095	
OX2	0.60	0.30	0,139		0.67	0.13	0.096	
OX3	0.20	0.17	0.005	*	0.53	0.27	0.072	
OX4	0.00	0.00	0.001	*	0.27	0.18	0.009	*
OX5	0.80	0.46	0.530		0.76	0.20	0.212	
OX6	0.78	0.21	0.232		0.56	0.10	0.041	*
OX7	0.85	0.09	0.272		0.98	0.15	0.886	
OX8	0.75	0.15	0.132		0.71	0.15	0.118	
OX9	0.70	0.17	0.101		0.80	0.27	0.357	
CTRL-OX	1.00	0.17	-		1.00	0.20	-	

Suppl. Table 5. Sequencing stats in the 4 edited mutants and in the WT San Marzano genotypes.

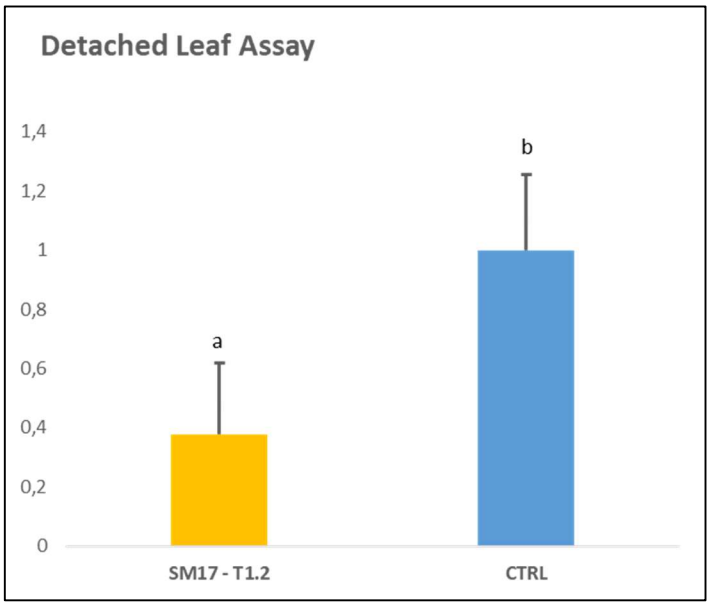
Sample	Raw reads	Raw data (Gb)	Effective (%)	Error (%)	Q20 (%)	Q30 (%)	GC (%)	Coverage
SM-6	271,306,910	40.70	99.33	0.03	97.34	93.06	36.39	52.01
SM-13	198,386,556	29.80	99.32	0.03	97.50	93.14	37.22	38.08
SM-17	227,401,788	34.10	99.35	0.03	97.52	91.15	37.14	43.58
SM-19	221,574,476	33.20	99.29	0.03	97.22	92.59	36.46	42.43
SM-CTRL	223,484,784	33.50	99.29	0.03	97.19	92.55	36.53	42.81
SM-WT-1	259,976,742	39.00	99.01	0.03	97.17	92.01	36.26	49.84
SM-WT-2	275,668,832	41.40	99.14	0.03	96.57	91.01	36.16	52.91

Suppl. Table 6. Off-target regions and sgRNA-like and SNP/indels found in the edited mutants and in the San Marzano genotype (CTRL).

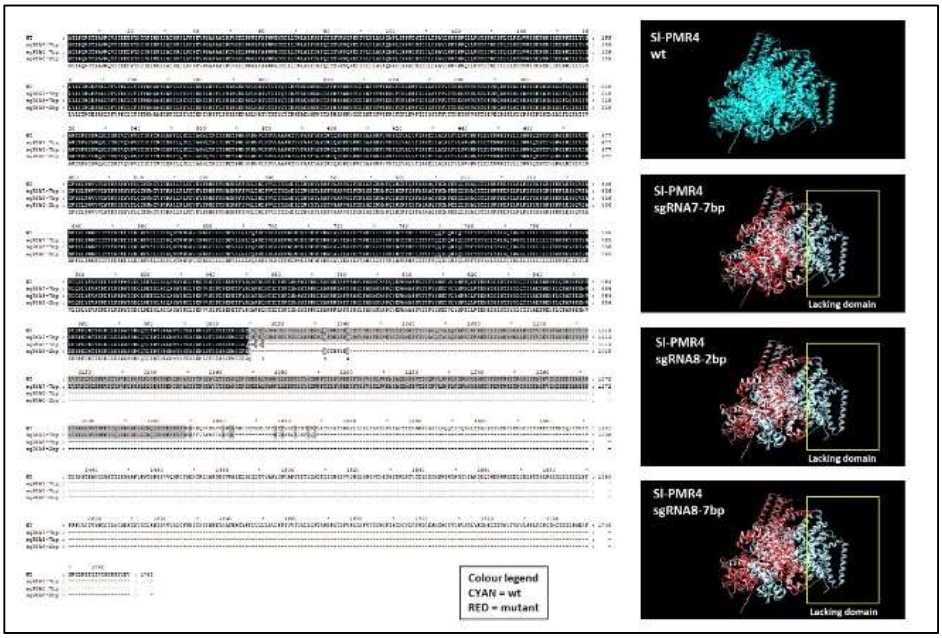
								N° SNP/indel				
Off-target genomic coordinates	strand	off-target sequence	PMR4 sgRNA	Type of mismatches	PAM	N° of mismatches	in CDS	SM6	SM13	SM17	SM19	CTRL
SL4.0ch02:40968952-40968975	-	GCtAAGGT_TGCtAGTGGtAA-TGGT	7	A21	A	3	yes	0	0	0	0	0
SL4.0ch09:32079813-32079836	-	GtCAAtGT_TGCCAaTGaCAA-TGGT	7	A22	A	4	-	0	0	0	0	0
SL4.0ch10:29190858-29190881	+	GtCAAGcT_TGaCAGTGGtAA-TGGT	7	A22	A	4	-	0	0	0	0	0
SL4.0ch12:27868456-27868479	+	GaCAAtGT_TGtCAGTtGCAA-TGGG	7	A22	A	4	-	0	0	0	0	0
SL4.0ch02:22578974-22578997	+	TTAcAGgA_GTCCCAaACTCc-GGGA	1	A22	A	4	yes	0	0	0	0	0
SL4.0ch04:33536140-33536163	+	TTgAAGCt_cTCCCATAgTCG-AGGC	1	A22	A	4	-	0	0	0	0	0
SL4.0ch05:27723956-27723979	-	TaAAAGgA_GTCCCATgCTCa-TGGA	1	A22	A	4	yes	0	0	0	0	0
SL4.0ch08:47322517-47322540	-	TTgAAGCg_GTgCCATACTCc-TGGT	1	A22	A	4	-	0	0	0	0	0
SL4.0ch09:3305746-3305769	-	TTAAAaCA_GTCgCATtCTCG-GGGA	1	A21	A	3	-	0	0	0	0	0
SL4.0ch01:85268760-85268783	-	GGATtTgA_GAGAAGtAcCAG-TGGG	8	A22	A	4	-	0	0	0	0	0
SL4.0ch02:15897577-15897600	+	GGtTATtA_GAGAAGGATCAt-GGGA	8	A12	A	3	-	0	0	0	0	0
SL4.0ch03:19724933-19724956	+	GGAaATaA_GAGAgGGATCAt-AGGG	8	A22	A	4	-	0	0	0	0	0
SL4.0ch05:15677408-15677431	-	GGtTgTCA_aAGAAGaATCAG-TGGA	8	A22	A	4	-	0	0	0	0	0
SL4.0ch07:29758891-29758914	-	GGATAaCg_GAGAAGGAgaAG-AGGA	8	A22	A	4	-	0	0	0	0	0
SL4.0ch10:49269193-49269216	+	GcAgATCA_cAGAAGtATCAG-AGGT	8	A22	A	4	-	0	0	0	0	0
SL4.0ch03:24947651-24947674	+	aTAaTGCC_CCACACTgTGta-AGGT	6	A32	A	5	-	0	0	0	0	0
SL4.0ch04:16536110-16536133	+	tTAtTGCC_CCAtACTCTcCa-TGGA	6	A32	A	5	-	0	0	0	0	0
SL4.0ch05:47540410-47540433	-	GTAtTtCC_tCACtCTCTtCG-AGGA	6	A32	A	5	-	0	0	0	0	0
SL4.0ch05:58288753-58288776	-	GaACTGtC_CCACACctTGCa-GGGA	6	A32	A	5	-	0	0	0	0	0
SL4.0ch05:64845772-64845795	-	GTgCTGtC_CCACttTCTGgG-AGGC	6	A32	A	5	yes	0	0	0	0	0
SL4.0ch07:10476402-10476425	+	GTACTGga_CCACtgTCTGCa-TGGA	6	A32	A	5	-	0	0	0	0	0
SL4.0ch08:58075701-58075724	+	GTACTGaa_CCtgAtTCTGCG-TGGT	6	A32	A	5	yes	0	0	0	0	0
SL4.0ch09:46513077-46513100	+	GTcgTGCC_CCcCAtTgTGCG-AGGA	6	A32	A	5	-	0	0	0	0	0
SL4.0ch09:64797758-64797781	-	tTAtTGCC_tCACACatTGCG-AGGC	6	A32	A	5	yes	0	0	0	0	0
SL4.0ch12:60823071-60823094	+	GTACTGat_CtAtACTCTcCG-CGGA	6	A32	A	5	yes	0	0	0	0	0



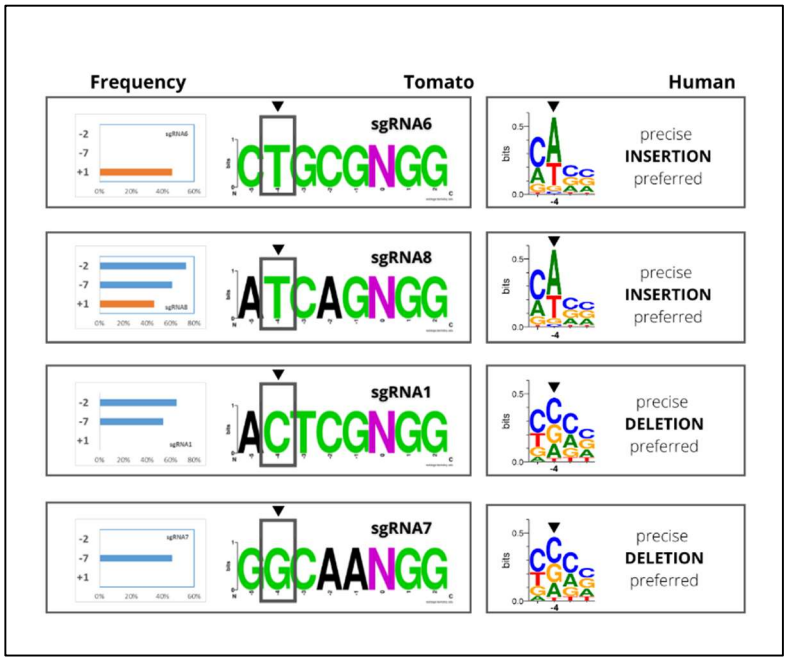
Suppl. Figure 1. Large deletion observed in the *PMR4* gene in the SM2 mutant. A) Scheme of the locus structure in SM2 and control plant. B) PCR amplification of the mutant with primers sg6F and sg7R.



Suppl. Figure 2. a) Detached-leaves assay with *Phytophthora infestans* performed on T1 *PMR4* San Marzano mutant (SM17-T1.2) and a wild type plant as a control group at 8 dpi. b) In the histogram, normalized LAD% values are reported for each genotype. The y-axis shows the mean ratio of the score of the mutant/control group; bars represent standard deviation (sd). Statistical differences among mutant/control were analyzed with a two-tailed t test ($P < 0.05$).



Suppl. Figure 3. Multi-alignment of PMR4 proteins carrying the indels observed in the edited mutants (SM6, 13, 17, 17-T1.2, 19), highlighting truncated versions of the WT protein. Pairwise juxtaposition of the 3D-protein models (WT and mutated PMR4) through the UCSF Chimera tool.



Suppl. Figure 4. Sequence logos for the precision core of the four sgRNAs used in this study. Left: Frequency of the most common indels (insertions or deletions) within the whole set of edited plants (SM and OX); details of the flanking PAM sequence with the −4 nucleotide position highlighted in a square box. Right: statistics reported in human for RNA-guided Cas9 nuclease (RGN) preferred indels (*Chakrabarti, Henser-Brownhill et al. 2019*).

Suppl. Figure 5. *pmr4* San Marzano mutants (SM6, 13, 17, and 19) and the WT plant in cultivation (9 months old).

Chapter 3 - Less is More: CRISPR/Cas9-based Mutations in DND1 Gene Enhance Tomato Resistance to Powdery Mildew with Low Fitness Costs

3.1 Abstract

Powdery mildew, caused by *Oidium neolycopersici*, is a devastating disease and a serious concern for plant productivity in tomato (*Solanum lycopersicum* L.). The presence of susceptibility (S) genes in plants facilitates pathogen proliferation and their disabling may help provide a broad-spectrum and durable type of tolerance/resistance. Previous research has shown that the *DND1* susceptibility gene's impairment enhances plant resistance to a wide range of pathogens, with the side-effect causing a detrimental impact on plant fitness. To check the possibility of reducing the negative consequences of *dnd1* mutation while bolstering plant response to diseases, a CRISPR-Cas9 vector containing four single guide RNAs targeting three *SlDND1* exons was designed and introduced via *Agrobacterium-tumefaciens*-mediated transformation into the market cultivar "Moneymaker" (MM). Three T1 lines (named E1, E3 and E4) were crossed with the Moneymaker wild-type genotype (T_1 x MM), to produce a T_{F1} generation in which all plants were susceptible to *Oidium neolycopersici*. T_{F1} were then selfed to produce a T_{F2} generation which was phenotyped, genotyped, and analysed for disease resistance. All the T_{F2} plants in homozygous state *dnd1*/*dnd1*, showed reduced disease symptoms compared to the heterozygous (*DND1*/*dnd1*) and control

(*DND1*/*DND1*) ones. Two events (E1 and E4) appeared as fully KO mutants, with trunked copies of DND1, exhibiting clear dwarf and auto-necrosis phenotypes, while one event (E3) showed a full-length protein with a 3 amino acids deletion, exhibiting a nearly normal height phenotype with fewer auto-necrosis spots. We thus compared the 3D structures of the reference and mutant proteins, observing a clear conformational change in the E3 derived mutant protein, likely affecting protein functionality. One *dnd1*/*dnd1* T_{F2} line (TV181848-9, E3) was whole-genome sequenced through Illumina for deeper analysis; no off-target effects were underlined in the selected genomic regions, neither traces of *Cas9* gene, which was eliminated by segregation. Our results confirmed, for the first time, reduced susceptibility to *Oidium neolycopersici* in tomato KO mutants. We also provided a special, full-length *dnd1* genotype (E3), which is resistant to powdery mildew and with much fewer fitness costs, indicating a possible way to breed with *dnd1* mutants.

3.2 Materials and methods

3.2.1 Experimental scheme

An experimental scheme (**Figure 1**) was employed to generate reliable mutants in the *DND1* gene with a reduced fitness cost. First, the genetic transformation of the cultivar 'Moneymaker' was conducted to introduce targeted mutations in the *dnd1* gene through CRISPR-based technology. T_1 events were then generated from the transformed plants. A cross was conducted between T_1 lines and the Moneymaker wild-type (WT) genotype (T_1 x MM), to produce a T_{F1} generation, in which all plants were heterozygous (*DND1*/*dnd1*, marked as A/a) at the target gene locus. T_{F1} generation were selfed to produce a T_{F2} generation. T_{F2} plants were phenotyped, genotyped, and analysed for disease resistance.

3.2.2 CRISPR/Cas-9-targeted mutagenesis of *SlDND1*

Four single guide RNAs (sgRNAs) (sgRNA10: GAAGCAAGC-GCGTGCAGAGA, sgRNA5: ATGTGTTTGGATGTCAATGG, sgRNA6: GTCAATGGACCATTTCCATA, sgRNA8: GCCACAAGCATACTTGA-GCC) were designed referring to the *DND1* homolog (solyc02g088560) from the Sol Genomics Network database (*Fernandez-Pozo, Menda et al. 2014*), on the website https://cctop.cos.uni-heidelberg.de/, and selected manually as described by (*Liang, Zhang et al. 2016, Chari, Yeo et al. 2017*). The program Cas-OFFinders (http://www.rgenome.net/cas-offinder/) was used to check for possible off-targets of the four sgRNAs of *SlDND1*. The mismatch number was set at 3 or less. A single

CRISPR/Cas9 construct containing four sgRNAs, the *NptII* resistance gene, and the *Cas9* gene was constructed. The plasmids were cloned using *E. coli* DH5α and transformed to *Agrobacterium* strain AGL1. The tomato cultivar 'Moneymaker' (MM, from the WUR-Plant Breeding seed collection) was used for genetic transformation according to the method described by McCormick (*McCormick, Niedermeyer et al. 1986*), according to Dutch legislation under GMO license 01–135. Primary transformants (T_1) were obtained from the in *vitro* cultivation, and the positive mutants, carrying mutant alleles, were selected via PCR amplification on both *NptII* and *Cas9* genes. These *dnd1* T_1 mutants were used to produce a T_{F1} generation obtained from the crossbreeding between T_1 and WT MM plants. T_{F1} lines were heterozygous and selfed to produce the T_{F2} progeny. All of the lines used in this study could be found in **Table 1**.

3.2.3 PCR-based characterization of mutation events and genotyping

DNA was extracted from the T_1, T_{F1} and T_{F2} genotypes with the modified CTAB DNA extraction method (*Porebski, Bailey et al. 1997*), quantified on the Qubit fluorometer (Thermofisher, USA), and NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermofisher, USA). We used 5 pairs of primers for flanking all the regions with possible mutations that could be visible via electrophoresis. The position of these primers and their flanking regions were shown in **Figure 2**, the primers were presented in **Table S1**. The distinction between homozygous, heterozygous, and WT

homozygous T_{F2} plants was identified by the same PCR method and primers as described above. The amplified fragments were Sanger sequenced and their allelic status (monoallelic, bi-allelic, and heterozygous) was determined by TIDE at: http://shinyapps.datacurators.nl/tide/.

3.2.4 *Sldnd1* mutant phenotype analysis and disease assay

Seeds of each T_{F1} and T_{F2} line were sown separately, and the number of the plants of each line obtained were presented in **Table S2**. The temperature in the greenhouse (Unifarm of Wageningen University & Research, The Netherlands) was set to 21 ℃ (day)/19 ℃ (night), with a relative humidity of 70% ± 15% and a day length of 16 hours. When the seedlings were 4-weeks old, a suspension of conidiospores of *Oidium neolycopersici* (*On*; obtained from leaves of infected tomato MM plants) was adjusted to a concentration of $3.5*10^4$ spores per ml and uniformly spray-inoculated on the plants (**Table S2**). Ten and 12 days after inoculation, the disease index (DI) scoring was carried out by visually observing the symptoms of powdery mildew with a scale from 0 to 3 (*Bai, Pavan et al. 2008*). WT MM plants were used as the control group (Ctrl).

3.2.5 Quantification of relative fungal biomass

The fourth true leaf of the infected tomato mutants and control plants were collected at 21 days post-inoculation (dpi). Plant and fungal genomic DNA (gDNA) was isolated from these materials with an adapted CTAB method (*Porebski, Bailey et al. 1997*). The primers used could be found in **Table**

S1, targeting the internal transcribed spacer sequence (ITS) of *On*, and the elongation factor 1α (*Ef1α*) of tomato, reported by Løvdal and Zheng (*Løvdal and Lillo 2009, Zheng, Appiano et al. 2016*). Quantitative real-time PCR (qRT-PCR) was performed using the $2^{-\Delta\Delta Ct}$ method (*Livak and Schmittgen 2001*) to calculate the fold-change ratio between fungal and tomato gDNA. The qPCR was performed in three biological replicates with a C_{1000} light cycler system (Bio-Rad) using SYBR Green mix (Bio-Rad).

3.2.6 Whole genome sequencing of TV181448 mutant line

One microgram of DNA was used to construct short-insert (length 350 bp) genomic library (Novogene, Hong Kong), which were sequenced using an Illumina sequencer (Illumina Inc., San Diego, CA, USA) with paired-end chemistry (2×150 bp). Raw reads were cleaned with fastp (https://github.com/OpenGene/fastp) to remove contaminant residual adapters and for the removal of reads with poor quality ends (Q < 30). A *de novo* genome assembly was performed using the MegaHit assembler (v1.2.9, https://github.com/voutcn/megahit), utilising specific assembly parameters (k-min = 27, k-max = 141, k-step = 10, cleaning-rounds = 1, and disconnect-ratio = 0). Metrics for assessing the quality of a genome assembly (e.g., N50, contig/scaffold number/size/length, and genome length) were obtained using the Perl script Assemblathon_stats.pl (https://github.com/ucdavis-bioinformatics/assemblathon2-analysis).

BLAST analysis was conducted on the assembled genomic sequence of the mutant to identify any possible insertions using the T-DNA sequence,

as a query. As preferential choice criteria, the e-value (e-value < $1 \times e^{-10}$), percentage similarity, and query coverage were considered.

3.2.7 Homology modelling of DND1 (WT and mutants) and comparison of 3D structures

WT and mutated DND1 proteins were reconstructed using the 'getorf' tool (http://emboss.sourceforge.net) and proteins were multi-aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). Homology models for both the WT and mutated proteins were built with the online tool SWISS-MODEL (https://swissmodel.expasy.org), using the HCN1 channel structure from *Homo sapiens* L. (SMTL ID 6uqf.1) as a template, and were subjected to validation using Molprobity (*Williams, Headd et al. 2018*) and QMEAN (*Benkert 2009*). Pairwise juxtaposition of models was carried out with UCSF Chimera (v1.16, https://cgl.ucsf.edu/chimera).

3.2.8 On- and off-target analyses and SNP statistics

In edited plants, the emergence of genomic variants and allele frequencies in the *SlDND1* locus was highlighted using CRISPResso2 (http://crispresso2.pinellolab.org) and SNP/indel analysis. Clean reads derived from the edited plants were mapped to the tomato reference genome (SL4.0, https://solgenomics.net) using the Burrows–Wheeler Aligner (v0.7.17, https://sourceforge.net/projects/bio-bwa/files) program and 'mem' command with the default parameters. BAM files were processed and used for SNP calling using Samtools (v1.9-166-g74718c2; Danecek et al., 2021) mpileup with default parameters, except for

minimum mapping quality (Q = 20). A variant call format (vcf) file was produced. The vcf file was inspected in the 200 bp window surrounding each sgRNA to highlight SNP/indels through bedtools intersect (https://bedtools.readthedocs.io). For off-target analysis, the CasOT script (https://github.com/audy/mirror-casot.pl) was used to identify any off-target regions in the tomato genome (SL4.0, https://solgenomics.net). All designed sgRNAs were considered as bait in a sgRNA mode, with default PAM type (NGG=A) and specific numbers of permitted mismatches in the seed (2), and non-seed (2) regions allowed. All the candidate off-target genomic region coordinates were intersected with the vcf file through bedtools for editing as well as for the control plants to filter-out monomorphic regions among the latter. The results were analysed using custom bash scripts.

3.3 Results

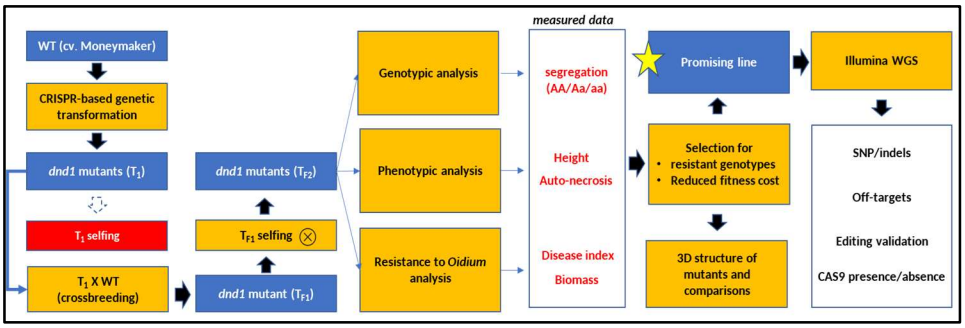


Figure 1. The experimental scheme used in this work for the identification of desirable mutants that exhibited increased disease resistance and the lowest fitness cost.

3.3.1 CRISPR/Cas-9-targeted mutagenesis of *SlDND1* and mutant generation

A single CRISPR/Cas9 construct containing four sgRNAs, the *NptII* resistance gene, and the *Cas9* gene was built and used to transform the tomato cultivar 'Moneymaker' (MM), susceptible to *O. neolycopersici*, via *Agrobacterium tumefaciens*. Four single guide RNAs (sgRNAs) were specifically designed to target the *DND1* homolog (*Solyc02g088560*) from the Sol Genomics Network database (*Fernandez-Pozo, Menda et al. 2014*), increasing the possibility to obtain large deletions between adjacent sgRNAs and destruct the gene structure and function. The position of the four sgRNAs in the *SlDND1* genomic sequence is depicted in **Figure 2**. Primary transformants (T_1) were obtained following genetic transformation and *in vitro* cultivation, and a total of 39 positive mutants were selected via PCR screening on both *NptII* and *Cas9* genes. A subset

of 12 transformants, showing a severe or mild *dnd1* phenotype (dwarf plants with auto-necrosis spots), were selected for further analysis.

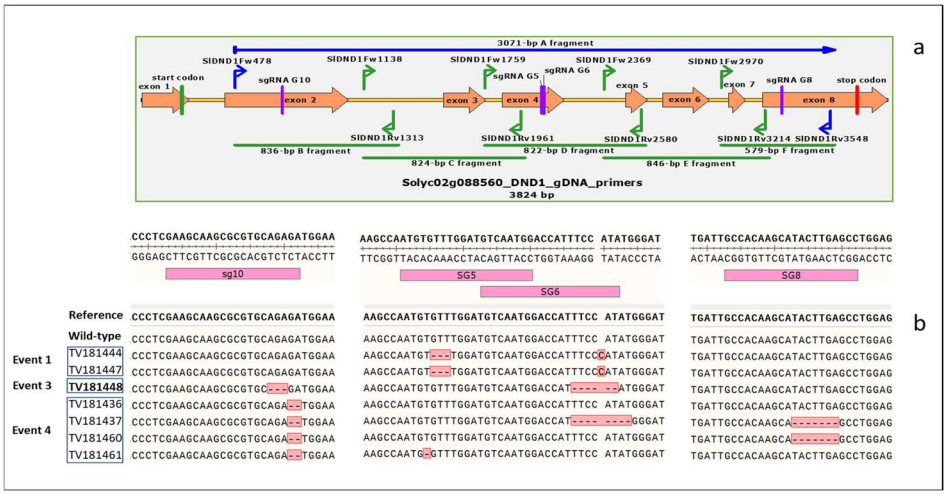


Figure 2. Position of target sites of the sgRNAs in *SlDND1* and editing details of events. a) Representation showing the locations of the sgRNAs' target sites and the regions that were partitioned for the mutation analysis in *SlDND1*(solyc02g088560). The 3071 bp region of *DND1* (fragment A) containing 4 sgRNAs was divided into fragments named B, C, D, E, and F for Sanger sequencing and identification of the mutations. b) Mutations in lines of T_{F2} generation from 3 different editing events. The results were obtained from Sanger sequencing.

3.3.2 Phenotypes of the *Sldnd1* mutants

Three specific T_1 mutants (E1, E3 and E4) were selected as representative ones based on their phenotypes: E1 and E4 exhibited clear dwarf and auto-necrosis phenotypes, while E3 showed a mild dwarf phenotype. These events were crossbred with wild-type (WT) plants of the cv. 'Moneymaker' (**Figure 1**), originating T_{F1} that once selfed generated T_{F2} progenies, segregating for the *DND1* locus. Among them, we observed distinct levels of *dnd1* phenotypes (**Table 1**). T_{F2} from E1

and E4 showed severely dwarfed or normal plants and showed necrotic spotting (**Figure 3a, 3b**), while plants derived from E3 were normal plants or exhibited a slightly dwarfed phenotype with fewer auto-necrosis spots, which appeared later than in E1 and E4 (**Figure 3c**).

Figure 3. Phenotypes of some T_{F2} *dnd1* mutants. a) Auto-necrosis phenotype of young seedlings of the TV181460 line (T_{F2}). Auto-necrosis spots were observed on the edge of its first pair of true leaves, the photo was taken 7 days after seeding. b) Dwarf phenotype observed on young seedlings, the photo was taken 15 days after seeding, homozygous compared with heterozygous mutant (TV181460) and wild-type (wt); line TV181460 was here selected as representative of two similar KO mutation events, E1 and E4). c) Phenotypes of mature plants. Height comparison among the two *dnd1* homozygous T_{F2} plants and the wt one month after seeding. On the left is shown a homozygous mutant of the TV181447 line exhibiting severe *dnd1* dwarf phenotype; in the middle is shown a homozygous mutant of the TV181448 line, exhibiting a slight *dnd1* phenotype (E3); on the right is shown the wt 'Moneymaker' plant.

3.3.3 Genotypes of the *Sldnd1* mutants

The T_{F2} mutants were fully characterised through Sanger sequencing and TIDE analyses allowed the reconstruction of the original editing events. Genotypes were amplified with primer sets (**Suppl. Table 1**) flanking the sgRNAs targeting regions (five regions: B, C, D, E, F; **Figure 2**). No large deletions were discovered at this step, compared with the length of the amplicons of WT. The T_{F1} plants showed to be completely heterozygous (*DND1*/*dnd1*, Aa). The T_{F2} lines derived from the 3 events showed different allelic profiles (**Table 1**), segregating at the *DND1* locus (AA, Aa and aa; **Table 1**). In particular, the T_{F2} plants deriving from the E1 event showed: a 3-bp deletion at sgRNA5 and 1-bp insertion at sgRNA6, the latter generating a trunked protein. The T_{F2} plant deriving from E3 event showed a 3-bp deletion at sgRNA10 and a 6-bp deletion at sgRNA6, introducing 3 amino acids deletion. The T_{F2} plants derived from the E4 event showed a 2-bp deletion at sgRNA10, generating a trunked protein,

followed by a 1bp or 8bp deletion at sgRNA6 and a 7-bp deletion at sgRNA8.

Table 1. Relationship among events and lines, with their phenotypes and mutations; * trunking mutations.

Event	Phenotype	protein length (aa)	B(sg10)	C	D(sg5)	D(sg6)	E
E1	severe dwarf, necrotic spotting	380	-	-	-3 bp	**+1 bp***	-
E3	no dwarf, necrotic spotting (edge of leaves)	full length	-3 bp	-	-	-6 bp	-
E4	severe dwarf, necrotic spotting	125	**-2 bp***	-	-	-1 bp/-8bp/ref	-

3.3.4 Resistance to powdery mildew in *Sldnd1* mutants

To evaluate the resistance of the *Sldnd1* T_{F2} mutants, we inoculated them with *Oidium neolycopersici* (*On,* **Figure 4**), assessing the disease index (DI) score (**Figure 5a**, **5b**). Additionally, we quantified the disease severity by measuring the relative *On* biomass in the mutants, complementing the DI observations (**Figure 5c**). WT MM plants were used as control. All the T_{F2} offsprings, heterozygous for *dnd1* (Aa), from E1, E3 and E4 showed to be susceptible to *On*, with no significant differences in the DI score or the relative fungal biomass with the controls and among them (**Figure 5**, **Suppl. Table 2**). All the T_{F2} offsprings, homozygous for *dnd1* (aa), from E1, E3 and E4, showed to be resistant to *On*, with a significant difference in the DI score or the relative fungal biomass with the controls. Surprisingly, the *dnd1/dnd1* offsprings from the E3 T_{F2} progeny, besides displaying an improved resistance (with low DI scores and fungal biomass; **Figure 5**), exhibited less dwarfism and auto-necrosis spots (**Figure 3c**).

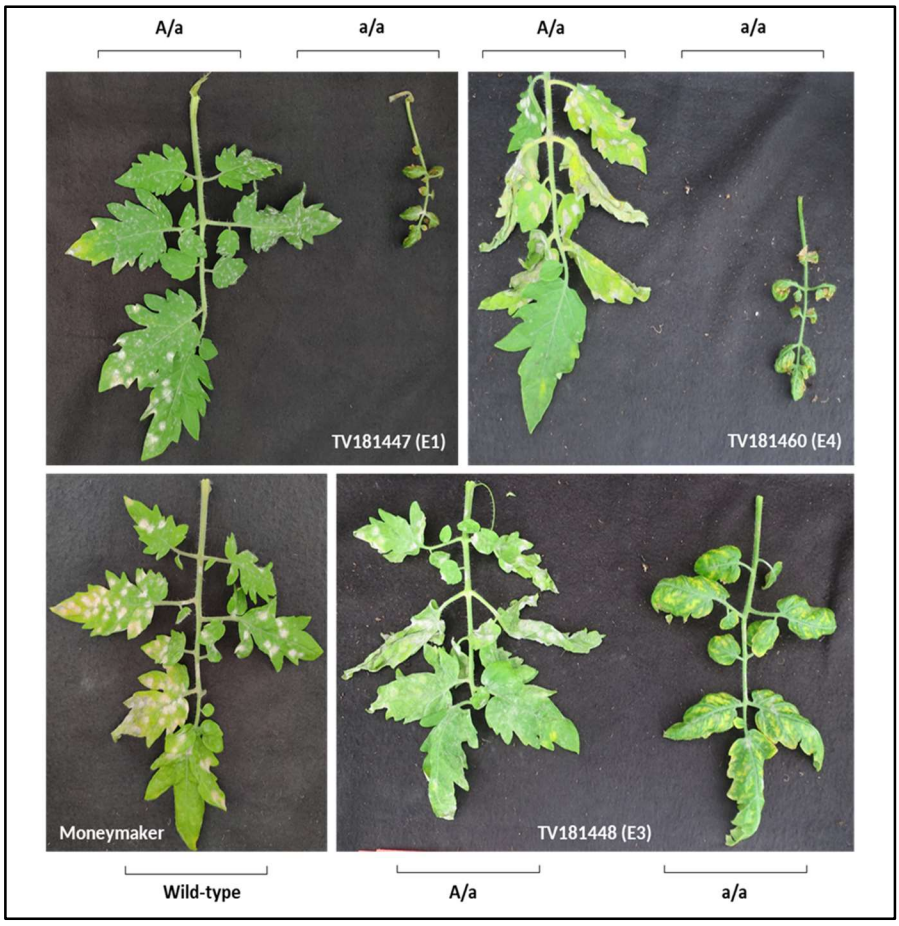


Figure 4. Phenotypic response to infection with *Oidium neolycopersici* of homozygous/heterozygous (a/a)/(A/a) *dnd1* mutants (T_{F2}). Powdery mildew symptoms were observed on the leaves of both homozygous and heterozygous mutants of each event (one line is given from each of the 3 mutation events; E1, E3, E4). Photos were taken 21 days post inoculation (dpi).

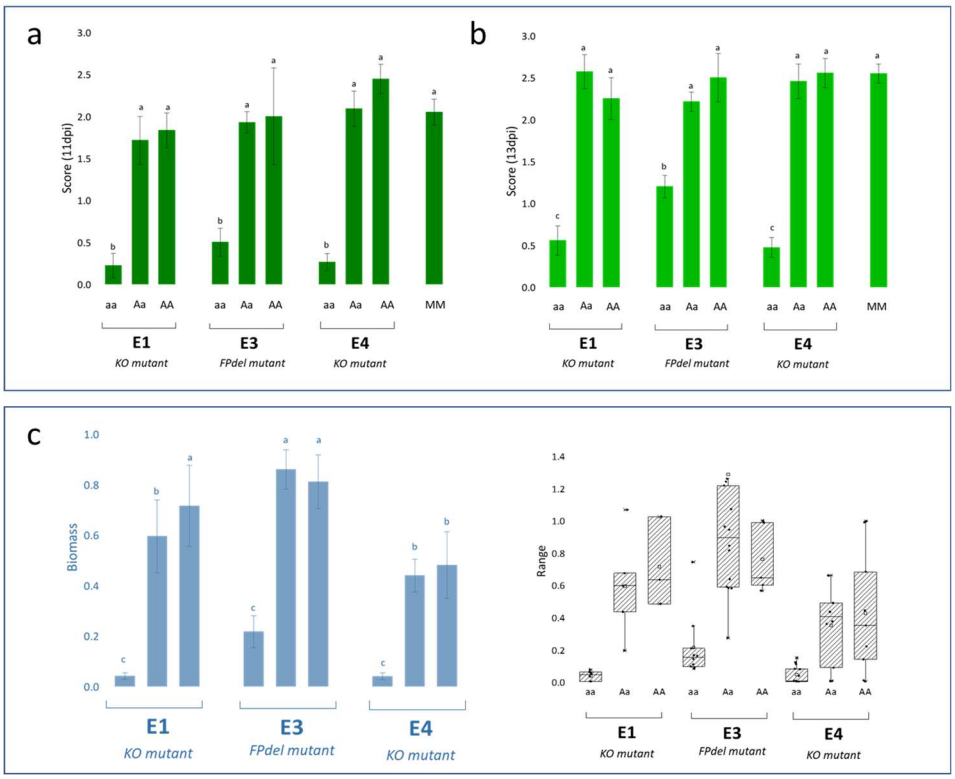


Figure 5. An average disease index score of the mutant lines in T_{F2} at 11 dpi (a) and 13 dpi (b). Homozygous mutants (aa) appeared significantly resistant in all the the assayed events (E1, E3, E4); however, heterozygous mutants (Aa) showed to be susceptible in all the *dnd1* assayed events (E1, E3, E4). Wild-type Moneymaker was used as control (MM). c) Relative fungal biomass quantification on at least three individual plants of the mutant lines (left, histograms with se bars; right, box plot). This is calculated as the ratio of fungal *ITS* gene amplification in comparison with tomato *EF1a* and normalized with the values of the wild-type Moneymaker. Samples for the biomass were taken at 21 days post inoculation (dpi). The y-axis shows the mean ratio of the score of the mutant/control group; bars represent standard error (se). Statistical differences were analyzed with a two-tailed t-test ($p < 0.05$).

3.3.5 Different mutation types impacted differently on the plant fitness

To determine if heterozygous mutants could lead to a reduced fitness cost while maintaining an acceptable degree of disease resistance, we

compared homozygous and heterozygous plants. Interestingly, all heterozygous mutants, including those from T_{F1} and T_{F2} generations, showed a reduced *dnd1* phenotype (less dwarfism) and did not display auto-necrosis spots. Considering the T_{F2} progeny, the homozygous mutants (aa) in E4 lines showed statistically significant dwarfism (mean: 28.81 ± 1.23 cm), whereas heterozygous plants (Aa) did not (mean: 61.15 ± 5.39 cm; **Figure 6**), being comparable to plants carrying WT alleles (AA, mean: 61.20 ± 5.23 cm) and controls (MM, mean: 66.30 ± 1.28 cm). Notably, homozygous mutants (aa) from E3 showed reduced dwarfism (mean: 61.10 ± 2.69 cm), with no statistical differences when compared with heterozygous plants (74.33 ± 1.57 cm) and with the ones containing the reference alleles (AA; 69.25 ± 3.81 cm).

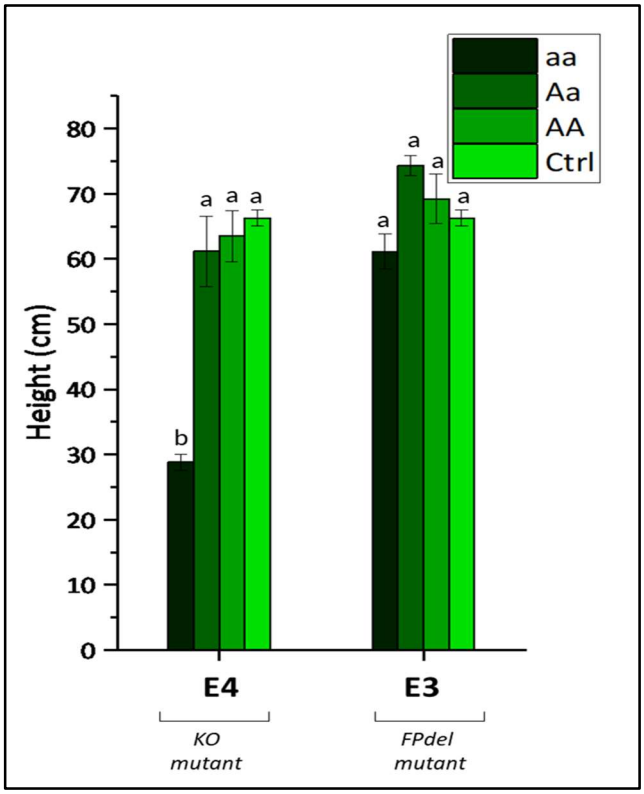


Figure 6. Height of homozygous/heterozygous *dnd1* mutants and WT plants (T_{F2}). A comparison of their height between a dwarf event (E4) and the slightly dwarfed event (E3) in three different allelic states (aa, Aa, AA), compared with WT Moneymaker plants used as control (Ctrl), is presented.

3.3.6 Homology modelling of DND1 in E3 event and 3D structure comparison

Preliminary sequence evaluation of the DND1 mutants was attempted. Multiple sequence alignment (**Figure 7**) of the reference protein (DND1, solyc02g088560) with 3 mutants (**Table 1**) revealed in 2 different editing outcomes: 1) trunked copies of the DND protein (E1, E4), being present KO mutations, a 2) full-length of the protein with 2+1 amino acids deletion

(E3). Multiple sequence alignment (**Figure 8**) of one specific line (E3, TV181448-9) with the reference protein (DND1, solyc02g088560) revealed that the editing outcome (a 3-bp deletion at sgRNA10 and a 6-bp deletion at sgRNA6) provoked amino acid deletions (110, Glutarnine, Q-del; 365-366, Phenylalanine, F, Proline, P, FP-del). This mutant codes for a full-length protein different from the ones from the other editing events (E1, E4) which appeared as trunked, putatively unfunctional, copy of the DND1 protein. The mutation impact analysis on E3 protein functionality was conducted through Polyphen. Following the deletion of the FP dipeptide in the TV181448-9 mutant, the F365 is replaced by a Y, while at P366 was substituted by a G. Overall, the amino acid (F) in position 365 showed to be crucial because if substituted leads to a possible damaging state of the protein (**Figure 8**). On the contrary, the substitution of the 366P showed a lower impact on protein function if substituted with other amino acids. For these reasons, we tried to reconstruct the 3D protein structures of both WT and mutants in order to evaluate any conformational change impacting function.

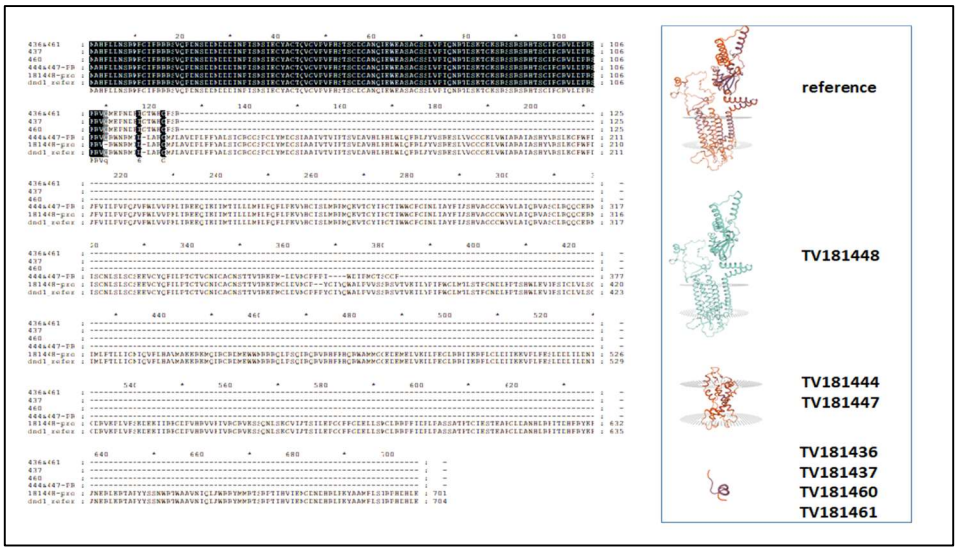


Figure 7. Sequence alignment of the DND1 proteins using the 'Moneymaker' (reference) sequence the predicted ones for the different mutants. Dashes represent lacking amino acids in the mutant. Superposed sequences of mutant (trunked protein, dark) and reference proteins (light blue).

Homology models for both the WT and mutated proteins were built and positively validated. The WT protein showed a QMEAN4 value of -2.27, and the Ramachandran plot showed that 90.51% of the residues were in favored regions. The QMEAN4 value for the mutated protein was -2.44 and 90.16% of the residues were in the favoured regions of the Ramachandran plot. The difference between the two models was analysed in the UCSF Chimera software (*Pettersen, Goddard et al. 2004*). A comparison of the 3D structures of both reference and mutant was attempted using the reconstructed 3D proteins in monomer form (**Figure 9**) and tetrameric form (**Figure 10**). Three observations were done: 1) the mutation (FP365-366del) is adjacent to the selective filter (**Figure 9** and

Figure 10); 2) the portion of the protein affected by the mutation changes conformation upstream of the selective filter and the pore helix (**Figure 8**); 3) this conformational change is sterically bulky (**Figure 9**). In the tetramer model, this conformational change is even more noticeable (**Figure 10**).

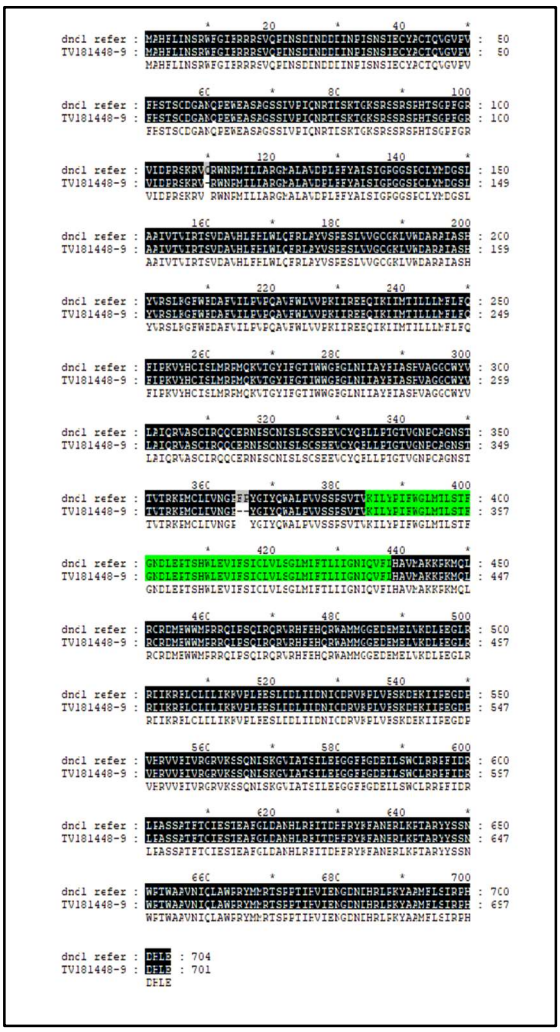


Figure 8. Sequence alignment of the DND1 proteins in 'Moneymaker' (reference) sequence and in the mutant (TV181448-9). Dashes represent lacking

amino acids in the mutant. In green “Selectively filter” and “Pore domain”.

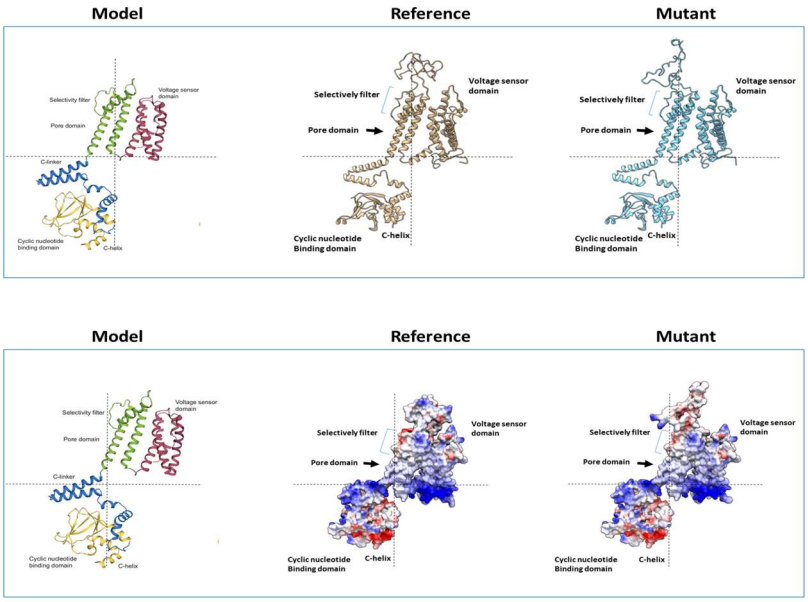


Figure 9. Comparison of 3D structures of DND1 (monomer) for the reference protein and the mutant. Top: ribbon model; Bottom: electrostatic mode (red: positive charges; blue: negative charges; white: no charges). The annotated model protein is from Rheinberger (*2018*).

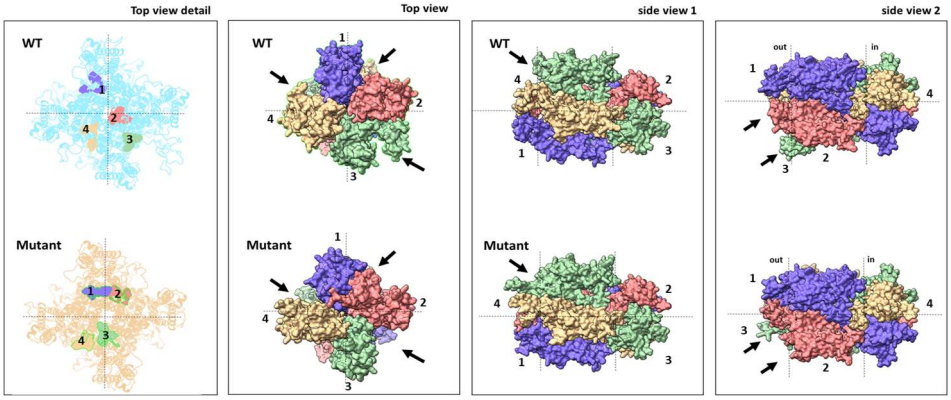


Figure 10. Comparison of 3D structures of DND1 (tetramer) indicating with the numbers (1 to 4) the four monomers of the DND1 protein. a - top view with highlighted 4 amino acids (YGIY) following the F365P366 dipeptide (deleted in the mutant). b - top view with conformational changes highlighted (black arrows). c - side view (1) with conformational

changes highlighted (black arrows). c - side view (2) with conformational changes highlighted (black arrows).

3.3.7 Whole genome sequencing of TV181448 (E3) and off-target effects

TV181448-9 and WT plants were subjected to Illumina whole-genome sequencing, generating 438 million raw paired-end reads (65.8 Gb; **Table S3**) with coverage ranging from 43.4X (TV181448-9) to 40.6X (WT). After filtering and trimming 436 million high-quality reads (99.46%), were obtained. The sequence data were deposited in the NCBI Short Read Archive under specific submission identifiers (*in submission*). A *de novo* genome assembly was carried out for the mutant line revealing no T-DNA insertions in the scaffolds, indicating a *Cas9* elimination by segregation. This line confirmed a 100% editing outcome in the *DND1* locus (Solyc02g088560) revealing a 3-bp deletion at sgRNA10 (110, Qdel) and a 6-bp deletion at sgRNA6 (365-366, FPdel), both in the homozygous state.

To verify that TV181448-9 displayed mutations solely in the *DND1* locus and to investigate potential off-target effects, we examined candidate off-target loci using the resequencing data. We identified a list of 28 potential off-targets for the four sgRNAs used to target the *DND1* locus, which all had more than 2 bp mismatches in respect of the gRNAs, and were located in both coding (2) and non-coding (26) regions (**Table S5 and S6**). We mapped the Illumina reads from the WT and TV181448-9 genomes to the tomato ‘Heinz 1706’ reference genome for off-target analysis. All 28 putative off-target regions were fully covered by Illumina reads in both the

WT and *dnd1* mutant, ruling out the possibility of large deletions (data not shown). Comparing DNA alignments in the WT and mutant, we found no SNPs/indels or significant deletions in the candidate off-target regions. While some indel/SNPs were present in the surrounding regions (SL4.0ch05:26816411-26816434 and SL4.0ch12:31973239-31973262), they did not indicate off-target effects, being conserved SNP/indels between mutants and WT or outside of the 20 bp window related to the gRNA-like sequence (200 bp window). These analyses confirm the specificity of Cas9-mediated *DND1* gene editing and demonstrate the absence of off-target effects. We identified 49,599 SNPs in TV181448-9 (90.7% of which were heterozygous) and 43,757 SNPs in WT (89.3% of which were heterozygous) using the Heinz tomato genome as a reference. The average SNP number and mutation rate were comparable across edited and unedited plants, showing an average mutation rate of 6.34×10^{-5} and 5.59×10^{-5}, respectively (**Table S4**).

3.4 Discussion

Plants with constitutive defense responses may result from DND1 function disability; however, real null *dnd1* plants show pleiotropic effects, are ineffective in production, and have low fitness (*Sun, Wolters et al. 2016*). In this study, we generated knock-out (truncated proteins) and 3-amino acid deleted (110Qdel; 365-366FPdel) *dnd1* mutants in a tomato cultivar susceptible to powdery mildew - 'Moneymaker' (MM) - by applying CRISPR/Cas9 technology. We investigated two ways for minimizing the negative effects of *dnd1* mutants for breeding: i) exploiting heterozygous knock-out mutants, to test the potential increased resistance towards *Oidium neolycopersici*, and ii) deepening the study of a unique full-length *dnd1* mutant, with 3 amino acid losses. The latter was also studied through whole-genome sequencing to exclude the emergence of any unintended OT effects and to assess its substantial equivalence with wild-type (WT) plants.

3.4.1 Resistance of *dnd1* mutants and their side effects

Plants cannot move to escape environmental challenges. In turn, they have evolved sophisticated mechanisms to perceive such attacks, and to translate that perception into an adaptive response. The *dnd1* mutant provides an example of gene-for-gene resistance without the hypersensitive response (HR) (*Clough, Fengler et al. 2000, Zachary Nimchuk, Thomas Eulgem et al. 2003*). In previous studies, in various plants (*Arabidopsis*, tomato and potato), *dnd1* mutants have been shown

to provide broad-spectrum resistance to pathogens (*Yu, Parker et al. 1998, Clough, Fengler et al. 2000, Chin, DeFalco et al. 2013, Sun, Wolters et al. 2016, Sun, van Tuinen et al. 2017*). The knockout or knockdown of *DND1* can result in severe dwarfism, auto-necrosis, and reduced male fertility in different plant species (*Clough, Fengler et al. 2000, Sun, Wolters et al. 2016, Sun, van Tuinen et al. 2017*).

Starting from primary editing events (E1, E3, E4), we generated various mutant genotypes through crossbreeding with WT MM plants (**Figure 1**), generating T_{F1} lines which were selfed to produce T_{F2} progenies showing segregation at the *DND1* locus. This approach was attempted to: i) mitigate unwanted effects, such as the difficulty to self the primary editing events, most probably due to *in vitro* plant culture side effects; ii) test the hypothesis that heterozygous *dnd1* mutants might provide adequate pathogen resistance without compromising fitness levels. Among the three editing events studied (E1; E3 and E4), we observed two distinct levels of *dnd1* phenotypes.

Homozygous T_{F2} mutants from E1 and E4, having trunked copies of the DND1 protein with a stop codon introduced, leading to a functional knock-out of *DND1*, were severely dwarfed and showed necrotic spotting (**Figure 3a**, **3b**; **Figure 4**). Our results confirmed a reduced pathogen susceptibility in *dnd1* tomato homozygous mutants, which was already previously observed through knock-down plants (i.e. via RNAi, Sun et al. 2017), supporting the hypothesis that the full disability of *DND1* (through

trunked mutants) can provide protection against *O. neolycopersici*. In previous studies, potato *dnd1* KD mutants showed a significantly slight *dnd1* phenotype, which could be related to its tetraploidy and thus an allelic dose effect (*Sun, Wolters et al. 2016, Sun, van Tuinen et al. 2017*). In our study, we thus considered the heterozygous mutants; however, by comparing the phenotype of the heterozygous with the control, no significant differences were observed (**Figure 5** and **6**), as highlighted by the analysis of the DI score and fungal biomass. We thus showed that the lack of resistance to *On* in *dnd1* heterozygous mutants was different from initially hypothesised.

In contrast, T_{F2} homozygous mutants from E3 line, having a mutated 3 amino acids deletion protein, surprisingly exhibited a slightly dwarfed phenotype producing plants (aa; e.g.: TV181448-9) with nearly normal growth (**Figure 5;** mean: 61.10 ± 2.69 cm), with no statistical differences when compared with heterozygous plants (74.33 ± 1.57 cm) and with the ones containing the WT alleles (AA; 69.25 ± 3.81 cm). Those plants (aa) also showed fewer auto-necrosis spots, which appeared later than the severe *dnd1* phenotype plants (**Figure 3c**). This plant promises to be useful in future breeding projects, representing the first example of induced mutagenesis in *DND1* generating tolerance with less negative pleiotropic effects. To assess the structural basis for this unique trait, homology modelling of DND1 in the E3 event as well as in the other events

(E1 and E4) and in the reference sequence was carried out and discussed below.

3.4.2 Homology modelling of DND1 and 3D structure comparison

DND1 is a Cyclic Nucleotide-Gated ion Channel (CNGC, *Kaplan, Sherman et al. 2007, Rheinberger, Gao et al. 2018*) playing a key role in plant defense (*Clough, Fengler et al. 2000*). These proteins sense changes in intracellular cNMP levels and regulate numerous cellular responses (*Duszyn, Świeżawska et al. 2019*). Data collected in recent years strongly suggest that cyclic nucleotide gated channels are the main cyclic nucleotides (cNMPs) effectors in plant cells. These channels are important cellular switches that transduce changes in intracellular concentrations of cyclic nucleotides into changes in membrane potential and ion concentrations.

Dnd1 mutants failed to produce a hypersensitive response (HR, Clough et al 2000). This was accomplished by sustaining high levels of salicylic acid, leading to the constitutive expression of pathogenesis-related (PR) genes, and other defense responses. The TV181448-9 mutant, representing E3, is capable of producing, during infection, autonecrotic spots on the leaves, so it might maintain the capability to produce HR. The failure in producing HR is typical of KO mutants. TV181448-9 is different and might counteract pathogens differently. The protein structure suggests an involvement of the region related to the cation sensing (selectively filter and pore domain), since the mutation impacts, from the

structural point of view, the part of the protein related to the pore-loop cation channel (**Figure 9** and **10**)**.** It would seem appropriate to investigate the role that "selectively filter" plays in the DND1 protein and the role that the conformational change of the TV181448-9 mutant might play on cation (Ca^{2+}) recognition. It would be interesting to conduct affinity experiments on different cations using the mutant gene and WT.

Dnd1 mutant exhibits a broad-spectrum resistance in absence of HR to several biotrophic and necrotrophic pathogens since HR is one of the most effective strategy to impede the growth of biotrophic pathogens, however, it is considered to facilitate the growth of necrotrophic pathogens like *B. cinerea* (*Govrin and Levine 2000, Sun, van Tuinen et al. 2017*). Considering the DND1 protein as a CNGC that plays a role in plant defense and senses changes in intracellular cNMP levels to regulate numerous cellular responses, including Ca^{2+} fluxes, the two-amino-acid deletion in the DND1 protein, underlined in the TV181448-9 mutant, may cause a conformational change in the protein's pore-loop cation channel, which affects the recognition of Ca^{2+} ions, leading to dysregulation of Ca^{2+} signalling. Dysregulated Ca^{2+} signalling may prevent the induction of PCD, which is a process that plants use to eliminate old, damaged, or unwanted cells in response to biotic and abiotic stresses. Thus, the hypothesis is that the TV181448-9 mutant's DND1 deletion causes dysregulated Ca^{2+} signalling, leading to a failure to induce PCD and HR in response to pathogen attack.

3.4.3 Whole genome sequencing of TV181448 (E3) and off-target effects

Although the CRISPR/Cas9 approach can result in random mutations at target loci that are functionally equivalent to natural mutations, it is not always easy to predict this equivalence. It has been suggested that the variations observed in edited lines are mostly induced by somaclonal variation during *in vitro* culture, inheritance from maternal plants, and pre-existing variation across the germline (*Sturme, van der Berg et al. 2022*). Whole genome sequencing (WGS) can be used to analyze the substantial equivalence of edited lines with their WT counterparts. WGS provides comprehensive information about genomic variations, such as indels, SNPs, other structural differences and the presence/position of Cas9 residual copies. Several studies have employed WGS analysis of WT and CRISPR/Cas9-edited lines to investigate the specificity of genome editing (*Hahn and Nekrasov 2019*). These studies observed that off-target mutations occur at a much lower level than background mutations due to pre-existing/inherent genetic or/and somaclonal variations (*Tang, Liu et al. 2018, Li, Liu et al. 2019, Wang, Tu et al. 2021, Li, Maioli et al. 2022, Sturme, van der Berg et al. 2022*).

In agreement with these observations, targeted deep sequencing of *SlDND1* (E3, TV181448-9) mutant line at putative 28 off-target loci confirmed the absence of significantly mutated off-targets (**Table S5** and **S6**). The average number of SNPs and mutation rate was comparable

between unedited and edited plants (6,34 x 10^{-5} for edited plants vs. 5,59 x 10^{-5} for un-edited plants, respectively, **Table S4**) and coherent with what was previously observed in tomato (*Li, Maioli et al. 2022*).

3.5 Conclusions

Our results demonstrated, for the first time, a reduced susceptibility to *Oidium neolycopersici* in tomato knock out *dnd1* mutants obtained through gene editing and provided a special, full-length *dnd1* resistant mutant, with reduced fitness costs. This plant lost any T-DNA insertion and showed the presence of a causal mutation (amino acids deletion) in the *DND1* locus, which was indistinguishable from a naturally occurring one. Notably, while *DND1* knockout can result in resistance to pathogens, but with the emergence of pleiotropic effects including dwarfism, the generation of a full-length mutated protein did not. The present findings underscore the importance of precision genetic engineering, as even small changes can have significant impacts on a plant's overall phenotype. These results align with the broader philosophy of "less is more," which emphasizes the importance of simplicity and essentiality in design and construction, a principle that can be applied not only to architecture but also to genetic engineering.

3.6 Supplementary materials

Table S1. List of used primers

target	primer names (forward)	sequence (5' - 3')	primer names (reverse)	sequence (5' - 3')
fragment A (sgRNA10 + sgRNA5 + sgRNA6 + sgRNA8)	SlDND1Fw478	ACGATGACGACATCAATCCA	SlDND1Rv3548	AACGTCGCCAAGCTA
fragment B (sgRNA10)	SlDND1Fw478	ACGATGACGACATCAATCCA	SlDND1Rv1313	TGACCACTTTTAACA
fragment C	SlDND1Fw1138	TTGATCGCCGTAGAAGAGAA	SlDND1Rv1961	CACCTCCTCTGAGCA
fragment D (sgRNA5 + sgRNA6)	SlDND1Fw1759	CATGTAAGTTTTGCCCTGCAT	SlDND1Rv2580	CCAATCAGCAAAGTC
fragment E	SlDND1Fw2369	TGGCCAGAGGAAAGGATATG	SlDND1Rv3214	AACCCTGTGCACTGG
fragment F (sgRNA8)	SlDND1Fw2970	TGGAAATATATTGATGTCTGGATTT	SlDND1Rv3548	AACGTCGCCAAGCTA
elongation factor 1alpha (EF1)	Ef-Fw	GGAACTTGAGAAGGAGCCTAAG	Ef-Rev	CAACACCAACAGCAA
Oidium ITS	Fw-On	CGCCAAAGACCTAACCAAAA	Rv-On	AGCCAAGAGATCCG

Table S2. Plant amount of each mutant/control line

T1 event (E)	TF2 - N° plants	TF2 - genotyped plants	TF2 - phenotyped plants
1	85	30	30
3	38	29	29
4	77	40	40
Moneymaker	20	20	20

Table S3. Sequencing statistics

Sample	Raw reads	Raw data (Gb)	Q20 (%)	Q30 (%)	GC (%)	Cleaned reads	Cleaned data (Gb)
TV181448_9	226.607.022	33,99	96,69	90,76	35,11	225.403.650	33,71
MM-WT	211.892.540	31,78	96,51	90,84	35,31	210.726.616	31,51

Table S4. SNPs statistics

Genotype	plant type	total	homozygous	heterozygous	SNP frequency	1 SNP every (bp)	SNP per Mb
P448_9	edited	49.599	4.622	44.977	6,34 x 10-5	15.776	63,39
MM-WT	in vitro	43.757	4.682	39075	5,59 x 10-5	17.882	55,92

Table S5. Off-target statistics

DND1 - sgRNAs	N° of off-target in genome	in coding	non coding	SNP/indels
10	3	1	2	0
5	15	1	14	0
6	6	0	6	0
8	4	0	4	0
total	28	2	26	0

Table S6. Off-target regions (details)

								N° SNP/indel (200 bp window)	
Off-target genomic coordinates	strand	off-target sequence	DND1 sgRNA	Type of mismatches	PAM	N° of mismatches	in CDS	P448_9	MM-WT
SL4.0ch03:2080748-2080771	-	GAAGagAG_CGCGTGCAcAGc-TGGC	10	A22	A	4	yes	0	0
SL4.0ch04:45774950-45774973	-	GAAaaAAG_gGCGTGaAGAGA-CGGA	10	A22	A	4	-	0	0
SL4.0ch09:58290594-58290617	+	ctAGCAAG_CGgGTGCAGAGc-GGGT	10	A22	A	4	-	0	0
SL4.0ch01:79091035-79091058	+	CCAAgGTG_TTTGaATaTCAA-TGGG	5	A21	A	3	-	0	0
SL4.0ch02:39620876-39620899	+	gCAATGTa_TTTGGATGTtAt-GGGA	5	A22	A	4	-	0	0
SL4.0ch05:10061979-10062002	+	aCAAcGTG_TTTGGATGgCAc-AGGT	5	A22	A	4	-	0	0
SL4.0ch05:25656577-25656600	-	CCAtTtTG_TTaGGATGTCAt-GGGT	5	A22	A	4	-	0	0
SL4.0ch05:26810411-26810434	+	CCcATcTG_TTTGGAccTCAA-TGGT	5	A22	A	4	-	5	5
SL4.0ch05:43518965-43518988	+	CgtATGTG_TTTtGATtTCAA-TGGA	5	A22	A	4	-	0	0
SL4.0ch05:55095463-55095486	-	ttAATGTG_cTTGGATcTCAA-TGGT	5	A22	A	4	-	0	0
SL4.0ch05:63126721-63126744	+	ttAATGTG_TTTGGATGaCcA-AGGA	5	A22	A	4	yes	0	0
SL4.0ch07:16706414-16706437	+	aaAATGTG_TTTaaATGTCAA-TGGT	5	A22	A	4	-	0	0
SL4.0ch08:51340309-51340332	+	CCAATtTt_TTTtGATGaCAA-GGGA	5	A22	A	4	-	0	0
SL4.0ch08:57220971-57220994	+	CtAATGTa_TTTGGAaGTCtA-GGGA	5	A22	A	4	-	0	0
SL4.0ch09:28518116-28518139	-	aCAATGTt_TTTGaATGaCAA-AGGA	5	A22	A	4	-	0	0
SL4.0ch09:41890708-41890731	+	CtAATGTc_TTTtGAaGTCAA-TGGT	5	A22	A	4	-	0	0
SL4.0ch09:46952216-46952239	-	CttATGTG_TaTGGAaGTCAA-AGGT	5	A22	A	4	-	0	0
SL4.0ch11:28106799-28106822	+	CCAATGca_TTTGGATcgCAA-CGGT	5	A22	A	4	-	0	0
SL4.0ch01:8187750-8187773	+	tTtAATGG_ACCAcTTCCAgA-TGGC	6	A22	A	4	-	0	0
SL4.0ch01:30267905-30267928	-	GTCAcgGG_ACCATcTCCAaA-AGGC	6	A22	A	4	-	0	0
SL4.0ch03:61906538-61906561	+	GaCAcTGG_AaCATgTCCATA-TGGT	6	A22	A	4	-	0	0
SL4.0ch04:8210434-8210457	-	GTCAATaa_AgCATTaCCATA-TGGT	6	A22	A	4	-	0	0
SL4.0ch11:48887513-48887536	+	GTtAATGt_ACCgTTTCCcTA-AGGA	6	A22	A	4	-	0	0
SL4.0ch12:31973239-31973262	+	aTCAATtG_ACCATTatCATA-TGGG	6	A22	A	4	-	1	1
SL4.0ch01:80615465-80615488	+	aCCACAAa_CATACTTtAGCa-AGGT	8	A22	A	4	-	0	0
SL4.0ch06:46370796-46370819	-	GCaACAAa_tATACTTaAGCC-TGGA	8	A22	A	4	-	0	0
SL4.0ch07:30450173-30450196	-	GCCAaAtG_CgcACTTGAGCC-CGGA	8	A22	A	4	-	0	0
SL4.0ch07:30468072-30468095	-	GCCAaAAG_CAcACTTaAGCC-CGGA	8	A21	A	3	-	0	0

Chapter 4 - Genomic Analysis Reveals Defective Susceptibility Genes in Tomato Germplasm

4.1 Abstract

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown vegetables in the world and is impacted by many diseases which cause yield reduction or even crop failure. Breeding for disease resistance is thus a key objective in tomato improvement. Since disease arises from a compatible interaction between plant and pathogen, a mutation which alters a plant susceptibility (S) gene facilitating compatibility may induce a broad-spectrum and durable plant resistance.

Here we report on a genome-wide analysis of a set of 360 tomato genotypes, with the goal to identify defective S-genes alleles as a potential source for breeding of resistance.

A whole of 125 gene homologues of 10 S-genes (*PMR4*, *PMR5*, *PMR6*, *MLO1*, *BIK1*, *DMR1*, *DMR6*, *DND1*, *CPR5*, *SR1*) were analysed. Their genomic sequences were examined and SNPs/indels were annotated using the SNPeff pipeline. A total of 54,000 SNPs/indels were identified, among which 1,300 estimated to have a moderate impact (non-synonymous variants), while 120 a high impact (e.g. missense/ nonsense/ frameshift variants). The latter were then analyzed for their effect on gene functionality. A total of 103 genotypes showed one high impact mutation in at least one of the scouted genes, while in 10 genotypes more than 4 high impact mutations in as many genes were detected. A set of 10 SNPs

were validated through Sanger sequencing. Three homozygous S-genes mutants were infected with *Oidium neolycopersici*, and two highlighted a significantly reduced susceptibility to the fungus. The existing mutations fall within the scope of a history of safe use (HoSU), and can be useful to guide risk assessment in evaluating the effect of new genomic techniques (NGTs). The current results provide a resource for tomato genomic-assisted breeding programs as well as tailored gene editing approaches for disease resistance.

4.2 Materials and methods

4.2.1 Data mining on S-Genes

A preliminary blastP (https://ftp.ncbi.nlm.nih.gov/blast) analysis allowed to identify the possible orthologs for susceptibility genes, using information from different plant species (*Schie and Takken 2014*; **Table S1**) and by considering as a preferential choice criterion the e-value (range 0 - $1e^{-10}$) and the percentage of similarity and the query coverage. Since many genes were present in multigene families, filtering criteria varied and previous functional annotations were used to filter out non appropriate candidates.

4.2.2 SNP/indel data

Genotypic data discussed in Lin et al. (2014) was retrieved from SGN (ftp://ftp.solgenomics.net/genomes/tomato_360), as raw vcf files. Data derived from 360 genotypes (**Table S2**) were divided in two merged datasets: 1) a collection of 168 big-fruited *S. lycopersicum* accessions (fruit weight = 111.33 ± 68.19) and 17 modern commercial hybrids (F_1), altogether called “BIG”; 2) the whole collection of 360 genotypes (namely “ALL”). A Principal Component Analysis (PCA) analysis was conducted using the R-based ClustVis suite (https://biit.cs.ut.ee/clustvis). Dataset used for PCA was the whole dataset pruned and filtered using vcftools (https://vcftools.github.io), using the option --max-missing=0.2, for filtering loci.

4.2.3 SNP annotation

SNP data were newly annotated using the v2.5 assembly with ITAG2.4 information. The SnpEff v5.0 program was adopted to infer functional annotation of any SNPs/indels and any potential deleterious effect on protein structure (*Cingolani, Platts et al. 2012*). The effect of each SNP/Indel was classified into four of classes of effects: 1) high effect, as variants changing frameshift thereby introducing/eliminating stop codons or modifying splice sites; 2) moderate effect, as variants altering the aminoacidic sequence; 3) low effect, as synonymous variants in coding regions; and 4) modifier effect, as variants located outside coding sequence (non-transcribed regions or introns). Annotated vcf files from each individual were merged in a single file to integrate the whole information. Bedtools intersect (https://github.com/arq5x/bedtools2) was used to screen for overlaps between the genomic features related to S-genes (in gff format) and the SNP positions emerged from the SnpEff analysis. Functionally annotated SNPs from both BIG and ALL datasets were inspected for different categories (high, moderate and low impact) and were considered and counted for each accession, through custom bash scripts. All the categories were decomposed in homozygous and heterozygous SNPs/indels. A subset of SNPs were validated through Sanger Sequencing (BMR Genomics Service, Padova, Italy) of PCR-amplified gene fragments using the primers listed in **Table S3.**

4.2.4 Single guide RNA (sgRNA) design on target genes

The CRISPR-PLANT v2 platform (http://omap.org/crispr2/CRISPRsearch.html) was used to design sgRNAs in S-genes using the gene code as a query for the scan of the SL2.5 genome. We selected sgRNAs only present in exons, discarding the ones with a high possibility to give off-targets. Then, the rest of the sgRNAs were selected using their quality, based on the mismatch score in their seed sequence. The sgRNAs were divided by the CRISPR-PLANT software into different quality classes (A0, B0, A0.1, B0.1, A1, B1, A2, B2), with A0 being the most specific and B2 being the least specific. The sgRNA sequence of each selected S-gene and the relative quality is reported in the **Table S4**; only A0, A0.1, B0, and B0.1 classes were reported in the output, as highly specific sgRNA for CRISPR-Cas9 mediated genome editing.

4.2.5 Disease assay

Thirty seeds of selected accession, three with mutations (M-82, Puno-I and Droplet) and two controls (VF-36, MoneyMaker) were sowed and then inoculated with the Wageningen University isolate of *O. neolycopersici* (*On*) by spraying 4 weeks-old plants with a suspension of conidiospores obtained from leaves of infected tomato Moneymaker plants and adjusted to a concentration of $3.5x10^4$ spores per ml. Moneymaker variety was used as susceptible control. Inoculated plants were grown at 20 ± 2 °C with 70 ± 15% relative humidity and day length of 16 h in a greenhouse of

Unifarm of Wageningen University & Research, The Netherlands. Disease index scoring was carried out 10 and 12 days after inoculation. Powdery mildew symptoms were scored visually using a scale from 0 to 3 as described by Huibers et al. (2013). Statistical differences between each variety and the control were analyzed using a two-tailed t-test (* $p < 0.05$).

4.3 Results and Discussion

In order to identify natural mutants alleles of tomato S genes, we analysed the genomic diversity of the cultivated tomato germplasm consisting of a set of 360 genotypes. We selected 10 S-genes (**Table S1**), of which some are known to reduce susceptibility to pathogens when knocked-out or knocked-down (*Schie and Takken 2014*). The selected S-genes include *PMR4*, *PMR5*, *PMR6*, *MLO1*, *BIK1*, *DMR1*, *DMR6*, *DND1*, *CPR5*, *SR1,* which facilitate host compatibility by being involved in host recognition and penetration, negative regulation of host immunity, or pathogen proliferation. This work represents the first examination at a genomic level of S-genes and existing defective alleles in the *Solanaceae* family.

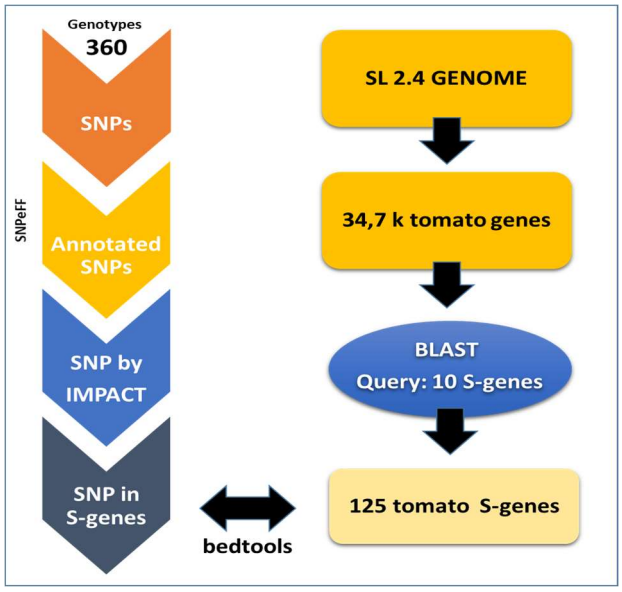


Figure 1. Flowchart of the high-impact SNP mining process within the available sequenced tomato germplasm (data were originally retrieved from *Lin, Zhu et al. 2014*)

Table 1. Statistics on SNP/indel within S-genes related to the 360 panel; the numbers are always formed by two values X/Y where X is the number of SNP observed in the 360 panel and Y in the tomato panel. BIG = 168 *S. lycopersicum* + 17 F1 hybrid genotypes*;* ALL = 168 *S. lycopersicum* + 17 F1 hybrid genotypes + *53 S. pi* + *112 S. cerasiforme* + *10* wild tomatoes.

S-Gene family	Ortholog	Genes	High impact		high impact (SNP/gene)		Moderate impact		low impact		N° variants (total)		Total SNP/gene	
			BIG	*ALL*	*BIG*	*ALL*	*BIG*	*ALL*	*BIG*	*ALL*	*BIG*	*ALL*	*BIG*	*ALL*
PMR4	**Solyc07g053980**	9	8	12	0.9	1.3	95	199	166	288	2,473	4,033	274.8	448.1
PMR5	**Solyc06g082070**	22	5	19	0.2	0.9	172	274	151	257	3,341	5,267	151.9	239.4
PMR6	**Solyc11g008140**	22	17	23	0.8	1.0	104	188	120	187	8,065	12,989	366.6	590.4
DMR1	**Solyc04g008760**	1	1	1	1.0	1.0	6	6	6	12	147	215	147.0	215.0
DMR6	**Solyc03g080190**	2	1	3	0.5	1.5	7	19	7	19	434	775	217.0	387.5
DND1	**Solyc02g088560**	3	2	2	0.7	0.7	16	38	18	46	410	806	136.7	268.7
MLO1	**Solyc04g049090**	13	6	16	0.5	1.2	67	120	60	121	5,309	7,787	408.4	599.0
CPR5	**Solyc04g054170**	1	0	0	0.0	0.0	2	6	6	9	653	873	653.0	873.0
BIK1	**Solyc10g084770**	51	18	41	0.4	0.8	237	452	272	500	12,789	21,376	250.8	419.1
SR1	**Solyc01g105230**	1	0	2	0.0	2.0	9	24	4	15	89	257	89.0	257.0
Total	-	125	58	119	-	-	715	1,326	810	1,454	33,710	54,378	-	-
Average	-	13	6	12	0.5	1.0	72	133	81	145	3,371	5,438	269.5	429.7

Initially, a blastP analysis was performed (**Figure 1**) to identify homologs from the 10 chosen genes. A total of 125 S-gene homologs were obtained and used for further analyses (**Table 1**). The genome sequences of 360 accessions (*Lin, Zhu et al. 2014*) were reanalyzed using bioinformatics, and 11,620,517 SNPs/indels were detected across approximately 30,000 tomato gene locations (**Table S2**, genotypes). SNPs over 185 accessions (BIG) were 7,744,233 (67%). In the 125 gene member subset (**Table 1**), 54,000 SNPs/indels were observed using the SNPeff pipeline. Among these, 51,000 had no effect on protein function, being synonymous SNPs or located in intergenic regions. A total of 1,500 SNPs had a low impact and 1,300 had a moderate impact. A total of 119 high-impact SNPs were observed. The distribution of these SNPs was studied among the 10 S-genes (**Figure 2**).

Despite differences in the number and type of genes considered, recent analyses on nucleotide diversity of S-genes in other species such as apple (*Pessina, Pavan et al. 2014, Tegtmeier, Pompili et al. 2020*) and grape

(*Pirrello, Zeilmaker et al. 2021*) have been conducted. The number and density of SNPs observed in grape (*V. vinifera*) was ~15 SNPs per Kb (1SNP every 66 bp), while in both wild species and hybrid/wild Vitis species was 18 SNPs per Kb (1 SNP every 55 bp, Pirrello et al 2021); in apple (*M. domestica),* in *Mlo1*-like genes, values of ~41 SNPs per Kb and 1SNP every 24 bp were observed (*Pessina, Palmieri et al. 2017*). These values were higher than the ones we obtained; i.e. 1 SNP every 1,031 bp in the whole dataset and 1 SNP every 472 bp in tomato (BIG), reflecting the different genetic structures of the species, the homozygosity level and their domestication history.

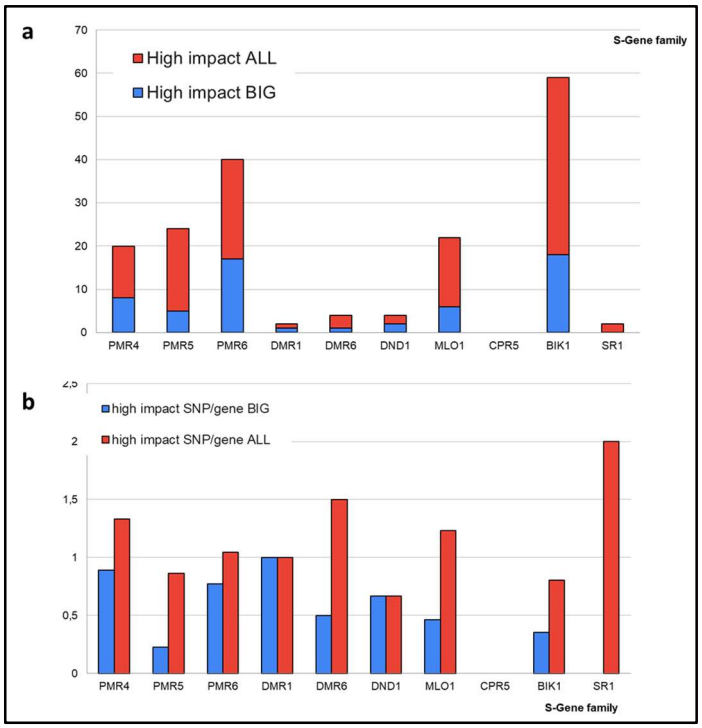


Figure 2. a) Distribution of high impact SNPs in the S-genes (y-axis scale= N° SNPs) and b) relative SNP density (y-axis scale= N° SNPs/gene);

Our analysis (**Table 1**) showed that when both wild and cultivated tomato genotypes were considered, the number of SNPs and their density were higher (119 SNPs with a density of 1 SNP per gene). However, when only "big tomato" genotypes were considered, the number of SNPs and their density was halved (58 SNPs with a density of 0.5 SNPs per gene); this suggests that there is a specific reservoir of S-gene alleles in the wild tomato germplasm that can be used for breeding. We analyzed the potential impact of 119 highly detrimental mutations, including frameshift-inducing mutations that result in major damage such as knock-out mutations. However, there are also many moderate-impact mutations (1326) that may lead to changes in protein conformation and function. Although we did not delve into these effects in details, they are worth monitoring in order to gain a deeper understanding of altered S-genes. Among the 119 SNPs, ten were validated in 10 genotypes readily available within the research group facilities (http://eurisco.ecpgr.org) through Sanger sequencing with a 90% validation rate (**Table S3**); indeed, some not validated SNPs were mutations detected in a heterozygous condition or possessed the same allelic profile as the reference; the emergence of such heterozygous/reference-like SNPs during the validation step can be explained by the high genetic diversity existing within the analyzed germplasm set (**Figure 3**), as observed in Li et al (2022).

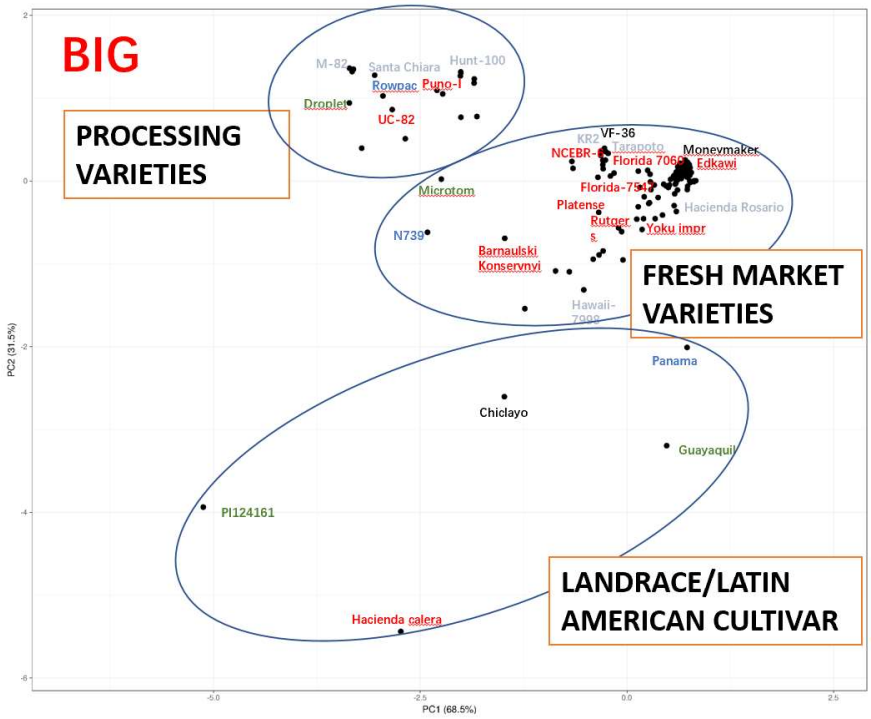


Figure 3. Genotypes accumulating multiple mutations in S-genes. In light blue are reported genotypes with 5 or more SNPs, in green genotypes with 4 SNPs, in gray genotypes with 3 SNPs, in red genotypes with 2 SNPs and in black the rest of the genoypes (0-1 SNPs).

The number of SNPs in each family was related to their length, but the SNP density appeared higher in certain genes (**Table 1**, *PMR 4*, *PMR5*, *PMR6*, *MLO1*, *BIK1*, *CPR5*) and lower in others (*DMR1*, *DND1*, *SR1*, DMR6). This difference might be due to the fact that some genes are single-copy, or present in a nodal position (hub) within the cell regulation network, hardly supporting deleterious SNPs (*Lenser and Theißen 2013*). On the contrary, the presence of multiple genes in a gene family may mitigate the impact of deleterious mutations (*Acquadro, Barchi et al. 2017*).

In specific cases, such as *DMR1*, a single-copy tomato gene exhibited a deleterious mutation (a gained stop codon) in homozygosity, but its potential impact on protein functionality was likely reduced, as the causative SNP was located in the last 6 codons of the gene (1129/1134) (**Table S6**). In some others (es. *BIK1*-like genes), many occurrences were observed since all the 51 serine-theronine kinases, belonging to the RLCK (clade VII) repertoire, were analysed.

4.3.1 Homozygous SNPs/indels

The number of genotypes with two SNPs was 174 (whole dataset) and 76 (BIG tomatoes, **table 2**), while those with three or more SNPs were 114 and 14 (**Figure 4**), respectively. This high representation can be explained by the presence of multigene families such as *BIK1*-like that might present some degree of redundancy. While examining those high-impact mutations, results revealed that certain mutations appeared frequently in the cultivated germplasm and were preserved across various genotypes, as displayed in **Tables S3** and **Table S6**. One example is *BIK1* (Solyc05g024290, SNP in chr5:31013858), which could be maintained under selective pressure in clustering genotypes within the germplasm materials (**Figure 3,** e.g.: Rowpac, M-82, Santa Chiara, Hunt101, Puno I, E-6203). The genotypes carrying a high number of SNPs (3 or more) were approximately a dozen (e.g. Panama, N 739, Rowpac, Micro-Tom, Guayaquil, Droplet, M-82, Hawaii 7998, KR2), and information about these SNPs is provided in **Table S5**. Certain mutations, such as *BIK1*-

like/Solyc01g008860 and *DMR1*-like/Solyc04g008760 in specific genotypes (e.g. N-739/TS-074), appeared to be of lower relevance as they were present in the final percentile of the sequence length (**Table S6**).

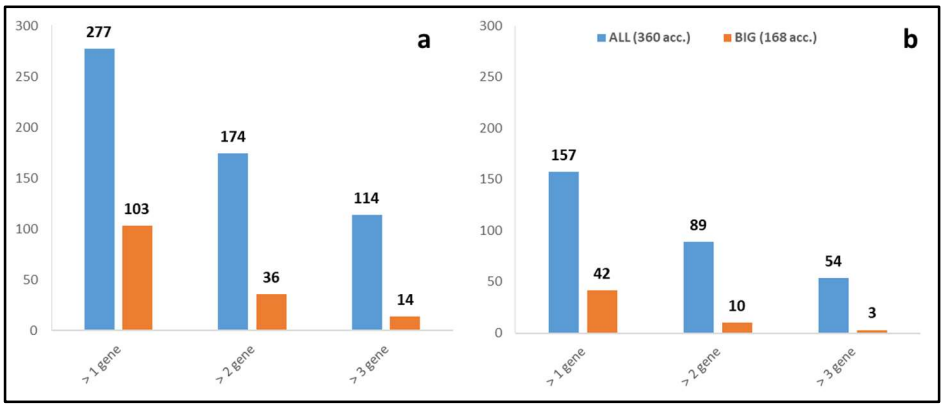


Figure 4. a) Genotypes accumulating mutations in s-genes in a) homozygous and in b) heterozygous state (y-axis scale= N° genotypes).

Table 2. Detailed statistics on the allelic richness the tomato genotypes (BIG) considering the high impact SNPs in the whole gene dataset and in the selected S-genes.

				High impact SNPs			High impact SNPs in S-genes		
Genotype	**Name**	**TGRC/PI-CGN/EA**	**Categories**	*Total*	*Homoz.*	*Heteroz.*	*Total*	*Homoz.*	*Heteroz.*
TS-214	Panama	-/-/-	Landrace	620	569	51	7	6	1
TS-074	N 739	-/-/-	Fresh Market	647	587	60	5	5	0
TS-186	Rowpac	LA3214/-/-	Modern Processing	445	423	22	5	5	0
TS-007	Micro-Tom	LA3911/-/-	Modern Fresh Market	901	724	177	4	4	0
TS-224	Guayaquil	LA0410/PI 258474/-	Landrace/Latin American cultivar	779	767	12	4	4	0
TS-296	Droplet	-/-/-	-	719	668	51	4	4	0
TS-409	-	-/PI124161/-	Landrace	1526	1263	263	4	4	0
TS-003	M-82	LA3475/-/-	Modern Processing	515	424	91	3	3	0
TS-004	Hawaii 7998	LA3856/-/-	Inbreed line	692	606	86	3	3	0
TS-011	KR2	-/-/-	Modern Fresh Market	565	392	173	5	3	2
TS-135	Hacienda Rosario	LA0466/PI 258469/-	Landrace/Latin American cultivar	334	301	33	3	3	0
TS-150	Tarapoto	LA2285/-/-	Landrace/Latin American cultivar	352	326	26	3	3	0
TS-190	Santa Chiara	-/-/-	cultivar	437	366	71	3	3	0
TS-277	Hunt100	LA3144/-/-	Modern Processing	266	236	30	3	3	0
TS-005	Edkawi	LA2711/-/-	Vintage Fresh Market	191	116	75	3	2	1
TS-012	yoku improvement	-/-/-	Modern Fresh Market	505	400	105	4	2	2
TS-078	-	-/-/EA02895	Processing tomato	300	273	27	2	2	0
TS-089	-	-/-/EA01185	Processing tomato	457	371	86	3	2	1
TS-090	-	-/-/EA02753	Cocktail tomato	368	286	82	2	2	0
TS-108	Puno I	-/-/EA01989	Processing tomato	334	312	22	2	2	0
TS-121	NC EBR-6	LA3846/-/-	Modern Fresh Market	267	225	42	2	2	0
TS-122	Rutgers	LA1090/-/-	Vintage Fresh Market	70	58	12	2	2	0
TS-127	Hacienda Calera	LA0113/-/-	Landrace/Latin American cultivar	1589	886	703	3	2	1
TS-143	Florida 7547	LA4025/-/-	Modern Fresh Market	182	163	19	2	2	0
TS-147	-	-/-/-	-	482	404	78	2	2	0
TS-171	UC-82	LA1706/-/-	Modern Processing	334	305	29	3	2	1
TS-204	Florida 7060	LA3840/-/-	Modern Fresh Market	247	202	45	2	2	0
TS-220	Barnaulski Konservnyi	-/-/-	cultivar	535	455	80	2	2	0
TS-225	-	-/PI330336/EA05747	Processing tomato	172	108	64	3	2	1
TS-226	Microtom	-/-/-	cultivar	436	400	36	3	2	1
TS-228	M-82	-/-/-	cultivar	398	369	29	2	2	0
TS-234	-	-/-/EA01371	Processing tomato	234	219	15	2	2	0
TS-237	Platense	LA3243/-/-	Vintage Fresh Market	190	145	45	2	2	0
TS-245	-	-/-/EA03126	Processing tomato	314	248	66	4	2	2
TS-276	-	-/-/EA03650	Cocktail/Processing tomato	160	124	36	3	2	1
TS-292	-	-/-/EA06902	Processing tomato	298	278	20	2	2	0
TS-002	Moneymaker	LA2706/-/-	Vintage Fresh Market	207	151	56	2	1	1
TS-008	E-6203	LA4024/-/-	Modern Processing	380	302	78	4	1	3
TS-009	Ailsa Craig	LA2838A/-/-	Vintage Fresh Market	182	128	54	2	1	1
TS-041	-	-/-/EA02435	Cocktail tomato	262	218	44	1	1	0
TS-043	Moneymaker	-/-/EA00840	Fresh Market	166	130	36	1	1	0
TS-045	-	-/PI303718/EA05578	Processing tomato	198	176	22	1	1	0
TS-047	-	-/-/EA01960	Processing tomato	144	125	19	1	1	0
TS-049	Earliana	LA3238/-/-	Vintage Processing	149	139	10	1	1	0
TS-051	-	-/-/-	-	127	100	27	1	1	0
TS-052	05-4126 (97-49-2)	-/-/-	cultivar	328	281	47	2	1	1
TS-055	-	-/-/EA00448	-	176	117	59	1	1	0
TS-058	-	-/-/EA03577	Processing tomato	131	119	12	1	1	0
TS-059	-	-/-/EA02898	Processing tomato	690	516	174	1	1	0
TS-068	Chiclayo	LA0395/-/-	Latin American cultivar	1640	185	1455	9	1	8
TS-069	Huachinango	LA1459/-/-	Latin American cultivar	247	231	16	1	1	0
TS-073	Quarantino	-/-/-	-	126	105	21	1	1	0
TS-076	-	-/-/EA01230	Processing tomato	156	129	27	1	1	0
TS-081	-	-/-/EA02761	Processing tomato	182	155	27	1	1	0
TS-085	-	-/-/-	-	474	237	237	3	1	2
TS-086	-	-/-/EA01684	-	139	118	21	1	1	0
TS-095	Moneymaker	-/-/-	Fresh Market	176	147	29	2	1	1
TS-100	-	-/-/EA03456	Processing	134	117	17	1	1	0
TS-112	-	-/-/EA03083	Processing tomato	175	148	27	1	1	0
TS-115	-	-/-/EA03426	Processing tomato	243	222	21	1	1	0
TS-117	Scatolone di bolsena	-/-/-	Landrace	214	104	110	1	1	0
TS-125	-	-/-/EA00422	Processing tomato	241	137	104	2	1	1
TS-128	Pearson	LA0012/-/-	Vintage Processing	245	214	31	1	1	0
TS-132	Primabel	LA3903/-/-	Vintage Fresh Market	136	116	20	1	1	0
TS-133	Peto95-43	LA3528/-/-	Modern Processing	307	264	43	1	1	0
TS-137	Spagnoletta	-/-/-	Landrace	305	136	169	1	1	0
TS-142	Roma	-/-/-	Vintage cultivar	136	122	14	2	1	1
TS-151	T-5	LA2399/-/-	Modern Fresh Market	625	529	96	2	1	1
TS-152	Santa Cruz B	LA1021/-/-	Landrace/Latin American cultivar	177	160	17	1	1	0
TS-155	Condine Red	LA0533/-/-	Vintage Fresh Market,Monogenic	130	119	11	1	1	0
TS-157	-	-/-/EA03648	Processing tomato	121	104	17	1	1	0
TS-160	-	-/-/EA03533	Processing tomato	221	185	36	1	1	0
TS-163	Marmande	LA1504/-/-	Vintage Fresh Market	129	114	15	1	1	0
TS-166	Piura	LA0404/-/-	Landrace/Latin American cultivar	178	163	15	2	1	1
TS-167	Tegucigalpa	LA0147/-/-	Landrace/Latin American cultivar	158	135	23	1	1	0
TS-168	-	-/-/-	Landrace	337	256	81	1	1	0
TS-174	-	-/-/EA00304	Processing tomato	212	191	21	1	1	0
TS-176	-	-/-/EA02669	Processing tomato	197	190	7	1	1	0
TS-177	-	-/-/EA01155	Processing tomato	127	108	19	1	1	0
TS-180	-	-/-/EA02728	Processing tomato	116	82	34	1	1	0
TS-183	-	-/-/EA02764	Processing tomato	154	133	21	1	1	0
TS-184	Tarapoto	LA2283/-/-	-	338	225	113	2	1	1
TS-193	Pantano dArdea	-/-/-	Landrace	170	121	49	1	1	0
TS-194	-	-/-/-	-	167	143	24	1	1	0
TS-197	Libanese	-/-/-	Landrace	165	122	43	1	1	0
TS-198	-	-/-/EA00512	-	153	129	24	1	1	0
TS-200	Hot set	LA3320/-/-	cultivar	187	135	52	1	1	0
TS-203	Bell pepper-like	-/-/-	Landrace	177	110	67	1	1	0
TS-206	Prince Borghese	LA0089/-/-	Vintage Fresh Market	26	22	4	1	1	0
TS-211	NC 84173	LA4354/-/-	Modern Fresh Market	425	366	59	1	1	0
TS-215	Vrbikanske Nizke	-/-/-	cultivar	183	126	57	2	1	1
TS-235	-	-/-/EA00892	Processing tomato	46	44	2	1	1	0
TS-239	NC EBR-5	LA3845/-/-	Modern Fresh Market	126	109	17	2	1	1
TS-242	Ayacucho	LA0134C/-/-	Latin American cultivar	530	332	198	1	1	0
TS-251	-	-/PI647249/EA04001	-	150	128	22	1	1	0
TS-256	-	LA2260/0/EA00744	Latin American cultivar	477	415	62	1	1	0
TS-261	-	LA1511/-/EA01444	Wild species	246	145	101	2	1	1
TS-263	Rio Grande	LA3343/-/-	Processing tomato	213	183	30	1	1	0
TS-264	King Humbert #1	LA0025/-/-	Vintage Fresh Market	134	119	15	1	1	0
TS-268	-	-/-/EA01915	cultivar	147	130	17	1	1	0
TS-274	-	-/-/EA03613	Cocktail/Processing tomato	266	241	25	1	1	0
TS-278	Early Santa Clara	LA0517/-/-	Vintage Processing	207	187	20	1	1	0
TS-400	-	-/-/-	inbred line	453	398	55	1	1	0

4.3.2 Heterozygous SNPs/indels

The incidence of deleterious SNPs in S-genes in a heterozygous condition was comparatively lesser than that of homozygous ones, as observed in both the complete germplasm collection (ALL) and the BIG tomato varieties (**Figure 4**). This frequency may be due to the genetic structure of tomato as an inbred species, which tends to have a low number of heterozygous mutations (*Li, Maioli et al. 2022*). However, the number appears relatively high because such mutations, although harmful, can be maintained in the genome if the normal allelic copy continues to function. This high frequency is particularly noticeable in the case of multiple member S-genes (e.g. BIK1-like) that may exhibit some redundancy and have no effects, or due to the position of the SNP within the gene (e.g. DMR1/Solyc04g008760 in TS-113; BIK1-like/Solyc01g008860 in Chiclayo, **Table S6**). If two SNPs are considered, the number of genotypes increases to 174 (ALL) and 76 (BIG), and if three SNPs are considered, the number of genotypes decreases to 114 (ALL) and 14 (BIG). Some heterozygous mutants for S-genes were also identified, which have a 50% chance of acquiring resistance through natural mutagenic effects (**Table 2**).

4.3.3 sgRNA design

Introgression of S genes' alleles through breeding into elite varieties is possible, but is a long and labour-intensive process and has limitations due to linkage drag. To address this issue, in analogy with the work from

(*Prajapati and Nain 2021*), sgRNA sequences were designed for eight of the proposed S-genes (**Table S4**) and made available to a wider audience through the creation of optimal gene editing constructs. In total, 113 sgRNA were designed, considering only the highly specific categories (A0, B0, A0.1, and B0.1) for CRISPR-Cas9-mediated genome editing to minimize off-target events. Specifically, 39 A0, 20 A0.1, 48 B0, and 6 B0.1 sgRNAs were designed. Each gene was equipped with at least one useful sgRNA, with *PMR4*, *PMR5*, *PMR6*, *MLO1*, and *BIK1* having the most sgRNAs at 13, 15, 20, 8, and 50, respectively.

4.3.4 Disease assay

As a preliminary assay, five genotypes, readily available within the research group facilities (http://eurisco.ecpgr.org), were selected for a disease assay to assess their resistance to *O. neolycopersici* (*On*). They included three varieties (Punol/TS-108, Droplet/TS-296, M82/TS-003) with deleterious SNPs and two varieties with no deleterious SNPs in the S-genes (VF-36/TS-01 and Moneymaker/TS-02). M-82 carried three mutated genes (*BIK1-like*: Solyc05g024290 and Solyc04g050970; *PMR4*-like/Solyc01g073750), which introduced a stop codon and produced truncated proteins. Puno-I carried two mutated genes (*BIK1*/Solyc05g024290) and *PMR4*/Solyc01g073750) in the middle of the gene, resulting in truncated proteins. Droplet had four high impact mutations, including one in the *BIK1-like* gene (Solyc04g050970), two in the *Mlo1-like* gene (Solyc02g077570), and one in the *PMR4-like* gene

(Solyc01g073750). These varieties showed sequences that predicted the presence of truncated susceptibility proteins in a homozygous state. To assess whether these selected varieties with deleterious SNPs in S-genes had higher resistance to PM, we inoculated all of them with *O. neolycopersici* and evaluated the disease index (**Table 3**). Two of them (Puno1 and M-82) showed reduced susceptibility to *O. neolycopersici* based on visual scoring of disease symptoms, while no significant differences in the disease index were observed in the others. The reason for this incomplete resistance may lie in the genes under consideration (*BIK1-like*: Solyc05g024290 and Solyc04g050970). The RLCK family encodes for a series (~50) of serine/threonine-protein kinases with a role in post-translational regulation through, in the case of BIK-1, the phosphorylation of FLS2 and BAK1 (*Lu et al. 2010, Xu et al. 2013*). The latter gene is involved in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) signaling, including calcium signaling, and defense responses downstream of FLS2. Being the RLCK- subfamily VII a large clade (46 members in Arabidopsis, 51 in the present work), whose members play a role both specifically or redundantly in immune signaling, some *BIK1*-like could have vicarious role in case of the emergence of mutant forms (e.g.: Solyc04g050970 (49.186.199 bp, chromosome 4) in M82 and Solyc05g024290 (31.013.858 bp, chromosome 5) in PunoI and M82 genotypes. Moreover, the genes *Mlo1-like* (Solyc02g077570) and *PMR4-like* (Solyc01g073750) genes were found to differ from the *SlMlo1* and *PMR4* genes (**Table 1**), which were previously known to provide

complete resistance in the presence of a loss-of-function allele. Our research was an extensive genomic study incorporating a small pilot study on the impact of mutations on pathogenesis. We carried out pathogenesis assays using plant material readily available in our academic institutions. However, restrictions imposed by the recent Nagoya protocol on plant material transfer and difficulties in obtaining material for phytosanitary reasons, limited our scope. We propose further research on accessions such as Panama, N739, and Rowpac (which have 6, 5, and 5 homozygous deleterious SNPs respectively) - a poorly characterized plant material that deserves further investigation. These materials should also be analyzed using different fungal pathogens (*Phytophthora infestans*, *Botrytis*, etc.) or bacteria (*Pseudomonas syringeae*).

Table 3. Disease assay with *On* performed on four varieties and a control variety (Moneymaker). Disease score values here reported were compared with the ones derived from the controls. Statistical differences among varieties/control were analyzed with a two-tailed t test (*, $p < 0.05$).

Variety	Code	Type	Disease Score (0-3)	Std. error	n	p-value	reduction (%)	class
VF-36	TS-1	control	3.00	0	20	-	-	a
Money Maker	TS-2	assayed	2.96	0.03	28	0.326189	1.2%	a
Droplet	TS-296	assayed	2.87	0.09	15	0.164318	4.4%	a
M-82	TS-003	assayed	2.42	0.14	33	0.000367	19.2%	b
Puno-I	TS-108	assayed	2.67	0.11	21	0.004900	11.1%	b

4.4 Conclusions

Here we performed a genomic survey of a wide set of tomato genotypes, with the goal to identify defective alleles of susceptibility genes. Our survey highlighted the presence of natural homozygous/heterozygous mutant alleles. Furthermore, we validated the identified SNPs through Sanger sequencing and found that some homozygous mutants exhibited a significantly reduced susceptibility to powdery mildew. Thus, our results provide a valuable resource for plant genetics, with potential applications in genomic-assisted breeding programs for resistance to biotic stresses. However, one cannot fail to consider that the introgression of desirable alleles into elite genotypes is a time-consuming process, often hindered by challenges such as linkage drag. For this reason we have also provided new insights on Single guide RNA (sgRNA) design for the application of a gene editing approach aimed at disabling the targeted genes, as it represents a powerful alternative for the obtainment of tomato elite genotypes resistant to biotic stresses. Additionally, this genomic survey can aid in guiding and proportionate risk assessment in the evaluation of the new genomic techniques (NGTs) by tracking existing alleles in the context of "History of Safe Use " (HoSU, *Organisms, Mullins et al. 2022a*).

4.5 Supplementary materials

Table S1. S-genes

Acc. Number	ITAG 2.5	Name	function	
O48814	Solyc10g084770	BIK1	Serine/threonine-protein kinase	OS=Arabidopsis thaliana
Q9LV85	Solyc04g054170	CPR5	Constitutive expresser of pathogenesis-related 5	OS=Arabidopsis thaliana
Q8L7R2	Solyc04g008760	DMR1	Homoserine kinase	OS=Arabidopsis thaliana
A0A178UM31	Solyc03g080190	DMR6	Plant invertase/pectin methylesterase inhibitor superfamily protein	OS=Arabidopsis thaliana
O65718	Solyc02g088560	DND1/CNGC2	Cyclic nucleotide-gated	OS=Arabidopsis thaliana
A0A097KYB3	Solyc04g049090	MLO1	MLO-like protein	OS=Solanum melongena
Q9ZT82	Solyc07g053980	PMR4	Callose synthase	OS=Arabidopsis thaliana
Q9LUZ6	Solyc06g082070	PMR5	Pectate lyase	OS=Arabidopsis thaliana
A0A178VCI0	Solyc11g008140	PMR6	Pectate lyase	OS=Arabidopsis thaliana
A0A178VUE5	Solyc01g105230	SR1	calmodulin-binding protein	OS=Arabidopsis thaliana

Table S2. Accessions

Individual code	Individual code	Group	TGRC	PI CGN#	EA #	Name	Botanical variety	Categories
TS-14	TS-014	PIM	LA1547	-	-	Chota to El Angel	*S. pimpinellifolium*	Wild species
TS-15	TS-015	PIM	LA2093	-	-		*S. pimpinellifolium*	Wild species
TS-16	TS-016	PIM	LA1246	PI365912	-		*S. pimpinellifolium*	Wild species
TS-17	TS-017	PIM	LA0373	-	-	Culebras	*S. pimpinellifolium*	Wild species
TS-18	TS-018	PIM	LA1579	-	-		*S. pimpinellifolium*	Wild species
TS-19	TS-019	PIM	LA1589	PI407545	EA01467		*S. pimpinellifolium*	Wild species
TS-20	TS-020	PIM	LA0442	-	-	Sechin	*S. pimpinellifolium*	Wild species
TS-21	TS-021	PIM	LA1375	PI365967	-		*S. pimpinellifolium*	Wild species
TS-22	TS-022	PIM	LA1269	PI365957	-	Pisiquillo	*S. pimpinellifolium*	Wild species
TS-23	TS-023	PIM	LA1521	-	-	El Pinon, Asia	*S. pimpinellifolium*	Wild species
TS-24	TS-024	PIM	-	-	-		*S. pimpinellifolium*	Wild species
TS-50	TS-050	PIM	LA0417	-	EA00565		*S. pimpinellifolium*	Wild species
TS-77	TS-077	PIM	LA1237	PI365910	-	Atacames	*S. pimpinellifolium*	Wild species
TS-79	TS-079	PIM	LA1924	-	-	Piedras Gordas	*S. pimpinellifolium*	Wild species
TS-92	TS-092	PIM	LA1582	PI407539	-	Punto Cuatro	*S. pimpinellifolium*	Wild species
TS-123	TS-123	PIM	LA0722	-	-	Trujillo	*S. pimpinellifolium*	Wild species
TS-124	TS-124	PIM	LA1245	PI365911	-	Santa Rosa	*S. pimpinellifolium*	Wild species
TS-144	TS-144	PIM	LA0411	PI251319	-	Pichilingue	*S. pimpinellifolium*	Wild species
TS-145	TS-145	PIM	LA1617	-	-	Tumbes south	*S. pimpinellifolium*	Wild species
TS-156	TS-156	PIM	LA2181	-	-	Balsa Huaico	*S. pimpinellifolium*	Wild species
TS-164	TS-164	PIM	LA1584	PI407541	-	Jayanca to La Vina	*S. pimpinellifolium*	Wild species
TS-182	TS-182	PIM	LA2183	-	-	Corral Quemado	*S. pimpinellifolium*	Wild species
TS-222	TS-222	PIM	-	-	-	Wva 700	*S. pimpinellifolium*	Wild species
TS-244	TS-244	PIM	LA1578	-	-	Santa Marta	*S. pimpinellifolium*	Wild species
TS-265	TS-265	PIM	LA0400	-	-	Hacienda Buenos Aires	*S. pimpinellifolium*	Wild species
TS-266	TS-266	PIM	LA1478	-	-	Santo Tome	*S. pimpinellifolium*	Wild species
TS-267*	TS-267	PIM	LA2660	-	-	San Ignacia de Moxos	*S. pimpinellifolium*	Wild species
TS-291	TS-291	PIM	LA1589	PI407545	EA01467	Viru to Galumga	*S. pimpinellifolium*	Wild species
TS-410	TS-410	PIM	-	PI370093	-		*S. pimpinellifolium*	Wild species
TS-411	TS-411	PIM	LA0480	-	-		*S. pimpinellifolium*	Wild species
TS-412	TS-412	PIM	LA0722	-	-		*S. pimpinellifolium*	Wild species
TS-413	TS-413	PIM	LA1242	-	-		*S. pimpinellifolium*	Wild species
TS-414	TS-414	PIM	LA1341	PI379020	-		*S. pimpinellifolium*	Wild species
TS-415	TS-415	PIM	LA1596	PI407552	-		*S. pimpinellifolium*	Wild species
TS-416	TS-416	PIM	LA1847	-	-		*S. pimpinellifolium*	Wild species
TS-417	TS-417	PIM	LA1933	-	-		*S. pimpinellifolium*	Wild species
TS-418	TS-418	PIM	LA2147	-	-		*S. pimpinellifolium*	Wild species
TS-419	TS-419	PIM	LA2173	-	-		*S. pimpinellifolium*	Wild species
TS-420	TS-420	PIM	LA2184	-	-		*S. pimpinellifolium*	Wild species
TS-421	TS-421	PIM	LA2187	-	-		*S. pimpinellifolium*	Wild species
TS-422	TS-422	PIM	LA2425	-	-		*S. pimpinellifolium*	Wild species
TS-424	TS-424	PIM	-	PI126947	-		*S. pimpinellifolium*	Wild species
TS-425	TS-425	PIM	-	PI126925	-		*S. pimpinellifolium*	Wild species
TS-429	TS-429	PIM	-	PI126954	-		*S. pimpinellifolium*	Wild species
TS-432	TS-432	PIM	-	PI270449	-		*S. pimpinellifolium*	Wild species
TS-433	TS-433	PIM	-	PI370093	-		*S. pimpinellifolium*	Wild species
TS-434	TS-434	PIM	LA1591	PI407547	-	SAL1871	*S. pimpinellifolium*	Wild species
TS-435	TS-435	PIM	LA1595	PI407551	-	SAL1875	*S. pimpinellifolium*	Wild species
TS-437	TS-437	PIM	LA1578	-	-	SAL1858	*S. pimpinellifolium*	Wild species
TS-438	TS-438	PIM	-	-	-	CN7542	*S. pimpinellifolium*	Wild species
TS-439	TS-439	PIM	LA2656	PI503524	-		*S. pimpinellifolium*	Wild species
TS-440	TS-440	PIM	LA2857	-	-		*S. pimpinellifolium*	Wild species
TS-441	TS-441	PIM	LA4431	-	-		*S. pimpinellifolium*	Wild species
TS-13	TS-013	CER	-	-	-	Pime-Bruce	*S. pimpinellifolium*	Wild species
TS-25	TS-025	CER	-	-	-	Clémentine	*S. lycopersicum var cerasiforme*	cultivar
TS-26	TS-026	CER	-	-	-		*S. lycopersicum var cerasiforme*	cultivar
TS-27	TS-027	CER	-	-	-	N135 Green Gage	*S. lycopersicum var cerasiforme*	cultivar
TS-28	TS-028	CER	-	-	-	N 347 Yablochnyi	*S. lycopersicum var cerasiforme*	
TS-29	TS-029	CER	-	-	-		*S. lycopersicum var cerasiforme*	cultivar
TS-30	TS-030	CER	LA1204	-	-		*S. lycopersicum var cerasiforme*	Wild species
TS-31	TS-031	CER	LA1464	-	-		*S. lycopersicum var cerasiforme*	Wild species
TS-32	TS-032	CER	LA0172	-	-	Santa Cruz	*S. lycopersicum var cerasiforme*	Latin American cultivar
TS-33	TS-033	CER	LA2137	-	-		*S. lycopersicum var cerasiforme*	Wild species
TS-34	TS-034	CER	LA2675	-	-		*S. lycopersicum var cerasiforme*	Wild species
TS-35	TS-035	CER	-	-	-	tomate Richters	*S. lycopersicum var cerasiforme*	uncertain
TS-36	TS-036	CER	-	PI187002-1	-		*S. lycopersicum var cerasiforme*	cultivar
TS-37	TS-037	CER	-	-	-	L. pimpinellifolium atypique, site 10 (F300045)	*S. lycopersicum var cerasiforme*	Wild Species
TS-38	TS-038	CER	-	PI129088	-		*S. lycopersicum var cerasiforme*	cultivar

TS-39	TS-039	CER	-	-	-	Cerise Gold	*S. lycopersicum var cerasiforme*	cultivar
TS-40	TS-040	CER	-	-	-	Cerise VFNT	*S. lycopersicum var cerasiforme*	cultivar
TS-53	TS-053	CER	LA2095	-	-		*S. lycopersicum var cerasiforme*	Wild species
TS-54	TS-054	CER	-	-	-	cerise rose	*S. lycopersicum var cerasiforme*	cultivar
TS-56	TS-056	CER	LA1320	PI365923	-		*S. lycopersicum var cerasiforme*	Wild species
TS-57	TS-057	CER	LA1307	PI378998	-		*S. lycopersicum var cerasiforme*	Wild species
TS-61	TS-061	CER	LA2670	-	-	Huayvaruni	*S. lycopersicum var cerasiforme*	Wild species
TS-62	TS-062	CER	-	-	EA02959			
TS-63	TS-063	CER	-	-	-			
TS-64	TS-064	CER	-	-	EA03525			Cocktail tomato
TS-65	TS-065	CER	LA1482	-	-		*S. lycopersicum var cerasiforme*	Wild species
TS-66	TS-066	CER	LA1388	PI379009	-	H707	*S. lycopersicum var cerasiforme*	Wild species
TS-67	TS-067	CER	-	-	EA02304			Cocktail tomato
TS-70	TS-070	CER	-	-	-	Pyriforme	*S. lycopersicum var cerasiforme*	cultivar
TS-71	TS-071	CER	-	-	-	Ohmiya SunCerise	*S. lycopersicum var cerasiforme*	cultivar
TS-72	TS-072	CER	-	-	-	Principe Borghese		Vintage cultivar
TS-75	TS-075	CER	-	-	-	Poire jaune	*S. lycopersicum var cerasiforme*	cultivar
TS-83*	TS-083	CER	-	-	EA01953		*S. lycopersicum var cerasiforme*	cultivar
TS-84	TS-084	CER	-	-	-			
TS-87	TS-087	CER	LA1701	-	-	Trujillo	*S. lycopersicum var cerasiforme*	Latin American cultivar; Wild species
TS-91	TS-091	CER	-	-	-	N 2759 Enano	*S. lycopersicum var cerasiforme*	cultivar
TS-94	TS-094	CER	-	-	-	Farthest North	*S. lycopersicum var cerasiforme*	cultivar
TS-96	TS-096	CER	LA1456	-	-		*S. lycopersicum var cerasiforme*	Wild species
TS-97	TS-097	CER	LA0154	-	-	Tiny tim	*S. lycopersicum var cerasiforme*	
TS-98	TS-098	CER	LA4355	-	-	Gold Nugget	*S. lycopersicum var cerasiforme*	cultivar
TS-99	TS-099	CER	-	-	-	Celsior	*S. lycopersicum var cerasiforme*	
TS-105	TS-105	CER	-	-	EA01448		*S. lycopersicum var cerasiforme*	Wild species
TS-106	TS-106	CER	-	-	-		*S. lycopersicum var cerasiforme*	cultivar
TS-107	TS-107	CER	LA1542	-	-	Turrialba	*S. lycopersicum var cerasiforme*	Wild species
TS-109	TS-109	CER	-	-	EA02660			Cocktail tomato
TS-116	TS-116	CER	-	-	-	N1565	*S. lycopersicum var cerasiforme*	cultivar
TS-118	TS-118	CER	LA0292	-	-	Santa Cruz, Galapagos	*S. lycopersicum var cerasiforme*	Latin American cultivar;Wild species
TS-119*	TS-119	CER	-	-	-	Wva 106	*S. lycopersicum var cerasiforme*	Wild species
TS-120	TS-120	CER	-	-	EA01356			
TS-129	TS-129	CER	LA2845	-	-	Moyobamba	*S. lycopersicum var cerasiforme*	Latin American cultivar; Wild species
TS-131*	TS-131	CER	LA1162	-	-	Cuba Plum	*S. lycopersicum var cerasiforme*	cultivar
TS-134	TS-134	CER	LA1429	-	-	La Estancilla	*S. lycopersicum var cerasiforme*	Wild species
TS-138	TS-138	CER	-	-	-	Malintka 101		cultivar
TS-148	TS-148	CER	LA1323	PI365924	-	Pfacchayoc	*S. lycopersicum var cerasiforme*	Wild species
TS-149	TS-149	CER	LA1425	PI379060	-	Villa Hermosa	*S. lycopersicum var cerasiforme*	Latin American cultivar
TS-154	TS-154	CER	LA1623	-	-	Muna	*S. lycopersicum var cerasiforme*	Wild species
TS-158	TS-158	CER	LA2626	-	EA01541	PE-67	*S. lycopersicum var cerasiforme*	Wild species
TS-165	TS-165	CER	LA1218	-	-	Veracruz	*S. lycopersicum var cerasiforme*	Latin American cultivar
TS-181	TS-181	CER	LA1457	-	EA01438		*S. lycopersicum var cerasiforme*	Wild species
TS-187	TS-187	CER	-	-	-	Monplaisir	*S. lycopersicum var cerasiforme*	cultivar
TS-189	TS-189	CER	-	-	-	Nagcarlang		cultivar
TS-202	TS-202	CER	LA2402	-	-		*S. lycopersicum var cerasiforme*	Latin American cultivar
TS-205	TS-205	CER	-	-	-	8 bis	*S. lycopersicum var cerasiforme*	cultivar
TS-209	TS-209	CER	-	-	-	Da serbo		landrace
TS-213	TS-213	CER	-	-	EA01784			
TS-216	TS-216	CER	-	-	-	Phyra	*S. lycopersicum var cerasiforme*	cultivar
TS-219	TS-219	CER	-	-	-	Mobalcon		landrace
TS-221	TS-221	CER	LA1569	-	-	Jalapa	*S. lycopersicum var cerasiforme*	Wild species
TS-223	TS-223	CER	LA2675	-	EA01557		*S. lycopersicum var cerasiforme*	Wild species
TS-227	TS-227	CER	-	-	-	Atom	*S. lycopersicum var cerasiforme*	cultivar
TS-229	TS-229	CER	LA1620	-	-		*S. lycopersicum var cerasiforme*	Wild species
TS-230	TS-230	CER	-	-	-	Cerise Ildi	*S. lycopersicum var cerasiforme*	cultivar
TS-231	TS-231	CER	-	-	-	L 285	*S. lycopersicum var cerasiforme*	cultivar
TS-233	TS-233	CER	LA1218	-	EA00602		*S. lycopersicum var cerasiforme*	Latin American cultivar
TS-238	TS-238	CER	LA1228	PI379047	-	Macas	*S. lycopersicum var cerasiforme*	Wild species
TS-240	TS-240	CER	LA3136	-	-	Arroyo Rico	*S. lycopersicum var cerasiforme*	Wild species
TS-243	TS-243	CER	-	-	EA04228			
TS-247	TS-247	CER	-	-	-	N 795 Pescio	*S. lycopersicum var cerasiforme*	cultivar
TS-248	TS-248	CER	-	-	-			
TS-250	TS-250	CER	-	-	EA03539			
TS-252	TS-252	CER	-	-	EA01802			cocktail tomato
TS-254	TS-254	CER	-	-	EA02979			
TS-257	TS-257	CER	-	-	-	Marpha N2	*S. lycopersicum var cerasiforme*	cultivar
TS-258	TS-258	CER	-	-	-	Minibel	*S. lycopersicum var cerasiforme*	cultivar
TS-260	TS-260	CER	-	-	-	Cerise Orange dUzès	*S. lycopersicum var cerasiforme*	cultivar
TS-262	TS-262	CER	LA1247	PI379058	EA01403		*S. lycopersicum var cerasiforme*	Wild species
TS-271	TS-271	CER	-	-	-	Linosa		landrace
TS-273	TS-273	CER	LA2640	-	EA01545	PE-63	*S. lycopersicum var cerasiforme*	Wild species
TS-280	TS-280	CER	-	-	-	Cerise du sud ouest N 2	*S. lycopersicum var cerasiforme*	Wild species
TS-281	TS-281	CER	LA1286	PI365920	-	San Martin de Pangoa	*S. lycopersicum var cerasiforme*	Wild species
TS-283	TS-283	CER	-	-	-	Cisterno	*S. lycopersicum var cerasiforme*	cultivar
TS-284	TS-284	CER	-	-	-	Cerazjnho		landrace
TS-286	TS-286	CER	-	-	-	Allungato piccolo		landrace
TS-287	TS-287	CER	-	-	EA00915			Cocktail tomato
TS-289*	TS-289	CER	LA1244	PI379044	EA00610		*S. lycopersicum var cerasiforme*	Latin American cultivar
TS-290	TS-290	CER	-	-	-	N 2257 Dikorastushil...	*S. lycopersicum var cerasiforme*	cultivar
TS-294	TS-294	CER	-	-	-	N 933	*S. lycopersicum var cerasiforme*	cultivar
TS-295*	TS-295	CER	LA0417	-	-	Puna	*S. lycopersicum var cerasiforme*	Latin American cultivar; Wild species
TS-298	TS-298	CER	LA2308	-	-	San Francisco	*S. lycopersicum var cerasiforme*	Wild species
TS-299	TS-299	CER	LA2131	-	-	Bomboiza	*S. lycopersicum var cerasiforme*	Wild species
TS-300	TS-300	CER	LA1231	PI379049	-	Tena	*S. lycopersicum var cerasiforme*	Wild species
TS-301	TS-301	CER	LA2688	-	-	Santa Cruz near Shintuyo	*S. lycopersicum var cerasiforme*	Wild species
TS-302	TS-302	CER	LA1543	-	-	Upper Parana	*S. lycopersicum var cerasiforme*	Wild species
TS-303	TS-303	CER	LA1461	-	-	Los Banos	*S. lycopersicum var cerasiforme*	Wild species
TS-304*	TS-304	CER	LA2307	-	-	Tarapoto	*S. lycopersicum var cerasiforme*	Latin American cultivar
TS-423*	TS-423	CER	LA2840	-	-		*S. lycopersicum var cerasiforme*	Wild species
TS-426*	TS-426	CER	LA1263	PI365918	-	SAL345	*S. lycopersicum var cerasiforme*	cultivar
TS-427*	TS-427	CER	-	PI126933	-		*S. lycopersicum var cerasiforme*	cultivar
TS-428*	TS-428	CER	-	PI126953	-		*S. lycopersicum var cerasiforme*	cultivar
TS-430*	TS-430	CER	-	PI127807	-	BL 587-S	*S. lycopersicum var cerasiforme*	cultivar
TS-431*	TS-431	CER	-	PI127807	-	BL 587	*S. lycopersicum var cerasiforme*	cultivar
TS-436*	TS-436	CER	-	-	-	BL 359	*S. lycopersicum var cerasiforme*	cultivar
TS-1	TS-001	BIG	LA0490	-	-	VF-36	*S. lycopersicum*	Vintage Processing
TS-2	TS-002	BIG	LA2706	-	-	Moneymaker	*S. lycopersicum*	Vintage Fresh Market
TS-3#	TS-003	BIG	LA3475	-	-	M-82	*S. lycopersicum*	Modern Processing
TS-4	TS-004	BIG	LA3856	-	-	Hawaii 7998	*S. lycopersicum*	Inbreed line

TS-5	TS-005	BIG	LA2711	-	-	Edkawi	*S. lycopersicum*	Vintage Fresh Market
TS-6	TS-006	BIG	LA3008	-	-	San Marzano	*S. lycopersicum*	Vintage Processing
TS-7	TS-007	BIG	LA3911	-	-	Micro-Tom	*S. lycopersicum*	Modern Fresh Market
TS-8#	TS-008	BIG	LA4024	-	-	E-6203	*S. lycopersicum*	Modern Processing
TS-9	TS-009	BIG	LA2838A	-	-	Ailsa Craig	*S. lycopersicum*	Vintage Fresh Market
TS-10	TS-010	BIG	LA0502	-	-	Marglobe	*S. lycopersicum*	Vintage Fresh Market
TS-11	TS-011	BIG	-	-	-	KR2	*S. lycopersicum*	Modern Fresh Market
TS-12	TS-012	BIG	-	-	-	yoku improvement	*S. lycopersicum*	Modern Fresh Market
TS-41	TS-041	BIG	-	-	EA02435		*S. lycopersicum*	Cocktail tomato
TS-42	TS-042	BIG	-	PI345565	EA05808		*S. lycopersicum*	Processing tomato
TS-43	TS-043	BIG	-	-	EA00840	Moneymaker	*S. lycopersicum*	Fresh Market
TS-44	TS-044	BIG	-	-	-	A pera abruzzese		Landrace
TS-45#	TS-045	BIG	-	PI303718	EA05578		*S. lycopersicum*	Processing tomato
TS-46	TS-046	BIG	-	-	EA01237			Processing tomato
TS-47	TS-047	BIG	-	-	EA01960		*S. lycopersicum*	Processing tomato
TS-48	TS-048	BIG	LA0146	-	-	Mexico City	*S. lycopersicum*	Landrace/Latin American cultivar
TS-49	TS-049	BIG	LA3238	-	-	Earliana	*S. lycopersicum*	Vintage Processing
TS-51	TS-051	BIG	-	-	-			
TS-52	TS-052	BIG	-	-	-	05-4126 (97-49-2)		cultivar
TS-55	TS-055	BIG	-	-	EA00448			
TS-58	TS-058	BIG	-	-	EA03577		*S. lycopersicum*	Processing tomato
TS-59#	TS-059	BIG	-	-	EA02898			Processing tomato
TS-60	TS-060	BIG	LA2009	-	-	New Yorker	*S. lycopersicum*	Vintage Fresh Market
TS-68	TS-068	BIG	LA0395	-	-	Chiclayo	*S. lycopersicum*	Latin American cultivar
TS-69	TS-069	BIG	LA1459	-	-	Huachinango	*S. lycopersicum*	Latin American cultivar
TS-73†	TS-073	BIG	-	-	-	Quarantino		
TS-74	TS-074	BIG	-	-	-	N 739	*S. lycopersicum*	Fresh Market
TS-76	TS-076	BIG	-	-	EA01230			Processing tomato
TS-78#	TS-078	BIG	-	-	EA02895		*S. lycopersicum*	Processing tomato
TS-80	TS-080	BIG	-	-	EA01020			Processing tomato
TS-81	TS-081	BIG	-	-	EA02761			Processing tomato
TS-82	TS-082	BIG	-	-	EA03274			Processing tomato
TS-85#	TS-085	BIG	-	-	-			
TS-86	TS-086	BIG	-	-	EA01684			
TS-88	TS-088	BIG	-	-	EA01804		*S. lycopersicum*	Cocktail tomato
TS-89	TS-089	BIG	-	-	EA01185			Processing tomato
TS-90	TS-090	BIG	-	-	EA02753		*S. lycopersicum*	Cocktail tomato
TS-93	TS-093	BIG	-	-	EA01002			Processing tomato
TS-95	TS-095	BIG	-	-	-	Moneymaker	*S. lycopersicum*	Fresh Market
TS-100	TS-100	BIG	-	-	EA03456		*S. lycopersicum*	Processing
TS-101	TS-101	BIG	-	-	EA00369		*S. lycopersicum*	
TS-102	TS-102	BIG	-	-	EA03673		*S. lycopersicum*	Processing tomato
TS-103	TS-103	BIG	-	-	EA00389		*S. lycopersicum*	
TS-104	TS-104	BIG	-	-	EA01756		*S. lycopersicum*	Processing tomato
TS-108#	TS-108	BIG	-	-	EA01989	Puno I	*S. lycopersicum*	Processing tomato
TS-110	TS-110	BIG	-	PI93302	EA04243		*S. lycopersicum*	
TS-111	TS-111	BIG	-	-	EA01270			Processing tomato
TS-112	TS-112	BIG	-	-	EA03083		*S. lycopersicum*	Processing tomato
TS-113	TS-113	BIG	-	-	EA01198			Processing tomato
TS-114	TS-114	BIG	-	-	EA01982		*S. lycopersicum*	Processing tomato
TS-115	TS-115	BIG	-	-	EA03426			Processing tomato
TS-117	TS-117	BIG	-	-	-	Scatolone di bolsena		Landrace
TS-121	TS-121	BIG	LA3846	-	-	NC EBR-6	*S. lycopersicum*	Modern Fresh Market
TS-122	TS-122	BIG	LA1090	-	-	Rutgers	*S. lycopersicum*	Vintage Fresh Market
TS-125	TS-125	BIG	-	-	EA00422			Processing tomato
TS-126	TS-126	BIG	-	-	EA01903		*S. lycopersicum*	
TS-127	TS-127	BIG	LA0113	-	-	Hacienda Calera	*S. lycopersicum*	Landrace/Latin American cultivar
TS-128	TS-128	BIG	LA0012	-	-	Pearson	*S. lycopersicum*	Vintage Processing
TS-130	TS-130	BIG	LA2413	-	-	Severianin	*S. lycopersicum*	Modern Fresh Market
TS-132	TS-132	BIG	LA3903	-	-	Primabel	*S. lycopersicum*	Vintage Fresh Market
TS-133#	TS-133	BIG	LA3528	-	-	Peto95-43	*S. lycopersicum*	Modern Processing
TS-135	TS-135	BIG	LA0466	PI 258469	-	Hacienda Rosario	*S. lycopersicum*	Landrace/Latin American cultivar
TS-136	TS-136	BIG	-	-	-	Vito		
TS-137	TS-137	BIG	-	-	-	Spagnoletta		Landrace
TS-139	TS-139	BIG	-	-	-	Red Setter		Vintage cultivar
TS-140	TS-140	BIG	-	-	-	149-77		cultivar
TS-141	TS-141	BIG	-	-	-	Saladette		cultivar
TS-142	TS-142	BIG	-	-	-	Roma		Vintage cultivar
TS-143	TS-143	BIG	LA4025	-	-	Florida 7547	*S. lycopersicum*	Modern Fresh Market
TS-147#	TS-147	BIG	-	-	-			
TS-150	TS-150	BIG	LA2285	-	-	Tarapoto	*S. lycopersicum*	Landrace/Latin American cultivar
TS-151	TS-151	BIG	LA2399	-	-	T-5	*S. lycopersicum*	Modern Fresh Market
TS-152	TS-152	BIG	LA1021	-	-	Santa Cruz B	*S. lycopersicum*	Landrace/Latin American cultivar
TS-153	TS-153	BIG	LA1544	-	-	Xol Languna	*S. lycopersicum*	Landrace/Latin American cultivar
TS-155	TS-155	BIG	LA0533	-	-	Condine Red	*S. lycopersicum*	Vintage Fresh Market,Monogenic
TS-157	TS-157	BIG	-	-	EA03648		*S. lycopersicum*	Processing tomato
TS-159	TS-159	BIG	-	-	EA03028			Processing tomato
TS-160	TS-160	BIG	-	-	EA03533		*S. lycopersicum*	Processing tomato
TS-161	TS-161	BIG	-	-	EA02586		*S. lycopersicum*	Processing tomato
TS-162#	TS-162	BIG	-	-	EA03463		*S. lycopersicum*	Processing tomato
TS-163	TS-163	BIG	LA1504	-	-	Marmande	*S. lycopersicum*	Vintage Fresh Market
TS-166	TS-166	BIG	LA0404	-	-	Piura	*S. lycopersicum*	Landrace/Latin American cultivar
TS-167	TS-167	BIG	LA0147	-	-	Tegucigalpa	*S. lycopersicum*	Landrace/Latin American cultivar
TS-168#	TS-168	BIG	-	-	-	Da appendere		Landrace
TS-169	TS-169	BIG	-	-	-	Cuor di bue di Albenga		Landrace
TS-170	TS-170	BIG	-	-	-	Cuor di bue		Landrace
TS-171#	TS-171	BIG	LA1706	-	-	UC-82	*S. lycopersicum*	Modern Processing
TS-172	TS-172	BIG	-	PI280060	EA05480		*S. lycopersicum*	Processing tomato
TS-173	TS-173	BIG	-	-	EA03611		*S. lycopersicum*	
TS-174	TS-174	BIG	-	-	EA00304			Processing tomato
TS-175	TS-175	BIG	-	-	EA03586		*S. lycopersicum*	Processing tomato
TS-176	TS-176	BIG	-	-	EA02669			Processing tomato
TS-177	TS-177	BIG	-	-	EA01155			Processing tomato
TS-178	TS-178	BIG	-	PI513036	EA06485		*S. lycopersicum*	Processing tomato
TS-179	TS-179	BIG	-	-	EA01027			Processing tomato
TS-180	TS-180	BIG	-	-	EA02728			Processing tomato
TS-183	TS-183	BIG	-	-	EA02764			Processing tomato
TS-184†	TS-184	BIG	LA2283	-	-	Tarapoto	*S. lycopersicum*	
TS-185	TS-185	BIG	LA4347	-	-	B-L-35	*S. lycopersicum*	Vintage Fresh Market
TS-186#	TS-186	BIG	LA3214	-	-	Rowpac	*S. lycopersicum*	Modern Processing

TS-188	TS-188	BIG	LA1506	-	-	Stone	*S. lycopersicum*	Vintage Fresh Market
TS-190#	TS-190	BIG	-	-	-	Santa Chiara		cultivar
TS-191	TS-191	BIG	-	-	-	Francescano		Landrace
TS-192	TS-192	BIG	-	-	-	Severianin		Vintage cultivar
TS-193	TS-193	BIG	-	-	-	Pantano dArdea		Landrace
TS-194	TS-194	BIG	-	-	-			
TS-195	TS-195	BIG	LA0516	-	-	Ace	*S. lycopersicum*	Vintage Fresh Market
TS-196	TS-196	BIG	-	-	EA00240	N020212		Processing tomato
TS-197	TS-197	BIG	-	-	-	Libanese		Landrace
TS-198	TS-198	BIG	-	-	EA00512			
TS-200	TS-200	BIG	LA3320	-	-	Hot set	*S. lycopersicum*	cultivar
TS-201	TS-201	BIG	LA1210	-	-	San Salvador	*S. lycopersicum*	Landrace/Latin American cultivar
TS-203	TS-203	BIG	-	-	-	Bell pepper-like		Landrace
TS-204	TS-204	BIG	LA3840	-	-	Florida 7060	*S. lycopersicum*	Modern Fresh Market
TS-206	TS-206	BIG	LA0089	-	-	Prince Borghese	*S. lycopersicum*	Vintage Fresh Market
TS-210	TS-210	BIG	LA3625	-	-	NC 265-1 (93)-3-3	*S. lycopersicum*	Modern Fresh Market
TS-211	TS-211	BIG	LA4354	-	-	NC 84173	*S. lycopersicum*	Modern Fresh Market
TS-212	TS-212	BIG	LA3242	-	-	Flora-Dade	*S. lycopersicum*	Modern Fresh Market
TS-214	TS-214	BIG	-	-	-	Panama		Landrace
TS-215	TS-215	BIG	-	-	-	Vrbikanske Nizke		cultivar
TS-218	TS-218	BIG	-	-	-	Santa Clara 5800		cultivar
TS-220	TS-220	BIG	-	-	-	Barnaulski Konservnyi		cultivar
TS-224	TS-224	BIG	LA0410	PI 258474	-	Guayaquil	*S. lycopersicum*	Landrace/Latin American cultivar
TS-225	TS-225	BIG	-	PI330336	EA05747		*S. lycopersicum*	Processing tomato
TS-226	TS-226	BIG	-	-	-	Microtom	*S. lycopersicum*	cultivar
TS-228#	TS-228	BIG	-	-	-	M-82	*S. lycopersicum*	cultivar
TS-232	TS-232	BIG	-	-	EA00951			Processing
TS-234	TS-234	BIG	-	-	EA01371			Processing tomato
TS-235	TS-235	BIG	-	-	EA00892			Processing tomato
TS-236	TS-236	BIG	-	-	EA02732			Processing tomato
TS-237	TS-237	BIG	LA3243	-	-	Platense	*S. lycopersicum*	Vintage Fresh Market
TS-239	TS-239	BIG	LA3845	-	-	NC EBR-5	*S. lycopersicum*	Modern Fresh Market
TS-241	TS-241	BIG	LA0126	-	-	Quito	*S. lycopersicum*	Latin American cultivar
TS-242	TS-242	BIG	LA0134C	-	-	Ayacucho	*S. lycopersicum*	Latin American cultivar
TS-245#	TS-245	BIG	-	-	EA03126		*S. lycopersicum*	Processing tomato
TS-246	TS-246	BIG	-	-	EA00983			Processing tomato
TS-249	TS-249	BIG	LA1462	-	-	Merida	*S. lycopersicum*	Landrace/Latin American cultivar
TS-251	TS-251	BIG	-	PI647249	EA04001		*S. lycopersicum*	
TS-253#	TS-253	BIG	LA4345	-	-	Heinz 1706-BG	*S. lycopersicum*	Modern Processing
TS-255	TS-255	BIG	-	-	EA03002			Processing tomato
TS-256	TS-256	BIG	LA2260		EA00744		*S. lycopersicum*	Latin American cultivar
TS-259	TS-259	BIG	-	-	EA01712			Processing tomato
TS-261*	TS-261	BIG	LA1511	-	EA01444		*S. lycopersicum*	Wild species
TS-263#	TS-263	BIG	LA3343	-	-	Rio Grande	*S. lycopersicum*	Processing tomato
TS-264	TS-264	BIG	LA0025	-	-	King Humbert #1	*S. lycopersicum*	Vintage Fresh Market
TS-268	TS-268	BIG	-	-	EA01915		*S. lycopersicum*	cultivar
TS-269	TS-269	BIG	-	-	-	Canestrino		Landrace
TS-270	TS-270	BIG	-	-	EA03174			
TS-272#	TS-272	BIG	-	-	EA06878			Processing tomato
TS-274#	TS-274	BIG	-	-	EA03613			Cocktail/Processing tomato
TS-275	TS-275	BIG	-	-	EA01049			Processing tomato
TS-276	TS-276	BIG	-	-	EA03650			Cocktail/Processing tomato
TS-277#	TS-277	BIG	LA3144	-	-	Hunt100	*S. lycopersicum*	Modern Processing
TS-278	TS-278	BIG	LA0517	-	-	Early Santa Clara	*S. lycopersicum*	Vintage Processing
TS-282*	TS-282	BIG	-	-	-	Ucra2		Landrace
TS-285	TS-285	BIG	-	PI303752	EA05612		*S. lycopersicum*	Processing tomato
TS-288	TS-288	BIG	-	-	EA04236		*S. lycopersicum*	Processing tomato
TS-292#	TS-292	BIG	-	-	EA06902		*S. lycopersicum*	Processing tomato
TS-293	TS-293	BIG	-	-	EA03439		*S. lycopersicum*	Cocktail/Processing tomato
TS-296	TS-296	BIG	-	-	-	Droplet	*S. lycopersicum*	
TS-297	TS-297	BIG	-	PI291344	EA05550		*S. lycopersicum*	
TS-400	TS-400	BIG	-	-	-		*S. lycopersicum*	inbred line
TS-401	TS-401	BIG	-	-	-		*S. lycopersicum*	inbred line
TS-409	TS-409	BIG	-	PI124161	-		*S. lycopersicum*	Landrace
TS-305	TS-305	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-306	TS-306	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-307	TS-307	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-308	TS-308	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-309	TS-309	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-310	TS-310	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-311	TS-311	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-312	TS-312	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-313	TS-313	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-314	TS-314	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-315	TS-315	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-316	TS-316	F1	-	-	-		*S. lycopersicum*	Fresh Market
TS-317	TS-317	F1	-	-	-		*S. lycopersicum*	Processing
TS-318	TS-318	F1	-	-	-		*S. lycopersicum*	Processing
TS-319	TS-319	F1	-	-	-		*S. lycopersicum*	Processing
TS-320	TS-320	F1	-	-	-		*S. lycopersicum*	Processing
TS-321	TS-321	F1	-	-	-		*S. lycopersicum*	Processing
TS-146	TS-146	wild	LA2133	-	-		*S. neorickii*	
TS-208	TS-208	wild	LA0528	-	-	Santa Cruz: Academy Bay	*S. galapagense*	
TS-199	TS-199	wild	LA0746	-	EA00587		*S. cheesmaniae*	
TS-207	TS-207	wild	LA1037	-	-		*S. cheesmaniae*	
TS-217	TS-217	wild	LA0429	-	EA00568		*S. cheesmaniae*	
TS-402	TS-402	wild	-	PI126935	-		*S. peruvianum*	
TS-403	TS-403	wild	-	PI128650	-		*S. peruvianum*	
TS-404	TS-404	wild	-	PI128657	-		*S. peruvianum*	
TS-408	TS-408	wild	LA1969	-	-		*S. chilense*	
TS-407	TS-407	wild	-	PI247087	-		*S. habrochaites*	

Data from in Lin et al. (2014)

Table S3. Primers

REF	ALT	protein effect	primer for	primer rev	Chiclayo	E-6203	VF-36	Micro-Tom	Droplet	GPT166090	M-82	Edkawi	Puno I	Barnaulski Konservnyi
CC	**TT**	STOP gained	ATGGAGGGAGTGAAGAAGGC	GGTAGAGTGTACGCAGACCA	**TT**	-	-	**TT***	-	**TT**	-	**CT****	-	**TT**
AA	**TT**	STOP gained	ACGTCCAGTGATAAGCTCCA	GCAGAATTTTGTGGCTCTTGG	-	**TT**	-	-	-	-	**TT**	-	**TT**	-
AA	**GG**	STOP lost	AGGAGCATACTTCTCTAGAGGAT	TCGGAAGTGGTCGATTGTGA	-	-	-	**GG**	-	-	-	-	-	-
CC	**TT**	STOP gained	TGTGGATTATTTTCGCGAGGG	TGGCATTCTTTGGACAAGACC	-	-	-	**TT**	-	-	-	-	-	-
GG	**AA**	STOP gained	TCCTTGTGTTCCTGTCCTGT	CATCCTTCCTCCTCACCACA	**GA**	-	-	-	**AA**	-	**AA**	-	-	-
GG	**TT**	intron-variant	TAGCTGCTTTTCTCATCGCG	CAATGTCCAACTCAAGCTACGT	-	-	-	-	**TT***	-	-	-	-	-
GG	**AA**	STOP gained	ACCTTTTGTGCTGACGTGAC	GCTTACCGTTGTCCATGCAA	-	-	-	-	**AA**	-	-	-	-	-
TT	**AA**	STOP lost	ACATCTTTGACATATCCACCACT	ACCGAACACACCATTAATGCA	-	-	-	-	-	**AA***	-	-	-	-
TT	**GG**	intron-variant	TGCAGTCTCCCAATTTGAAGAG	TCTGAACTTGCCTCAAATCTCA	-	-	-	-	-	-	-	**GG**	-	-
AA	**GG**	STOP lost/intron-variant	CATGAAGATGTGTGTGTTTTCCA	AGAGTTGCTATCGGGAAAACG	-	-	-	-	-	-	-	-	-	-

Table S4. sgRNAs

GENE FAMILY	GENE NAME	SeqID	minMM_NGG	minMM_NAG	Spacer seq (5'->3')	PAM (5'->3')	strand	location	class
PMR4	Solyc07g056260	Chr7:61414763-61414782:rc	4+	4+	TTGCCTTCGTACCGACAGGC	TGGGGCATTC	-	exon	A0
	Solyc07g056260	Chr7:61416034-61416053:rc	4+	4+	GAGAGGATTTCGCACGGCCC	TGGGTGATTT	-	exon	A0
	Solyc07g056260	Chr7:61420927-61420946	4+	4+	ACTACGGTCTCGCTGTTCAC	TGGACTTCTT	+	exon	A0
	Solyc07g056260	Chr7:61433018-61433037	4+	4+	TGTTGCTTGTCACAGCCCGG	CGGAAACCTG	+	exon	A0
	Solyc07g056260	Chr7:61415913-61415932:rc	4+	3+	GGTTCTAAATATCGAGCTAC	TGGTCGTGGC	-	exon	A0.1
	Solyc07g056260	Chr7:61420526-61420545	4+	3+	TCCCCAATTTTAGGCGGGCC	AGGAAGCTTG	+	exon	A0.1
	Solyc01g006370	Chr1:989950-989969	4+	3+	AGCACCTCCTCCGTGTAGTA	TGGGGTCAAG	+	exon	A0.1
	Solyc01g006370	Chr1:1004015-1004034	4+	3+	CGCCGCTGCAGCGACGGCTC	CGGTCCCCCT	-	exon	A0.1
	Solyc01g073750	Chr1:25499-25518	4+	4+	TTGGGCTAGTGAAGCCCGGA	GGGCAAGAAG	+	exon	A0
	Solyc01g073750	Chr1:25559-25578	4+	4+	GGATTCTAATGAGGCCCGACC	CGGCCCGTAA	+	exon	A0
	Solyc07g061920	Chr7:62028763-62028782:rc	4+	4+	TACAAGTTTAAGAACCGCTG	AGGTGATGAA		exon	A0
	Solyc01g006350	Chr1:950346-950365	4+	3+	TCACGGACAGGTCGTGTTAC	AGGCATCTCC	+	exon	A0.1
	Solyc01g006350	Chr1:934803-934822	4+	4+	CCAGCCCTCGACTGAACGCT	TGGTTGAACA	+	exon	A0
PMR5	Solyc06g007170	Chr6:1236821-1236840:rc	4+	3+	TGCCGGTGGTCCAATCGCTT	GGGCTGATAT	-	exon	A0.1
	Solyc06g007170	Chr6:1236835-1236854:rc	4+	4+	TGACGAGGTCGACGTGCCGG	TGGTCCAATC	-	exon	A0
	Solyc06g007170	Chr6:1236838-1236857:rc	4+	3+	TGATGACGAGGTCGACGTGC	CGGTGGTCCA	-	exon	A0.1
	Solyc06g007170	Chr6:1236895-1236914:rc	4+	4+	CGTGCCCGGATACGCCGTGC	CGGTCATCGG	-	exon	A0
	Solyc06g007170	Chr6:1236887-1236906	4+	4+	GATGACCGGCACGGCGTATC	CGGGCACGTT	+	exon	A0
	Solyc06g007170	Chr6:1236888-1236907	4+	4+	ATGACCGGCACGGCGTATCC	GGGCACGTTC	+	exon	A0
	Solyc07g053350	Chr7:59133544-59133563	4+	4+	ACGTCTTGATTTTCAACAGC	TGGCATTGGT	+	exon	B0
	Solyc02g082950	Chr2:41139723-41139742:rc	4+	4+	GCTAGATAACGAGCTTATCT	GGGTCTGCTT	-	exon	B0
	Solyc02g082950	Chr2:41139779-41139798	4+	4+	CAATCTCGCCCTCTGTATAA	TGGGACAAAT	+	exon	B0
	Solyc03g006220	Chr3:850023-850042:rc	4+	3+	CGCAGAAATTGATTCTTTTC	TGGTAAAAGA	-	exon	B0.1
	Solyc03g006220	Chr3:851816-851835	4+	3+	ATATGTTGATTTTCAATTCT	TGGCATTGGT	+	exon	B0.1
	Solyc03g006220	Chr3:851849-851868	4+	3+	CTCACAAGGGAAAATCTCAA	GGGTAATTAC	+	exon	B0.1
	Solyc03g006220	Chr3:853546-853565:rc	4+	3+	GTAGATGATGGTAATCCTCC	TGGATATGTT	-	exon	B0.1
	Solyc03g006220	Chr3:853663-853682	4+	3+	GCATATAGTGGACAACACCC	TGGTAATGAT	+	exon	B0.1
	Solyc03g006220	Chr3:853723-853742:rc	4+	3+	AGAAGTTGGTTCCAAGTGTC	TGGTAGACCA	-	exon	B0.1
PMR6	Solyc05g055510	Chr5:64277408-64277427	4+	3+	GCAATCCGATCGACGACTGC	TGGCGGTGCG	-	exon	A0.1
	Solyc05g055510	Chr5:64277511-64277530	4+	4+	GAAAGGCGGCCGGTATTACC	TGGTTAGCGA	+	exon	A0
	Solyc05g055510	Chr5:64277586-64277605:rc	4+	4+	CATCCTGGATCACCGCGTGC	CGGAGGGTTC	-	exon	A0
	Solyc05g055510	Chr5:64277571-64277590	4+	4+	CCCTGGAACCCTCCGGCACG	CGGTGATCCA	-	exon	A0
	Solyc05g055510	Chr5:64277698-64277717	4+	4+	GCGAACGTCCACGTCACGGG	TGGTGGATGT	+	exon	A0
	Solyc05g055510	Chr5:64279151-64279170	4+	3+	GAACAACCATTGCCGCCAAC	CGGAGCAGTA	-	exon	A0.1
	Solyc02g093580	Chr2:48972366-48972385:rc	4+	4+	GGCTATTTATCGTGCGGATC	AGGGAATCCA	-	exon	A0
	Solyc03g111690	Chr3:56396908-56396927	4+	4+	GGGTATTTATCGTGTGGTAC	CGGAAATCCA	+	exon	A0
	Solyc03g111690	Chr3:56397076-56397095	4+	4+	AATGCCGTCACTCCGAAGCC	GGGGACTCTC	-	exon	A0
	Solyc01g010740	Chr1:5779527-5779546:rc	4+	3+	CATTGGCAAATGTACGCGAT	TGGTGGCAGT	-	exon	A0.1
	Solyc01g010740	Chr1:5779615-5779634:rc	4+	3+	TGCATTCAATCGGTTCGGGG	AGGGATTGAT	-	exon	A0.1
	Solyc01g010740	Chr1:5780203-5780222:rc	4+	3+	TGAATCAGATGATAACGTGC	AGGAACCAAA	-	exon	A0.1
	Solyc02g087670	Chr2:44602283-44602302	4+	3+	GATAAGACCATTGACGGCCG	TGGAGTTAAT	+	exon	A0.1
	Solyc06g071020	Chr6:40015322-40015341:rc	4+	4+	TTCACCATATCGGTGTCCCC	AGGGTCAGTT	-	exon	A0
	Solyc06g071020	Chr6:40016420-40016439	4+	4+	GGGCAGGATCAGACGCAGGC	AGGCTAACAA	+	exon	A0
	Solyc02g080910	Chr2:39588428-39588447:rc	4+	3+	ATTGCTCCAAAATACACGAG	AGGACAATCG	-	exon	A0.1
	Solyc02g080910	Chr2:39593032-39593051:rc	4+	4+	CGGGATAGTCCGGTGCATGC	CGGCCATCGT	-	exon	A0
	Solyc02g080910	Chr2:39593294-39593313	4+	4+	GGTTGACCGGGTCATCGGAC	GGGTTAGTAA	+	exon	A0
	Solyc02g067450	Chr2:32223021-32223040:rc	4+	4+	GGCTTCGGTAGCAAGGCTAC	TGGTGGTAAA	-	exon	A0
	Solyc02g067450	Chr2:32223028-32223047:rc	4+	3+	TGCAATGGGCTTCGGTAGCA	AGGCTACTGG	-	exon	A0.1
DMR1	Solyc04g008760	Chr4:2413054-2413073	4+	3+	CTCCGTCAAGTCGTTTGCTC	CGGCCACTGT	-	exon	A0.1
	Solyc04g008760	Chr4:2413621-2413640	4+	3+	GAGGGCGGTATTGCCATCGG	AGGTGACAAT	+	exon	A0.1
DMR6	Solyc03g080190	Chr3:45630168-45630187	4+	4+	TTCCGATAGACGTGGTCTAT	CGGATTCGGG	-	exon	A0
DND1	Solyc02g088560	Chr2:45186848-45186867	4+	4+	ATGAACACAACCCTGTGCAC	TGGATCTCCT	-	exon	A0
	Solyc02g088560	Chr2:45189378-45189397:rc	4+	4+	GGCACACATCGGGGCCGTTC	GGGCGTGTAT	-	exon	A0
	Solyc02g088560	Chr2:45189361-45189380	4+	4+	GGGTCTAATACACGCCCGAA	CGGCCCCGAT	-	exon	A0
	Solyc02g088560	Chr2:45189393-45189412	4+	4+	GTGCCGGCTGCGACTGGACC	GGGATTTTCC	+	exon	A0
MLO1	Solyc06g082820	Chr6:44782014-44782033	4+	3+	AGACTACAGAGCGCTTCGTT	TGGGATTCAT	+	exon	A0.1
	Solyc06g010010	Chr6:4699905-4699924:rc	4+	4+	CGGTCTTCCTATGCTTTTTG	AGGCACTTCA	-	exon	B0
	Solyc06g010010	Chr6:4699920-4699939	4+	4+	GACCGCTTTGTTTGAAGCTG	TGGAGAAACT	+	exon	B0
	Solyc06g010010	Chr6:4703380-4703399	4+	4+	ATTACGCCATGGCTTCATTA	CGGTCAGTAT	-	exon	B0
	Solyc02g077570	Chr2:37045142-37045161:rc	4+	4+	ACAACAGCAATAGCCCATGT	TGGTGTTGTC	-	exon	B0
	Solyc02g077570	Chr2:37048615-37048634	4+	4+	GACTACTTAACTCTCCGGCA	TGGATTCATA	+	exon	B0
	Solyc02g077570	Chr2:37048626-37048645	4+	4+	TCTCCGGCATGGATTCATAA	TGGTATTTAC	-	exon	B0
	Solyc02g077570	Chr2:37049063-37049082:rc	4+	4+	AAGGAAGCCAATAGGTGCTA	TGGAACACTA	-	exon	B0
BIK1	Solyc06g005500	Chr6:527496-527515	4+	3+	GTTCATGCCTCGGGGCAGCT	TGGAAAACCA	+	exon	A0.1
	Solyc06g005500	Chr6:528652-528671:rc	4+	4+	TGGATAGGCACCGCAGGGCA	AGGTTAGCTA	-	exon	A0
	Solyc06g005500	Chr6:528657-528676:rc	4+	4+	ATCTTTGGATAGGCACCGCA	GGGCAAGGTT	-	exon	A0
	Solyc12g077390	Chr12:56897543-56897562:rc	4+	4+	AACCTGCTGCGTTCGTGCGC	GGGATCAAGT	-	exon	A0
	Solyc01g028830	Chr1:33506328-33506347	4+	4+	GTAAGGTTTTGTTCTCGGGC	TGGTTTCGAT	+	exon	A0
	Solyc01g028830	Chr1:33508836-33508855	4+	3+	TTGTAGCTGAGCTACGACGC	AGGTCTTCTA	+	exon	A0.1
	Solyc01g008860	Chr1:2886152-2886171:rc	4+	4+	GGTGCTCGGTCGATCTCCCG	GGGGCGGAAC	-	exon	A0
	Solyc06g083500	Chr6:45195231-45195250:rc	4+	4+	ACATTCCTTTGGGCCGAATC	GGGTAAAGGG	-	exon	A0
	Solyc01g067400	Chr1:68270871-68270890	4+	4+	GCTTGGTAAAGTCCTCGCAT	TGGGTAACGA	+	exon	A0
	Solyc01g067400	Chr1:68278348-68278367:rc	4+	4+	TCGGCTGGAAAGCACCGGAC	AGGTAGGTGT	-	exon	A0
	Solyc05g024290	Chr5:31021658-31021677:rc	4+	4+	AGGGCAACTACCTAGCGGTC	AGGTAAATTC	-	exon	A0
	Solyc04g050970	Chr4:48334204-48334223	4+	4+	GCAGGGCGAGCAACCGGGAC	AGGCTTGTCC	-	exon	A0
	Solyc05g007050	Chr5:1639931-1639950	4+	4+	GGTTTCGGATGTGTCTTCAA	GGGGTGGATC	+	exon	B0
	Solyc05g007050	Chr5:1639932-1639951	4+	4+	GTTTCGGATGTGTCTTCAAG	GGGTGGATCG	-	exon	B0
	Solyc05g007050	Chr5:1639935-1639954	4+	4+	TCGGATGTGTCTTCAAGGGG	TGGATCGAAG	+	exon	B0
	Solyc05g007050	Chr5:1640182-1640201:rc	4+	4+	TAGGGTGAACGAGATCACCC	AGGAAATTCA	-	exon	B0
	Solyc05g007050	Chr5:1642719-1642738:rc	4+	4+	ACATGTGTCTTATCACCCTC	AGGACCATCT	-	exon	B0
	Solyc05g007050	Chr5:1643414-1643433:rc	4+	4+	CTCATCATAGGCCTAACTTT	GGGATCACGA	-	exon	B0
	Solyc05g007050	Chr5:1643561-1643580	4+	4+	CAGGGATCATTCTCGAGGAA	TGGACAACAA	-	exon	B0
	Solyc01g008870	Chr1:2893616-2893635:rc	4+	4+	AGGGAGTTCAGACTATACCT	TGGGCTACAC	-	exon	B0
	Solyc01g008870	Chr1:2894121-2894140	4+	4+	CGATGCCCTTGGAAGCTTTC	GGGCTTAAGT	+	exon	B0
	Solyc01g008870	Chr1:2894245-2894264	4+	4+	CGCCAAGAAGGCTATCAGCT	CGGAAGTTCC	-	exon	B0
	Solyc02g087830	Chr2:44669663-44669682:rc	4+	4+	TACCCTTAGTAACAAGCAGA	AGGGGAAACG	-	exon	B0
	Solyc02g087830	Chr2:44669658-44669677	4+	4+	CCCCTTCTGCTTGTTACTAA	GGGTATAGCT	+	exon	B0
	Solyc02g087830	Chr2:44669683-44669702	4+	4+	TAGCTAGCCGGTGACGCATT	TGGACCAGTT	+	exon	B0
	Solyc02g087830	Chr2:44669692-44669711	4+	4+	GGTGACGCATTTGGACCAGT	TGGAGAATTG	+	exon	B0
	Solyc02g087830	Chr2:44669914-44669933	4+	4+	GAGAAATTGCCACCAAGACG	AGGATCTATA	+	exon	B0
	Solyc02g087830	Chr2:44670340-44670359:rc	4+	4+	CTCGCCAGAGATGGGCCAGA	GGGAGATCAG	-	exon	B0
	Solyc02g087830	Chr2:44670341-44670360:rc	4+	4+	ACTCGCCAGAGATGGGCCAG	AGGGAGATCA	-	exon	B0
	Solyc02g087830	Chr2:44670371-44670390	4+	4+	ATAGCTTCGCGTTGTAGTCC	TGGAATGACA	+	exon	B0
	Solyc02g087830	Chr2:44670544-44670563:rc	4+	4+	ATTAGGGGCAGCACAAGGCC	TGGCTTATCT	-	exon	B0
	Solyc02g087830	Chr2:44670567-44670586	4+	4+	ATTCTTATCCTCGTGCACCA	CGGAAGAACC	-	exon	B0
	Solyc02g087830	Chr2:44670709-44670728:rc	4+	4+	GTCTACGAGTTTATGGCTCG	TGGGAGTCTG	-	exon	B0
	Solyc02g087830	Chr2:44671116-44671135:rc	4+	4+	AACCACTCCTGATAGAGGCA	TGGTACAGGA	-	exon	B0
	Solyc02g087830	Chr2:44671133-44671152	4+	4+	GTTCGAGCAGTTTCAGCGGA	AGGGCCTCCG	+	exon	B0
	Solyc06g062920	Chr6:36106969-36106988	4+	4+	TCGTTCGGAGGCGAAGGTGA	TGGAGAACAG	+	exon	B0
	Solyc06g062920	Chr6:36107520-36107539:rc	4+	4+	TCGAAAATAGTTGATCCTCG	AGGTTTCCCC	-	exon	B0
	Solyc06g062920	Chr6:36108171-36108190:rc	4+	4+	GAGGGGGCGTTTCTTGTCCA	TGGCTCGTCG	-	exon	B0
	Solyc06g062920	Chr6:36108184-36108203	4+	4+	CCCCCTCAAAGAGCGAATCT	TGGTGGATTG	-	exon	B0
	Solyc06g062920	Chr6:36108193-36108212	4+	4+	AGAGCGAATCTTGGTGGATT	GGGCAAGACC	-	exon	B0
	Solyc06g062920	Chr6:36108224-36108243:rc	4+	4+	TTGTGTGGATCCCTTAGCAT	TGGTCTTGCC	-	exon	B0
	Solyc06g062920	Chr6:36108283-36108302	4+	4+	CTCGACACAAGGAGCCAAGA	AGGTAGCTGC	-	exon	B0
	Solyc06g062920	Chr6:36108345-36108364:rc	4+	4+	GCTCATAGTAGGCCTAGACC	TGGGGTGGTG	-	exon	B0
	Solyc06g062920	Chr6:36108567-36108586	4+	4+	AGCAACGGAGAGTCGGACAC	AGGTATAAAC	+	exon	B0
	Solyc08g077560	Chr8:58708412-58708431	4+	4+	ACTCAATACACTTGGATTGC	AGGTATAAAC	-	exon	B0
	Solyc08g077560	Chr8:58709414-58709433	4+	4+	CTGGGATATTGCGCTACAGA	TGGCGAAAGA	+	exon	B0
	Solyc08g077560	Chr8:58709511-58709530	4+	4+	AAGCCGTACCTGTAGTTCCT	TGGAGAACAA	+	exon	B0
	Solyc08g077560	Chr8:58709557-58709576:rc	4+	4+	CCATCCCTTGTGCGGCACCA	AGGATAATTT	-	exon	B0
	Solyc08g077560	Chr8:58709565-58709584:rc	4+	4+	CAGATAAGCCATCCCTTGTG	CGGCACCAAG	-	exon	B0
	Solyc08g077560	Chr8:58709584-58709603	4+	4+	GCACGAGGGACTAGAAGTCC	AGGTATGCTT	-	exon	B0

Table S5. Number of SNPs

1 SNP every bp (cumulative s-genes)	
BIG	**ALL**
1,031	472

S-Gene family	Genes		Gene Length (bp)	N° variants (total)		High impact		1 SNP every bp		Moderate impact		1 SNP every bp		low impact		1 SNP every bp		gene coordinates	
				BIG	ALL	BIG	ALL	BIG	ALL	BIG	ALL	BIG	ALL	BIG	ALL	BIG	ALL	start	end
PMR4	**Solyc07g053980**	Glucan synthase like 1 (D8R536_SELML)	6,838	125	221	0	0	0	0	1	8	6,838	855	27	37	253	185	62,388,193	62,395,031
	Solyc02g078230	Glucan synthase like 1 (D8R536_SELML)	7,811	137	229	0	0	0	0	15	40	521	195	20	41	391	191	42,980,821	42,988,632
	Solyc11g005980	Glucan synthase like 3 (D8QQF0_SELML)	13,729	119	259	0	0	0	0	13	30	1,056	458	20	46	686	298	776,828	790,557
	Solyc01g006370	Glucan synthase like 3 (D8R885_SELML)	21,825	284	462	1	2	21,825	10,913	9	14	2,425	1,559	8	15	2,728	1,455	983,795	1,005,620
	Solyc07g056260	Glucan synthase like 3 (D8R885_SELML)	25,711	377	662	2	3	12,856	8,570	18	31	1,428	829	10	24	2,571	1,071	64,090,068	64,115,779
	Solyc01g073750	Glucan synthase like 3 (D8R885_SELML)	27,742	365	576	1	3	27,742	9,247	16	32	1,734	867	17	25	1,632	1,110	81,118,565	81,146,307
	Solyc07g061920	Glucan synthase like 3 (D8R885_SELML)	11,780	157	262	3	3	3,927	3,927	14	28	841	421	23	48	512	245	64,801,005	64,812,785
	Solyc03g111570	Glucan synthase like 7 (D8TE42_SELML)	25,071	350	496	0	0	0	0	4	10	6,268	2,507	22	30	1,140	836	62,185,065	62,210,136
	Solyc01g006350	Glucan synthase like 7 (D8TE42_SELML)	17,956	559	866	1	1	17,956	17,956	5	6	3,591	2,993	19	22	945	816	934,429	952,385
	total		158,463	2,473	4,033	8	12	19,808	13,205	95	199	1,668	796	166	288	955	550		
PMR5	**Solyc06g082070**	Glucan synthase-like 1	2,997	117	180	0	0	0	0	1	5	2,997	599	8	19	375	158	48,008,357	48,011,354
	Solyc06g007170	annotation not available.	6,139	324	410	1	1	6,139	6,139	19	22	323	279	30	32	205	192	1,231,308	1,237,447
	Solyc03g006220	annotation not available.	5,384	242	335	1	2	5,384	2,692	9	15	598	359	9	15	598	359	848,503	853,887
	Solyc10g008030	annotation not available.	3,193	154	255	0	1	0	3,193	16	21	200	152	6	10	532	319	2,196,279	2,199,472
	Solyc02g077080	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR	4,357	141	218	0	0	0	0	2	8	2,179	545	4	9	1,089	484	42,135,296	42,139,653
	Solyc07g062210	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	3,359	165	243	0	1	0	3,359	8	10	420	336	5	11	672	305	65,008,011	65,011,370
	Solyc07g053350	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	5,351	167	266	2	2	2,676	2,676	4	4	1,338	1,338	4	6	1,338	892	61,806,270	61,811,621
	Solyc12g014360	protein trichome birefringence-like 41	5,033	486	589	0	0	0	0	4	9	1,258	559	1	4	5,033	1,258	5,213,918	5,218,951
	Solyc07g062220	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	4,742	172	246	0	1	0	4,742	13	16	365	296	11	22	431	216	65,012,381	65,017,123
	Solyc11g006990	Trichome birefringence-like 36 (AHRD V3.3 *** A0A061FQB5_THECC)	2,565	62	191	0	0	0	0	2	12	1,283	214	8	12	321	214	1,487,075	1,489,640
	Solyc10g007450	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	3,142	117	161	0	1	0	3,142	16	19	196	165	1	2	3,142	1,571	1,809,659	1,812,801
	Solyc12g044450	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	3,547	174	327	0	0	0	0	9	11	394	322	5	8	709	443	38,447,988	38,451,535
	Solyc01g068420	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	2,521	110	189	0	0	0	0	10	15	252	168	7	15	360	168	77,672,583	77,675,104
	Solyc07g055780	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	4,945	117	209	0	0	0	0	3	8	1,648	618	2	5	2,473	989	63,714,067	63,719,012
	Solyc07g055800	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	2,907	116	212	0	2	0	1,454	4	8	727	363	2	4	1,454	727	63,724,680	63,727,587
	Solyc01g068430	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	3,214	82	153	0	0	0	0	1	2	3,214	1,607	2	7	1,607	459	77,679,243	77,682,457
	Solyc07g055790	Trichome birefringence-like protein (AHRD V3.3 *** G7JPR2_MEDTR)	2,263	80	158	0	2	0	1,132	4	11	566	206	2	5	1,132	453	63,721,278	63,723,541
	Solyc07g053330	Protein trichome birefringence (AHRD V3.3 *** A0A199W0G2_ANACO)	1,897	104	163	0	1	0	1,897	16	22	119	86	9	13	211	146	61,793,525	61,795,422
	Solyc10g078910	Protein trichome birefringence (AHRD V3.3 *** TBR_ARATH)	3,608	156	232	0	0	0	0	4	10	902	361	13	25	278	144	60,561,209	60,564,817
	Solyc01g060270	Protein trichome birefringence (AHRD V3.3 *** TBR_ARATH)	1,801	19	122	0	3	0	600	0	14	#DIV/0!	129	3	7	600	257	69,894,965	69,896,766
	Solyc02g082950	Trichome birefringence-like protein (AHRD V3.3 *** G7JKP3_MEDTR)	2,300	190	255	1	1	2,300	2,300	25	27	92	85	19	21	121	110	46,561,600	46,563,900
	Solyc01g068440	Trichome birefringence-like protein (AHRD V3.3 *** G7IKB9_MEDTR)	3,754	46	153	0	1	0	3,754	2	5	1,877	751	0	5	#DIV/0!	751	77,689,619	77,693,373
	total		79,019	3,341	5,267	5	19	5,338	1,405	172	274	459	288	151	257	523	307		
PMR6	**Solyc11g008140**	Pectate lyase family protein (D7LXH4_ARALY)	3,892	693	1,347	0	1	0	3,892	4	8	973	487	2	4	1,946	973	2,407,048	2,410,940
	Solyc03g071570	Pectate lyase-like protein (Q6U6I9_BRANA)	4,163	185	344	0	0	0	0	5	9	833	463	1	1	4,163	4,163	18,202,566	18,206,729
	Solyc05g055510	Pectate lyase family protein (D7LXH4_ARALY)	2,913	178	234	1	1	2,913	2,913	4	6	728	486	13	16	224	182	65,130,199	65,133,112
	Solyc09g008380	Pectate lyase-like protein (Q56XU8_ARATH)	5,393	206	285	0	0	0	0	1	1	5,393	5,393	9	12	599	449	1,844,382	1,849,775
	Solyc09g091430	Pectate lyase 1-27 (B9TU35_9ROSI)	4,371	197	349	0	0	0	0	2	5	2,186	874	6	8	729	546	70,703,546	70,707,917
	Solyc06g083580	Pectate lyase 1-27 (B9TU35_9ROSI)	6,027	85	193	0	0	0	0	6	15	1,005	402	4	13	1,507	464	48,943,373	48,949,400
	Solyc05g014000	Pectate lyase (D3IZZ1_GOSHE)	2,806	326	407	0	0	0	0	7	9	401	312	4	4	702	702	7,501,147	7,503,953
	Solyc02g093580	Tomato 9612 mRNA (manually curated)	2,000	84	123	1	2	2,000	1,000	6	11	333	182	4	4	500	500	54,392,803	54,394,803
	Solyc03g111690	Pectate lyase (Q2Z1Y4_PRUMU)	2,151	322	425	1	1	2,151	2,151	7	9	307	239	1	2	2,151	1,076	62,343,287	62,345,438
	Solyc09g061890	Pectate lyase 1-27 (B9TU35_9ROSI)	3,693	934	1,319	0	0	0	0	1	1	3,693	3,693	7	8	528	462	60,618,611	60,622,304
	Solyc05g007080	Pectate lyase family protein (D7M6R5_ARALY)	3,019	37	138	0	0	0	0	4	8	755	377	5	9	604	335	1,665,367	1,668,386
	Solyc01g010740	Pectate lyase family protein (D7M6R5_ARALY)	3,970	273	414	1	1	3,970	3,970	8	12	496	331	4	11	993	361	5,778,009	5,781,979
	Solyc02g087670	Pectate lyase family protein (D7M6R5_ARALY)	1,993	107	174	4	4	498	498	8	21	249	95	8	10	249	199	50,023,499	50,025,492
	Solyc03g113150	Pectate lyase family protein (D7LQ55_ARALY)	2,329	96	168	0	0	0	0	4	8	582	291	12	17	194	137	63,409,275	63,411,604
	Solyc06g071020	Pectate lyase (B6TSP4_MAIZE)	2,019	36	108	2	2	1,010	1,010	5	11	404	184	3	11	673	184	43,624,823	43,626,842
	Solyc03g058890	Pectate lyase family protein (D7LQ55_ARALY)	2,726	1,370	1,914	0	0	0	0	3	5	909	545	3	5	909	545	26,591,514	26,594,240
	Solyc02g080910	Pectate lyase family protein (D7ME84_ARALY)	5,301	117	197	3	4	1,767	1,325	10	13	530	408	10	13	530	408	45,010,413	45,015,714
	Solyc02g080910	Pectate lyase family protein (D7ME84_ARALY)	5,301	117	197	3	4	1,767	1,325	10	13	530	408	10	13	530	408	45,010,413	45,015,714
	Solyc06g071840	Pectate lyase (Q2Z1Y4_PRUMU)	3,467	98	200	0	0	0	0	1	8	3,467	433	4	7	867	495	44,255,013	44,258,480
	Solyc03g058910	Pectate lyase (B6TPL1_MAIZE)	1,734	2,041	3,134	0	0	0	0	4	7	434	248	3	6	578	289	26,603,230	26,604,964
	Solyc02g067450	Pectate lyase family protein (D7M6R5_ARALY)	5,642	263	359	1	2	5,642	2,821	4	5	1,411	1,128	6	9	940	627	37,643,757	37,649,399
	Solyc12g044750	Pectate lyase family protein (D7M222_ARALY)	1,956	300	960	0	1	0	1,956	0	3	#DIV/0!	652	1	4	1,956	489	37,875,581	37,877,537
	total		76,866	8,065	12,989	17	23	1,793	1,325	104	188	739	409	120	187	641	411		
DMR1	**Solyc04g008760**	Homoserine kinase (D2DKF1_SOYBN)	1,133	147	215	1	1	1,133	1,133	6	6	189	189	6	12	189	94	2,412,878	2,414,011
	total		1,133	147	215	1	1	49,708	49,708	6	6	189	189	6	12	189	94		
DMR6	**Solyc03g080190**	Flavanone 3-hydroxylase-like protein (Q9FLV0_ARATH)	4,939	386	550	1	2	4,939	2,470	4	5	1,235	988	3	5	1,646	988	52,078,938	52,083,877
	Solyc06g073080	Flavonol synthase/flavanone 3-hydroxylase (FLS_PETHY)	2,957	48	225	0	1	0	2,957	3	14	986	211	4	14	739	211	45,017,961	45,020,918
	total		7,896	434	775	1	3	89,212	29,737	7	19	1,128	416	7	19	1,128	416		
DND1	**Solyc02g088560**	Cyclic nucleotide gated channel (A9CRE4_MALDO)	3,823	182	327	1	1	3,823	3,823	3	7	1,274	546	5	13	765	294	50,608,396	50,612,219
	Solyc12g005400	Cyclic nucleotide gated channel (A9CRE4_MALDO)	5,932	116	228	0	0	0	0	2	6	2,966	989	9	18	659	330	236,128	242,060
	Solyc10g006800	Cyclic nucleotide gated channel (A9CRE4_MALDO)	6,447	112	251	1	1	6,447	6,447	11	25	586	258	4	15	1,612	430	1,240,517	1,246,964
	total		16,202	410	806	2	2	56,655	56,655	16	38	1,013	426	18	46	900	352		
MLO1	**Solyc04g049090**	SlMlo1 (manually curated)	5,506	846	1,688	0	0	0	0	0	2	#DIV/0!	2,753	1	1	5,506	5,506	39,552,675	39,558,181
	Solyc03g095650	MLO-like protein 17 (C6EWF0_VITVI)	8,144	105	166	0	3	0	2,715	1	4	8,144	2,036	2	4	4,072	2,036	56,833,316	56,841,460
	Solyc06g010030	MLO-like protein 3 (C6EWE6_VITVI)	6,064	2,325	2,596	0	0	0	0	4	4	1,516	1,516	10	11	606	551	4,786,764	4,792,828
	Solyc11g069220	MLO-like protein 3 (C6EWE6_VITVI)	7,193	167	256	0	0	0	0	7	11	1,028	654	5	11	1,439	654	53,856,033	53,863,226
	Solyc02g082430	MLO-like protein 3 (C6EWE6_VITVI)	6,387	116	257	0	2	0	3,194	2	4	3,194	1,597	2	5	3,194	1,277	46,116,758	46,123,145
	Solyc00g007200	MLO-like protein 3 (C6EWE6_VITVI)	6,525	490	667	0	0	0	0	0	1	#DIV/0!	6,525	0	9	#DIV/0!	725	6,816,892	6,823,417
	Solyc06g010010	Mlo like protein (Q0WWA7_ARATH)	7,019	307	345	2	2	3,510	3,510	20	22	351	319	13	15	540	468	4,699,552	4,706,571
	Solyc08g067760	MLO-like protein 3 (C6EWE6_VITVI)	5,822	295	657	0	3	0	1,941	1	10	5,822	582	1	6	5,822	970	56,747,912	56,753,734
	Solyc08g015870	MLO-like protein 7 (C6EWE8_VITVI)	4,943	189	396	0	1	0	4,943	6	13	824	380	3	10	1,648	494	6,173,940	6,178,883
	Solyc01g102520	MLO-like protein 1 (Q94CG7_MAIZE)	3,579	108	174	0	0	0	0	4	10	895	358	8	14	447	256	91,311,060	91,314,639
	Solyc07g063260	MLO-like protein 14 (B6SWK8_MAIZE)	7,555	141	227	0	0	0	0	1	5	7,555	1,511	1	9	7,555	839	65,771,745	65,779,300
	Solyc06g082820	MLO-like protein 1 (C6EWE5_VITVI)	4,362	86	157	2	3	2,181	1,454	5	10	872	436	6	12	727	364	48,489,673	48,494,035
	Solyc02g077570	MLO-like protein 6 (C6EWE7_VITVI)	4,842	134	201	2	2	2,421	2,421	16	24	303	202	8	14	605	346	42,467,244	42,472,086
	total		77,941	5,309	7,787	6	16	8,506	3,190	67	120	1,163	650	60	121	1,299	644		
CPR5	**Solyc04g054170**	CPR5 (Fragment) (B0ZWT2_ARATH)	7,534	663	873	0	0	0	0	2	6	3,767	1,256	6	9	1,256	837	51,873,591	51,881,125
	total		7,534	653	873	0	0	0	0	2	6	3,767	1,256	6	9	1,256	837		
BIK1	**Solyc10g084770**	Receptor-like protein kinase At3g21340 (RLK6_ARATH)	2,806	89	141	0	0	0	0	3	10	935	281	5	10	561	281	64,191,839	64,194,645
	Solyc04g011520	Serine/threonine kinase-like protein ABC1063 (Q1KMP8_HORVD)	4,625	159	291	0	0	0	0	0	1	#DIV/0!	4,625	8	14	578	330	3,956,690	3,961,315
	Solyc09g010850	ATP binding / serine-threonine kinase (C5DB71_VITVI)	2,976	310	512	0	0	0	0	3	4	992	744	4	7	744	425	4,169,558	4,172,534
	Solyc08g005500	tomato protein kinase 1b (manually curated)	3,182	223	304	2	2	1,591	1,591	11	12	289	265	3	4	1,061	796	525,975	529,157
	Solyc05g053930	ATP binding / serine-threonine kinase (C5DB71_VITVI)	3,077	167	248	0	0	0	0	5	9	615	342	7	10	440	308	63,954,180	63,957,257
	Solyc05g007140	Receptor-like protein kinase At3g21340 (RLK6_ARATH)	3,685	204	643	0	1	0	3,685	1	4	3,685	921	2	6	1,843	614	1,733,186	1,736,871
	Solyc01g010660	Receptor-like protein kinase At3g21340 (RLK6_ARATH)	6,629	179	327	0	0	0	0	1	5	6,629	1,326	1	2	6,629	3,315	5,673,997	5,680,626
	Solyc05g007050	ATP binding / serine-threonine kinase (C5DB71_VITVI)	6,020	48	240	1	1	6,020	6,020	3	7	2,007	860	2	11	3,010	547	1,637,910	1,643,930
	Solyc01g010780	ATP binding / serine-threonine kinase (C5DB71_VITVI)	5,574	135	247	0	0	0	0	2	5	2,787	1,115	2	4	2,787	1,394	5,831,702	5,837,276
	Solyc07g007980	ATP binding / serine-threonine kinase (C5DB71_VITVI)	3,386	63	235	0	0	0	0	1	6	3,386	564	1	1	3,386	3,386	2,672,425	2,675,811
	Solyc03g032150	Serine/threonine kinase-like protein ABC1063 (Q1KMP8_HORVD)	5,169	75	184	0	2	0	2,585	5	9	1,034	574	9	15	574	345	4,653,491	4,658,660
	Solyc11g062400	ATP binding / serine-threonine kinase (C5DB71_VITVI)	3,008	466	672	0	0	0	0	1	1	3,008	3,008	4	8	752	376	49,403,389	49,406,397
	Solyc00g007060	Serine/threonine kinase-like protein ABC1063 (Q1KMP8_HORVD)	5,561	209	330	0	0	0	0	2	2	2,781	2,781	3	3	1,854	1,854	6,674,808	6,680,369
	Solyc01g008870	ATP binding / serine-threonine kinase (C5DB71_VITVI)	2,943	107	176	1	1	2,943	2,943	4	7	736	420	1	7	2,943	420	2,892,754	2,895,697
	Solyc01g112220	Serine/threonine protein kinase-like (Q5XWQ1_SOLTU)	2,488	166	215	0	0	0	0	5	5	498	498	6	6	415	415	98,271,488	98,273,976
	Solyc10g074710	ATP binding / serine-threonine kinase (C5DB71_VITVI)	3,013	306	526	0	0	0	0	0	0	#DIV/0!	#DIV/0!	0	2	#DIV/0!	1,507	58,327,353	58,330,366
	Solyc12g009310	ATP binding / serine-threonine kinase (C5DB71_VITVI)	3,616	224	301	0	0	0	0	5	7	723	517	6	8	603	452	2,586,788	2,590,404
	Solyc04g082500	ATP binding / serine-threonine kinase (C5DB71_VITVI)	4,167	256	350	0	0	0	0	5	9	833	463	5	9	833	463	66,152,655	66,156,822
	Solyc07g042590	ATP binding / serine-threonine kinase (C5DB71_VITVI)	5,310	209	344	0	5	0	1,062	2	4	2,655	1,328	1	3	5,310	1,770	56,055,036	56,060,346
	Solyc12g077390	ATP binding / serine-threonine kinase (C5DB71_VITVI)	2,031	287	591	1	1	2,031	2,031	0	5	#DIV/0!	406	2	3	1,016	677	18,845,982	18,848,013
	Solyc05g025820	ATP binding / serine-threonine kinase (C5DB71_VITVI)	1,930	835	1,145	0	1	0	1,930	3	8	643	241	2	4	965	483	36,956,235	36,958,165
	Solyc02g087830	ATP binding / serine-threonine kinase (C5DB71_VITVI)	2,858	61	126	1	2	2,858	1,429	9	22	318	130	4	17	715	168	50,091,700	50,094,558
	Solyc07g041940	ATP binding / serine-threonine kinase (C5DB71_VITVI)	2,257	328	622	0	0	0	0	6	10	376	226	1	7	2,257	322	54,672,988	54,675,245
	Solyc12g049360	Receptor-like protein kinase At3g21340 (RLK6_ARATH)	2,490	330	576	0	0	0	0	2	5	1,245	498	3	5	830	498	36,987,083	36,989,573
	Solyc01g028830	ATP binding / serine-threonine kinase (C5DB71_VITVI)	3,822	1,512	2,622	1	1	3,822	3,822	1	2	3,822	1,911	0	2	#DIV/0!	1,911	41,232,211	41,236,033
	Solyc08g074980	ATP binding / serine-threonine kinase (C5DB71_VITVI)	2,984	113	330	0	1	0	2,984	4	12	746	249	5	14	597	213	59,107,456	59,110,440
	Solyc08g061250	ATP binding / serine-threonine kinase (C5DB71_VITVI)	2,070	453	715	0	0	0	0	1	4	2,070	518	2	3	1,035	690	48,305,701	48,307,771
	Solyc06g062920	auxin-regulated dual specificity cytosolic kinase (manually curated)	2,194	179	247	2	2	1,097	1,097	2	3	1,097	731	1	3	2,194	731	39,716,883	39,719,077
	Solyc01g008860	ATP binding / serine-threonine kinase (C5DB71_VITVI)	2,535	135	213	2	2	1,268	1,268	10	17	254	149	4	14	634	181	2,886,127	2,888,662
	Solyc06g005520	ATP binding / serine-threonine kinase (C5DB71_VITVI)	3,884	339	467	0	0	0	0	3	3	1,295	1,295	10	11	388	353	546,476	550,360
	Solyc01g088690	ATP binding / serine-threonine kinase (C5DB71_VITVI)	3,071	139	209	1	1	3,071	3,071	14	15	219	205	9	13	341	236	83,437,740	83,440,811
	Solyc09g075720	ATP binding / serine-threonine kinase (C5DB71_VITVI)	2,141	179	219	0	0	0	0	12	15	178	143	21	23	102	93	67,338,513	67,340,654
	Solyc08g077560	ATP binding / serine-threonine kinase (C5DB71_VITVI)	4,303	147	217	1	1	4,303	4,303	8	10	538	430	3	6	1,434	717	61,541,667	61,545,970
	Solyc06g083500	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	3,773	109	201	1	1	3,773	3,773	1	6	3,773	629	7	14	539	270	48,904,883	48,908,656
	Solyc09g008010	ATP binding / serine-threonine kinase (C5DB71_VITVI)	5,059	148	229	0	0	0	0	3	6	1,686	843	6	7	843	723	1,486,900	1,491,959
	Solyc04g082510	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	3,336	120	175	0	0	0	0	1	5	3,336	667	2	4	1,668	834	66,163,398	66,166,734
	Solyc12g094680	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	2,757	150	218	0	0	0	0	7	12	394	230	12	24	230	115	64,835,508	64,838,265
	Solyc10g006340	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	2,658	75	134	0	1	0	2,658	6	14	443	190	11	30	242	89	972,310	974,968
	Solyc01g067400	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	9,561	561	1,114	2	4	4,781	2,390	3	17	3,187	562	4	11	2,390	869	75,879,603	75,889,164
	Solyc09g061330	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	2,747	590	792	0	0	0	0	26	29	106	95	32	35	86	78	58,717,095	58,719,842
	Solyc11g072660	Receptor protein kinase-like protein (Q9LJU5_ARATH)	4,155	142	223	0	0	0	0	10	18	416	231	4	10	1,039	416	55,884,048	55,888,203
	Solyc12g005160	Receptor protein kinase-like protein (Q9LJU5_ARATH)	2,010	95	138	0	0	0	0	12	17	168	118	16	23	126	87	117,402	119,412
	Solyc05g024290	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	9,913	635	988	1	1	9,913	9,913	1	4	9,913	2,478	1	4	9,913	2,478	31,012,552	31,022,465
	Solyc10g085990	Receptor protein kinase-like protein (Q9LJU5_ARATH)	4,455	129	187	0	0	0	0	1	2	4,455	2,228	5	7	891	636	64,979,123	64,983,578
	Solyc09g007170	Serine/threonine kinase (Q32SF8_HORVD)	6,476	100	170	0	0	0	0	0	1	#DIV/0!	6,476	12	15	540	432	793,123	799,599
	Solyc06g075550	Serine/threonine kinase (Q32SF8_HORVD)	3,741	35	130	0	1	0	3,741	2	8	1,871	468	7	13	534	288	46,912,732	46,916,473
	Solyc10g005300	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	2,457	86	166	0	5	0	491	6	14	410	176	6	10	410	246	220,068	222,525
	Solyc07g064340	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	3,443	73	142	0	1	0	3,443	11	20	313	172	3	10	1,148	344	66,532,119	66,535,562
	Solyc01g079340	Receptor-like kinase (A7VM33_MARPO)	7,480	145	442	0	1	0	7,480	1	15	7,480	499	2	15	3,740	499	78,433,449	78,440,929
	Solyc02g094380	Serine/threonine-protein kinase (C6ZRV0_SOYBN)	2,593	61	117	0	0	0	0	7	17	370	153	2	8	1,297	324	54,921,482	54,924,075
	Solyc04g050970	Receptor protein kinase-like protein (Q9LRY1_ARATH)	2,533	903	1,225	1	2	2,533	1,267	5	9	507	281	3	5	844	507	49,184,225	49,186,758
	total		194,952	12,789	21,376	18	41	2,394	1,051	237	452	823	431	272	500	717	390		
SR1	**Solyc01g105230**	Calmodulin-binding transcription activator 2 (Q0WNN4_ARATH)	10,821	89	257	0	2	0	5,411	9	24	1,202	451	4	15	2,705	721	93,464,755	93,475,576
	total		10,821	89	257	0	2	0	36,209	9	24	1,202	451	4	15	2,705	721		

Table S6. vcf data

Name		TGRC	PI CGN#	EA #	Name	Botanical variety	Categories													nt mutated	position in nt
Panama	TS-214	-	-	-	Panama	0	Landrace	homoz	TS-214	BIG	BIK1	Solyc06g083500	SL2.50ch06	48908349	T	A	stop_gained	c.136A>T	p.Lys46*	136/1303	18.63%
Panama					Panama			homoz	TS-214	BIG	BIK1	Solyc01g067400	SL2.50ch01	75887538	A	G	stop_lost	c.424T>C	p.Ter142Glnext*?	712/2051	34.71%
Panama					Panama			homoz	TS-214	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
Panama					Panama			homoz	TS-214	BIG	DMR6	Solyc03g080190	SL2.50ch03	52083445	A	G	stop_lost	c.229T>C	p.Ter77Glnext*?	349/1479	23.60%
Panama					Panama			homoz	TS-214	BIG	MLO1	Solyc06g082820	SL2.50ch06	48492973	T	C	stop_lost	c.1123T>C	p.Ter375Glnext*?	1225/1945	62.98%
Panama					Panama			homoz	TS-214	BIG	PMR4	Solyc07g056260	SL2.50ch07	64110648	A	C	stop_lost	c.828T>G	p.Ter276Trpext*?	984/5895	16.69%
Panama					Panama			heteroz	TS-214	BIG	MLO1	Solyc06g082820	SL2.50ch06	48490999	A	C	stop_lost	c.342A>C	p.Ter114Tyrext*?	444/1945	22.83%
N 739	TS-074	-	-	-	N 739	S. lycopersicu	Fresh Market	homoz	TS-074	BIG	BIK1	Solyc06g062920	SL2.50ch06	39717979	T	A	stop_lost	c.694T>A	p.Ter232Argext*?	818/1801	45.42%
N 739					N 739			homoz	TS-074	BIG	BIK1	Solyc01g008860	SL2.50ch01	2886129	A	G	stop_lost&splice_region_variant	c.1231T>C	p.Ter411Glnext*?	1231/1233	99.84%
N 739					N 739			homoz	TS-074	BIG	BIK1	Solyc01g067400	SL2.50ch01	75887538	A	G	stop_lost	c.424T>C	p.Ter142Glnext*?	712/2051	34.71%
N 739					N 739			homoz	TS-074	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
N 739					N 739			homoz	TS-074	BIG	DMR6	Solyc03g080190	SL2.50ch03	52083445	A	G	stop_lost	c.229T>C	p.Ter77Glnext*?	349/1479	23.60%
Rowpac	TS-186	LA3214	-	-	Rowpac	S. lycopersicu	Modern Processing	homoz	TS-186	BIG	BIK1	Solyc01g008860	SL2.50ch01	2886129	A	G	stop_lost&splice_region_variant	c.1231T>C	p.Ter411Glnext*?	1231/1233	99.84%
Rowpac					Rowpac			homoz	TS-186	BIG	BIK1	Solyc05g024290	SL2.50ch05	31013858	A	T	stop_gained	c.674T>A	p.Ser225*	911/1957	46.55%
Rowpac					Rowpac			homoz	TS-186	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
Rowpac					Rowpac			homoz	TS-186	BIG	MLO1	Solyc02g077570	SL2.50ch02	42468063	G	T	splice_donor_variant&intron_variant	c.380+1G>T			
Rowpac					Rowpac			homoz	TS-186	BIG	PMR6	Solyc02g067450	SL2.50ch02	37648327	G	A	splice_donor_variant&intron_variant	c.721+2C>T			
Micro-Tom	TS-007	LA3911	-	-	Micro-Tom	S. lycopersicu	Modern Fresh Market	homoz	TS-007	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Micro-Tom					Micro-Tom			homoz	TS-007	BIG	DMR6	Solyc03g080190	SL2.50ch03	52083445	A	G	stop_lost	c.229T>C	p.Ter77Glnext*?	349/1479	23.60%
Micro-Tom					Micro-Tom			homoz	TS-007	BIG	PMR4	Solyc01g073750	SL2.50ch01	81135674	C	T	stop_gained	c.2791C>T	p.Arg931*	2926/5487	53.33%
Micro-Tom					Micro-Tom			homoz	TS-007	BIG	PMR6	Solyc03g111690	SL2.50ch03	62344382	C	T	stop_gained	c.658C>T	p.Gln220*	952/1844	51.63%
Guayaquil	TS-224	LA0410	PI 258474	-	Guayaquil	S. lycopersicu	Landrace/Latin American cul	homoz	TS-224	BIG	BIK1	Solyc06g083500	SL2.50ch06	48908349	T	A	stop_gained	c.136A>T	p.Lys46*	308/1653	18.63%
Guayaquil					Guayaquil			homoz	TS-224	BIG	BIK1	Solyc01g067400	SL2.50ch01	75887538	A	G	stop_lost	c.424T>C	p.Ter142Glnext*?	712/2051	34.71%
Guayaquil					Guayaquil			homoz	TS-224	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
Guayaquil					Guayaquil			homoz	TS-224	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Droplet	TS-296	-	-	-	Droplet	S. lycopersicu	0	homoz	TS-296	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
Droplet					Droplet			homoz	TS-296	BIG	MLO1	Solyc02g077570	SL2.50ch02	42468063	G	T	splice_donor_variant&intron_variant	c.380+1G>T			
Droplet					Droplet			homoz	TS-296	BIG	MLO1	Solyc02g077570	SL2.50ch02	42471760	G	A	stop_gained	c.899G>A	p.Trp300*	899/1128	79.70%
Droplet					Droplet			homoz	TS-296	BIG	PMR4	Solyc01g073750	SL2.50ch01	81135674	C	T	stop_gained	c.2791C>T	p.Arg931*	2926/5487	53.33%
0	TS-409	-	PI124161	-	0	S. lycopersicu	Landrace	homoz	TS-409	BIG	BIK1	Solyc01g008860	SL2.50ch01	2886129	A	G	stop_lost&splice_region_variant	c.1231T>C	p.Ter411Glnext*?	1231/1233	99.84%
0					1			homoz	TS-409	BIG	BIK1	Solyc01g067400	SL2.50ch01	75887538	A	G	stop_lost	c.424T>C	p.Ter142Glnext*?	712/2051	34.71%
0					2			homoz	TS-409	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
0					3			homoz	TS-409	BIG	PMR5	Solyc03g006220	SL2.50ch03	852558	T	A	stop_lost	c.703T>A	p.Ter235Lysext*?	800/1288	62.11%
M-82	TS-003	LA3475	-	-	M-82	S. lycopersicu	Modern Processing	homoz	TS-003	BIG	BIK1	Solyc05g024290	SL2.50ch05	31013858	A	T	stop_gained	c.674T>A	p.Ser225*	911/1957	46.55%
M-82					M-82			homoz	TS-003	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
M-82					M-82			homoz	TS-003	BIG	PMR4	Solyc01g073750	SL2.50ch01	81135674	C	T	stop_gained	c.2791C>T	p.Arg931*	2926/5487	53.33%
Hawaii 7998	TS-004	LA3856	-	-	Hawaii 7998	S. lycopersicu	Inbreed line	homoz	TS-004	BIG	BIK1	Solyc06g005500	SL2.50ch06	527016	T	G	splice_donor_variant&intron_variant	c.376+2T>G			
Hawaii 7998					Hawaii 7999			homoz	TS-004	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
Hawaii 7998					Hawaii 8000			homoz	TS-004	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
KR2	TS-011	-	-	-	KR2	S. lycopersicu	Modern Fresh Market	homoz	TS-011	BIG	MLO1	Solyc02g077570	SL2.50ch02	42468063	G	T	splice_donor_variant&intron_variant	c.380+1G>T			
KR2					KR3			homoz	TS-011	BIG	MLO1	Solyc02g077570	SL2.50ch02	42471760	G	A	stop_gained	c.899G>A	p.Trp300*	899/1128	79.70%
KR2					KR4			homoz	TS-011	BIG	PMR4	Solyc01g006350	SL2.50ch01	952385	T	C	start_lost	c.1A>G	p.Met1?	1/3672	0.03%
KR2					KR5			heteroz	TS-011	BIG	BIK1	Solyc05g007050	SL2.50ch05	1640061	T	G	splice_donor_variant&intron_variant	c.519+2T>G			
KR2					KR6			heteroz	TS-011	BIG	BIK1	Solyc08g077560	SL2.50ch08	61543477	C	T	stop_gained	c.493C>T	p.Gln165*	900/1896	47.47%
Hacienda Rosari	TS-135	LA0466	PI 258469	-	Hacienda Rosari	S. lycopersicu	Landrace/Latin American cul	homoz	TS-135	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Hacienda Rosario					Hacienda Rosario			homoz	TS-135	BIG	DND1	Solyc02g088560	SL2.50ch02	50610274	A	T	stop_gained	c.983T>A	p.Leu328*	1187/2482	47.82%
Hacienda Rosario					Hacienda Rosario			homoz	TS-135	BIG	MLO1	Solyc02g077570	SL2.50ch02	42468063	G	T	splice_donor_variant&intron_variant	c.380+1G>T			
Tarapoto	TS-150	LA2285	-	-	Tarapoto	S. lycopersicu	Landrace/Latin American cul	homoz	TS-150	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
Tarapoto					Tarapoto			homoz	TS-150	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Tarapoto					Tarapoto			homoz	TS-150	BIG	DND1	Solyc02g088560	SL2.50ch02	50610274	A	T	stop_gained	c.983T>A	p.Leu328*	1187/2482	47.82%
Santa Chiara	TS-190	-	-	-	Santa Chiara	0	cultivar	homoz	TS-190	BIG	BIK1	Solyc05g024290	SL2.50ch05	31013858	A	T	stop_gained	c.674T>A	p.Ser225*	911/1957	46.55%
Santa Chiara					Santa Chiara			homoz	TS-190	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
Santa Chiara					Santa Chiara			homoz	TS-190	BIG	PMR4	Solyc01g073750	SL2.50ch01	81135674	C	T	stop_gained	c.2791C>T	p.Arg931*	2926/5487	53.33%
Hunt100	TS-277	LA3144	-	-	Hunt100	S. lycopersicu	Modern Processing	homoz	TS-277	BIG	BIK1	Solyc01g008860	SL2.50ch01	2886129	A	G	stop_lost&splice_region_variant	c.1231T>C	p.Ter411Glnext*?	1231/1233	99.84%
Hunt100					Hunt101			homoz	TS-277	BIG	BIK1	Solyc05g024290	SL2.50ch05	31013858	A	T	stop_gained	c.674T>A	p.Ser225*	911/1957	46.55%
Hunt100					Hunt102			homoz	TS-277	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Edkawi	TS-005	LA2711	-	-	Edkawi	S. lycopersicu	Vintage Fresh Market	homoz	TS-005	BIG	BIK1	Solyc06g005500	SL2.50ch06	527016	T	G	splice_donor_variant&intron_variant	c.376+2T>G			
Edkawi					Edkawi			homoz	TS-005	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Edkawi					Edkawi			heteroz	TS-005	BIG	BIK1	Solyc05g007050	SL2.50ch05	1640061	T	G	splice_donor_variant&intron_variant	c.519+2T>G			
yoku improvemen	TS-012	-	-	-	yoku improvemen	S. lycopersicu	Modern Fresh Market	homoz	TS-012	BIG	MLO1	Solyc02g077570	SL2.50ch02	42468063	G	T	splice_donor_variant&intron_variant	c.380+1G>T			
yoku improvement					yoku improvement			homoz	TS-012	BIG	MLO1	Solyc02g077570	SL2.50ch02	42471760	G	A	stop_gained	c.899G>A	p.Trp300*	899/1128	79.70%
yoku improvement					yoku improvement			heteroz	TS-012	BIG	BIK1	Solyc05g007050	SL2.50ch05	1640061	T	G	splice_donor_variant&intron_variant	c.519+2T>G			
yoku improvement					yoku improvement			heteroz	TS-012	BIG	BIK1	Solyc08g077560	SL2.50ch08	61543477	C	T	stop_gained	c.493C>T	p.Gln165*	900/1896	47.47%
0	TS-078	-	-	EA02895	0	S. lycopersicu	Processing tomato	homoz	TS-078	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
0					0			homoz	TS-078	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
0	TS-089	-	-	EA01185	0	0	Processing tomato	homoz	TS-089	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
0					0			homoz	TS-089	BIG	DND1	Solyc02g088560	SL2.50ch02	50610274	A	T	stop_gained	c.983T>A	p.Leu328*	1187/2482	47.82%
0					0			heteroz	TS-089	BIG	MLO1	Solyc06g082820	SL2.50ch06	48492973	T	C	stop_lost	c.1123T>C	p.Ter375Glnext*?	1225/1945	62.98%
0	TS-090	-	-	EA02753	0	S. lycopersicu	Cocktail tomato	homoz	TS-090	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
0					0			homoz	TS-090	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Puno I	TS-108	-	-	EA01989	Puno I	S. lycopersicu	Processing tomato	homoz	TS-108	BIG	BIK1	Solyc05g024290	SL2.50ch05	31013858	A	T	stop_gained	c.674T>A	p.Ser225*	911/1957	46.55%
Puno I					Puno I			homoz	TS-108	BIG	PMR4	Solyc01g073750	SL2.50ch01	81135674	C	T	stop_gained	c.2791C>T	p.Arg931*	2926/5487	53.33%
NC EBR-6	TS-121	LA3846	-	-	NC EBR-6	S. lycopersicu	Modern Fresh Market	homoz	TS-121	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
NC EBR-6					NC EBR-6			homoz	TS-121	BIG	MLO1	Solyc02g077570	SL2.50ch02	42468063	G	T	splice_donor_variant&intron_variant	c.380+1G>T			
Rutgers	TS-122	LA1090	-	-	Rutgers	S. lycopersicu	Vintage Fresh Market	homoz	TS-122	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Rutgers					Rutgers			homoz	TS-122	BIG	PMR6	Solyc02g093580	SL2.50ch02	54394569	C	T	splice_acceptor_variant&intron_variant	c.102-1G>A			
Hacienda Calera	TS-127	LA0113	-	-	Hacienda Calera	S. lycopersicu	Landrace/Latin American cul	homoz	TS-127	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Hacienda Calera					Hacienda Calera			homoz	TS-127	BIG	MLO1	Solyc02g077570	SL2.50ch02	42468063	G	T	splice_donor_variant&intron_variant	c.380+1G>T			
Hacienda Calera					Hacienda Calera			heteroz	TS-127	BIG	PMR5	Solyc03g006220	SL2.50ch03	852558	T	A	stop_lost	c.703T>A	p.Ter235Lysext*?	800/1288	62.11%
Florida 7547	TS-143	LA4025	-	-	Florida 7547	S. lycopersicu	Modern Fresh Market	homoz	TS-143	BIG	MLO1	Solyc02g077570	SL2.50ch02	42471760	G	A	stop_gained	c.899G>A	p.Trp300*	899/1128	79.70%
Florida 7547					Florida 7547			homoz	TS-143	BIG	PMR5	Solyc07g053350	SL2.50ch07	61809964	A	G	stop_lost	c.506A>G	p.Ter169Trpext*?	548/1473	37.20%
Chiclayo	TS-068	LA0395	-	-	Chiclayo	S. lycopersicu	Latin American cultivar	homoz	TS-068	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Chiclayo					Chiclayo			heteroz	TS-068	BIG	BIK1	Solyc06g062920	SL2.50ch06	39717979	T	A	stop_lost	c.694T>A	p.Ter232Argext*?	818/1801	45.42%
Chiclayo					Chiclayo			heteroz	TS-068	BIG	BIK1	Solyc01g008860	SL2.50ch01	2886129	A	G	stop_lost&splice_region_variant	c.1231T>C	p.Ter411Glnext*?	1231/1233	99.84%
Chiclayo					Chiclayo			heteroz	TS-068	BIG	BIK1	Solyc01g067400	SL2.50ch01	75887538	A	G	stop_lost	c.424T>C	p.Ter142Glnext*?	712/2051	34.71%
Chiclayo					Chiclayo			heteroz	TS-068	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
Chiclayo					Chiclayo			heteroz	TS-068	BIG	PMR5	Solyc02g082950	SL2.50ch02	46562565	A	T	stop_lost	c.674A>T	p.Ter225Leuext*?	778/1488	52.28%
Chiclayo					Chiclayo			heteroz	TS-068	BIG	PMR6	Solyc06g071020	SL2.50ch06	43625286	A	T	stop_gained	c.256A>T	p.Lys86*	293/1588	18.45%
Chiclayo					Chiclayo			heteroz	TS-068	BIG	PMR6	Solyc02g080910	SL2.50ch02	45010864	A	C	stop_lost	c.1984T>G	p.Ter662Gluext*?	1984/2345	84.61%
Chiclayo					Chiclayo			heteroz	TS-068	BIG	PMR6	Solyc02g080910	SL2.50ch02	45010864	A	C	stop_lost	c.1984T>G	p.Ter662Gluext*?	1984/2345	84.61%
E-6203	TS-008	LA4024	-	-	E-6203	S. lycopersicu	Modern Processing	homoz	TS-008	BIG	BIK1	Solyc05g024290	SL2.50ch05	31013858	A	T	stop_gained	c.674T>A	p.Ser225*	911/1957	46.55%
E-6203					E-6203			heteroz	TS-008	BIG	BIK1	Solyc05g007050	SL2.50ch05	1640061	T	G	splice_donor_variant&intron_variant	c.519+2T>G			
E-6203					E-6203			heteroz	TS-008	BIG	BIK1	Solyc06g062920	SL2.50ch06	39717410	T	G	splice_donor_variant&intron_variant	c.402+2T>G			
E-6203					E-6203			heteroz	TS-008	BIG	PMR4	Solyc01g073750	SL2.50ch01	81135674	C	T	stop_gained	c.2791C>T	p.Arg931*	2926/5487	53.33%
0	TS-245	-	-	EA03126	0	S. lycopersicu	Processing tomato	homoz	TS-245	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
0					0			homoz	TS-245	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
0					0			heteroz	TS-245	BIG	BIK1	Solyc05g007050	SL2.50ch05	1640061	T	G	splice_donor_variant&intron_variant	c.519+2T>G			
0					0			heteroz	TS-245	BIG	BIK1	Solyc01g028830	SL2.50ch01	41235068	G	C	stop_lost	c.670G>C	p.Ter224Glnext*?	1024/1671	61.28%
0	TS-085	-	-	-	0	0	0	homoz	TS-085	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
0					0			heteroz	TS-085	BIG	BIK1	Solyc05g024290	SL2.50ch05	31013858	A	T	stop_gained	c.674T>A	p.Ser225*	911/1957	46.55%
0					0			heteroz	TS-085	BIG	PMR4	Solyc01g073750	SL2.50ch01	81135674	C	T	stop_gained	c.2791C>T	p.Arg931*	2926/5487	53.33%
0	TS-113	-	-	EA01198	0	0	Processing tomato	heteroz	TS-113	BIG	BIK1	Solyc04g050970	SL2.50ch04	49186199	G	A	stop_gained	c.211C>T	p.Lys71*	453/2123	21.34%
0					0			heteroz	TS-113	BIG	DMR1	Solyc04g008760	SL2.50ch04	2414006	C	T	stop_gained	c.1129C>T	p.Arg377*	1129/1134	99.56%
Vito	TS-136	-	-	-	Vito	0	0	heteroz	TS-136	BIG	PMR6	Solyc02g080910	SL2.50ch02	45010864	A	C	stop_lost	c.1984T>G	p.Ter662Gluext*?	1984/2345	84.61%
Vito					Vito			heteroz	TS-136	BIG	PMR6	Solyc02g080910	SL2.50ch02	45010864	A	C	stop_lost	c.1984T>G	p.Ter662Gluext*?	1984/2345	84.61%
San Salvador	TS-201	LA1210	-	-	San Salvador	S. lycopersicu	Landrace/Latin American cul	heteroz	TS-201	BIG	PMR6	Solyc02g080910	SL2.50ch02	45011463	G	T	stop_gained	c.1562C>A	p.Ser521*	1562/2345	66.61%
San Salvador					San Salvador			heteroz	TS-201	BIG	PMR6	Solyc02g080910	SL2.50ch02	45011463	G	T	stop_gained	c.1562C>A	p.Ser521*	1562/2345	66.61%

Chapter 5 - Conclusions and Future Perspectives

Genomic surveys and gene editing approaches are powerful tools for obtaining tomato genotypes resistant to biotic stresses. By using genomic surveys to identify defective alleles of susceptibility genes, researchers can develop strategies to improve tomato plants' resistance to biotic stresses. The application of gene editing technologies, such as CRISPR/Cas9, has been successful in obtaining tomato genotypes with reduced susceptibility. In particular, our studies involving the knock-out of susceptibility genes, such as *PMR4* and *DND1*, have demonstrated the potential for reduced susceptibility to late blight and powdery mildew, seperately.

PMR4 is a susceptibility gene that encodes for a plasma membrane-localized protein involved in the regulation of callose deposition in the plant cell wall. Studies have shown that PMR4 plays a role in providing broad-spectrum protection against pathogens in tomato plants (*Huibers, Loonen et al. 2013, Santillán Martínez, Bracuto et al. 2020, Li, Maioli et al. 2022*). In my study, we investigated that full knock-out of the *PMR4* gene through CRISPR/Cas9 editing in two widely cultivated Italian tomato cultivars, ‘San Marzano’ and ‘Oxheart’, can reduce susceptibility to late blight, a devastating disease caused by *P. infestans*. We also selected four tomato *pmr4* mutants and, following the whole genome resequencing, assessed the overall editing efficiency, types of induced mutations, as well as the emergence of any unintended out-target effects. The results towards the late blight confirmed the role of knocked-out *PMR4* in

providing protection against late blight in two tomato cultivars, and proved the reliability of gene editing technology, CRISPR/Cas9.

DND1 is another susceptibility gene that encodes for a nuclear-localized protein involved in the regulation of programmed cell death in plant cells (*Yu, Parker et al. 1998, Clough, Fengler et al. 2000, Chin, DeFalco et al. 2013*). Studies have shown that *DND1* plays a crucial role in plant defense against pathogens, however, it was hard to use in tomato breeding because of its side effects, including dwarfism and autonecrosis spots (*Sun, Wolters et al. 2016, Sun, van Tuinen et al. 2017*). The research conducted demonstrated, for the first time, a reduced susceptibility to *Oidium neolycopersici* in tomato knockout *dnd1* mutants obtained through CRISPR/Cas9 gene editing approach, and also provided a special, full-length *dnd1* resistant mutant, with reduced fitness costs. With whole genome sequencing it was possible to confirm that this special genotype lost all T-DNA insertions and showed the presence of a causal mutation (amino acids deletion) in the *DND1* locus that was indistinguishable from a naturally occurring one. Because even small changes can have a big impact on a plant's overall phenotype, the current results highlight the value of precision genetic engineering and demonstrate the potential of gene editing in plant breeding.

The use of gene editing technologies to develop resistant tomato genotypes has the potential to revolutionize plant breeding programs, allowing for the development of more resistant varieties in a shorter period

of time. However, it is essential to consider the potential risks associated with the use of gene editing approaches, including unintended off-target effects and the emergence of pleiotropic effects. The identification of natural homozygous/heterozygous mutant alleles in tomato genotypes provides a valuable resource for plant genetics and breeding programs. With the aim of locating defective variants of susceptibility genes, we conducted a genomic study on a diverse range of tomato genotypes. The presence of naturally occurring homozygous/heterozygous mutant alleles was emphasized by our survey. Three identified homozygous tomato mutants showed a noticeably decreased susceptibility to powdery mildew. Thus, our results provide a valuable resource for plant genetics, with potential applications in genomic-assisted breeding programs for resistance to biotic stresses.

However, one cannot fail to consider that the introgression of desirable alleles into elite genotypes is a time-consuming process, often hindered by challenges such as linkage drag. In order to acquire tomato genotypes resistant to biotic stresses, we have also given new insights on the application of a gene editing method, as it represents a potent alternative. With the second and even third generation sequencing technologies development, the ability to map and identify genome-wide allelic variation has been enhanced by the expanded genome sequence information in crops (*Varshney, Bohra et al. 2021*). The speeding up of gene and trait discovery as a result has improved the precision and effectiveness of crop breeding initiatives. In additon, our ability to make precise and speedy

changes to plant genomes has considerably increased thanks to advancements in genome editing. With the help of cutting-edge tools and technologies, we have been able to better comprehend the function of the genome and the genetic basis for significant trait architectures. As a result of our ability to quantify and utilize trait variation in elite varieties, our germplasm repositories, and novel variation produced using targeted genetic recombination and genome editing, we expect continued improvement in the rate of genetic gains in crop breeding programs.

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Figure of Gatta. Professional full body shot. It's so hard to choose the best photo, so let's choose the most formal one

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Papers

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