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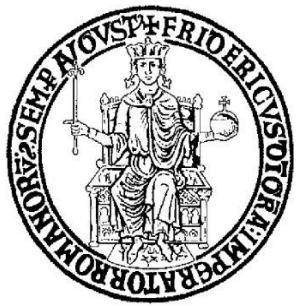

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TITLE:

GENOMIC APPROACHES TO IMPROVE TOMATO RESPONSE TO MULTIPLE STRESS

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SUMMARY

CHAPTER III

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ABSTRACT

Climate changes are reducing cultivable areas and available natural resources, affecting food security in many countries. In this light, producing more food for a growing population represent a critical challenge for humanity in the coming decades. Tomato (Solanum lycopersicum) is among the most widespread horticultural crop and is commonly used as a model plant for genetic studies due to its short biological cycle, the availability of genetic and genomic resources and the recently fully sequenced genome. Globally, many biotic and abiotic stresses adversely affect tomato growth, production and quality, inducing tremendous economic and yield losses. The introduction of R-genes in cultivated tomatoes could enhance resistance to different stresses. However, this process is tedious and time-consuming and results in high costs over the long term. Therefore, developing novel strategies to obtain tomato cultivars with enhanced resilence to multiple stress conditions is critical for plant scientists.

This work was aimed to develop tomato cultivars resistant to multiple stresses using innovative genome editing approaches. As a starting point, ten tomato cultivars of interest for the company were evaluated for their phenotypical traits, the presence or absence of molecular markers associated with resistance loci, and the *in vitro* regeneration rates. The regeneration step is crucial for producing stable genomic-transformed plants. Phenotypical and molecular analysis comparisons allowed to select SanMarzano2 as the most interesting line for the genetic improvement program, due to its strategic importance for the company, the lack of genetic resistances, and the good number of regenerated shoots. Successively, we conducted a large-scale literature analysis, exploring dozens of trancriptomic studies, to identify suitable target genes for the CRISPR/Cas9 editing. Twelve studies were selected, and the raw transcriptomic data of tomato plants exposed to different biotic and abiotic stress were retrieved and analyzed de *novo* using the bioinformatic platform A.I.R.

DEGs induced by each stress were compared for the identification of genes involved in response to different stress. Data cross-comparison allowed the identification of several pathways activated in response to fungi, bacteria, virus, pest, and different abiotic stress such as drought, salinity, cold and oxidative stress. In particular, pathogen recognition, signaling, hormone metabolism, transcription, defensive proteins, and other important cell compounds resulted perturbed after stress recognition. One-hundred and twenty-six genes were identified to be involved in

response to different biotic and abiotic stresses. This list was used as a query against several tomato-stress studies reported in the literature. Interestingly, forty-nine genes have also been reported in other tomatoes-stress interactions, while seventy-seven DEGs have never been characterized in tomato before. These genes could be used for further investigations using genetic engineering techniques for assessing their role in plant multiple stress response. In addition, a bibliographic search with specific keywords allowed the identification of twenty-seven genes involved in tomato resistance or susceptibility to vascular pathogens or multiple stress responses.

The cross-examination of comparative transcriptomic data and bibliographical research allowed the selection of *WATI* and *HyPRPI* as optimal gene targets for the genome editing experiment. To this scope, the CRISPR/Cas9 system was used to produce deleterious deletions on the two genes in order to increase tomato resistance to vascular pathogens and abiotic stress. A large number of SanMarzano2 and MoneyMaker genotypes edited plants were obtained. Transformed plants were checked at the molecular level and transferred *in vivo* to obtain the next selfing generations and to assess basic phenotypic traits. The gene knockout produced *slwatI* plants with dwarf and stunted phenotypes compared with *slhyprpI* plants, while the double mutated plants *slwatI:slhyprpI* showed a tall intermediate phenotype. Further analyses will be conducted to test tolerance to biotic and abiotic stress.

CHAPTER I

INTRODUCTION

1.1 Economic importance of tomato: a brief overview

With more than 100 genera and 2000 species, the Solanaceae family is one of the most prominent plant families worldwide (Filipowicz and Renner 2012). It is considered one of the most economically important families in the plant kingdom, only second to Graminaceae and Fabaceae since it includes several cultivated plants (Ghatak et al., 2017). In particular, *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Solanum melongena* (eggplant), and *Capsicum annuum* (pepper) play a central role in human nutrition and healthcare. Globally, tomato is considered one of the main pillars of the agricultural scene. FAO estimated that in 2019, tomato production reached the global value of 182 million tons. A large part of tomato production is concentrated in Asia (more than 60%), while Europe (13.5%), followed by America and Africa (13.4% and 11.8%), complete the remainder of global production. It has been estimated that 4.8 million hectares are cultivated globally (FAOSTAT, 2019). Over the past ten years, tomato gross production values have constantly increased (Figure 1A). Globally, in 2008 tomato gross production was estimated at around 75.7 billion dollars, while in 2018, gross production values rose to 93.4 billion dollars.

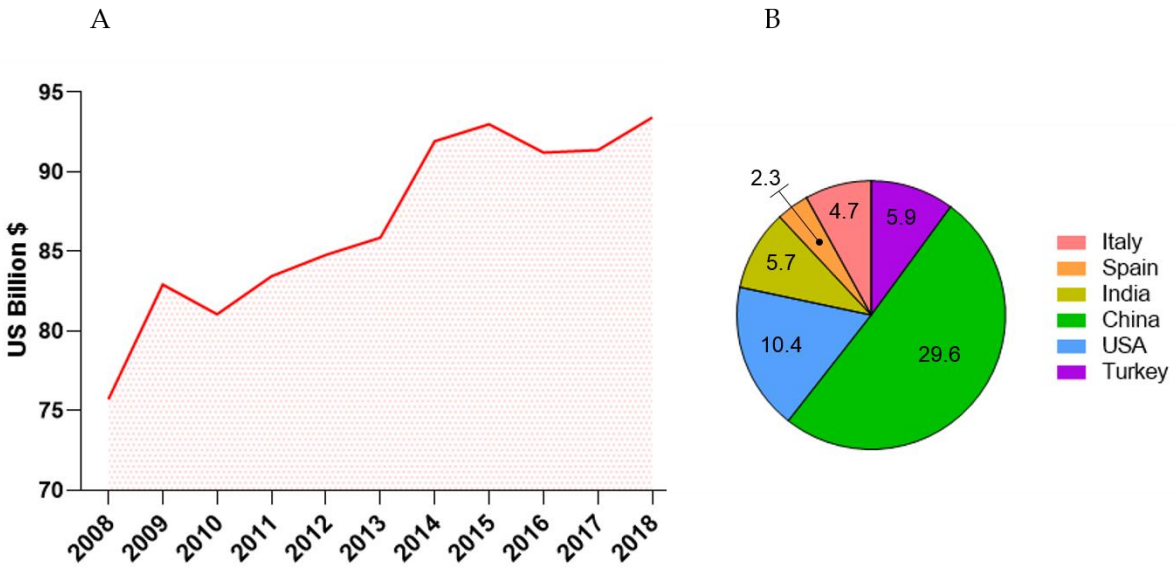


Figure 1. Global tomatoes production. A) Worldwide estimated values of gross tomato production from 2008 to 2018. Panel; B) Value of total tomato production in China, USA, India, Italy, Spain, and Portugal during 2019. Numbers are referred to as Billion dollars (US$) (FAOSTAT, 2019).

As mentioned above, most tomato production is carried out in Asia, America, and Europe. However, only six countries are responsible for more than half of the total gross production. In 2019, China and USA, followed by India, Turkey, Italy, and Spain, generated outputs for a total value of 58.6 billion dollars (62.7% of the global value of 2018) (Figure 1B). Hence, for its economic importance, tomato is widely studied and used as a model crop to investigate plant molecular processes related to development, metabolism, and stress response (Quinet et al., 2019; Li et al., 2018). It has been estimated that on a global scale, tomato stress can lead to massive lost productions and decreases in fruit quality, leading to losses of billions of dollars (Severin et al., 2001). Reduction in yields and quality are due to biotic and abiotic stress, which can occur during the growing cycle (leading to 30–40% percent of losses) or in post-harvest (6–20% of total losses) (FAO, 2005).

1.2 Tomato biotic stress

Worldwide, tomatoes productions are affected by several diseases, including fungi, bacteria, viruses, nematodes, and pests (Lukyanenko, 1991). Favorable environmental conditions during tomato growth can increase disease severity and spread. For example, infections of *Xanthomonas campestris, Pseudomonas syringae,* and *Phytophthora infestans* can be favored by humid and cool environmental conditions, leading to tremendous yield losses (Ohlson and Foolad, 2015; Costache et al., 2007; Tamir-Ariel et al., 2007). On the other hand, high temperatures and moisture can favor *Alternaria solani* infections, causing yield losses up to 80% (Yadav and Dabbas, 2012). *Cladosporium fulvum* in optimal environmental conditions (temperature around 20C° and high relative humidity) can induce mild to severe production losses (Medina et al., 2015). Other pathogens such as Powdery mildew (caused by the disease agent *Leveillula taurica*) require low humidity levels accompanied by hot temperatures with yields reduction of around 40% when Powdery mildew infections are untreated (Aegerter et al., 2014). This pathogen results widely diffused all around the world, causing tremendous yield losses in both field and greenhouses conditions (Mosquera et al., 2019). Table 1 shows the most common tomato pathogens, with their respective optimal development conditions, the average induced yield reductions, and their respective sources of resistance. Often tomato diseases are managed with fungicides and pesticides. However, chemicals overuse can result in environmental contaminations, crop-damaging, and human health toxicity (Ahmad et al., 2021). Hence, one of the toughest challenges for breeders worldwide is the introgression of new resistance genes (R-genes) in cultivated varieties to increase crop resistances and limit production losses (Foolad et al., 2008). To date, several of R-genes against various tomato pathogens such as *Phytophthora infestans*, *Leveillula taurica*, *Septoria lycopersici*, *Fusarium oxysporum*, *Verticillium dahlia*, *Pyrenochaeta lycopersici*, *Stemphylium solani*, *Pseudomonas syringae, Cladosporium fulvum*, *TYLC*, *ToMoV*, *ToMV,* and *TSWV* and the most common nematodes have been introgressed in commercial varieties (Qi et al., 2021; Kaushal et al., 2020; Gill et al., 2019; Medina et al., 2015; Doganlar et al., 1998). In addition, several QTLs (Quantitative/Qualitative Trait Loci) have been identified conferring resistance against several biotic stress (Liu et al., 2021; Kaushal et al., 2020; Mosquera et al., 2019; Ohlson and Foolad, 2015) (Table 1).

Table 1. Most relevant tomato fungi, bacteria, nematodes, and viruses. From left to right: scientific name, common name, average tomato yield losses, pathogen optimal development conditions, and resistance traits identified

Fungi	Common name	yield reductions	Environmental conditions	Resistance loci
Phytophthora infestans	Late blight	40–100%	Cold and humid	*Ph-1* and *Ph-2*
Alternaria solani	Early blight	up to 80%	Moderate or warm and high humidity (>90%)	Polygenes and QTLs regions
Leveillula taurica	Powdery mildew	up to 40%	Warm and dry air	*Lv* (dominant resistance gene), *ol-2* (recessive gene), and several QTLs
Septoria lycopersici	Septoria leaf spot	Around 50%	Persistent humidity and warm	A single dominant gene *Se*
Cladosporium fulvum	Leaf mold	40–90%	Temperatures around 20C° and humidity	24 *Cf* genes
F. oxysporum f. sp. Lycopersici	Fusarium wilt	10–50%	Warm (28C°) humid weather and pH of 5.5	*I, I-2, I-3,* and a partial dominant gene (*I-7*)
Verticillium dahlia	Verticillium wilt	40–50%	Soil pH (7-9) and temperatures of 25C°	*Ve-1* and *Ve-2*
C. coccodes and *C. dematium*	Anthracnose	Post-harvest and rot fruit	Temperatures from 25 to 30C°, humidity (>90%), and light	Polygenes with epistatic interactions
Pyrenochaeta lycopersici	Corky root	70—75%	Cool temperatures (15–20°C)	*Py-1*
Stemphylium solani	Grey Leaf Spot	up to 100%	High temperature and humidity	Dominant *Sm* closely associated with *I* gene
Nematodes				
M. arenaria, M. incognita and M. javanica	Root-knot nematodes	20 to 85%	Temperate or tropical temperatures	*Mi-1*, *Mi-3*, *Mi-5*, *Mi-9* (heat-stable)
Bacteria				
Ralstonia solanacearum	Bacterial wilt	up to 75–100%	High temperatures and high rainfall, soil pH < 7.0	Polygenes and qualitative traits
Pseudomonas syringae PV. tomato	Bacterial speck	15% to 100%	Cool and moist	*Pto-1*, *Pto-2*, *Pto-3,* and *Pto-4*and polygenes
Clavibacter michiganensis	Bacterial canker	up to 90%	Warm temperatures and medium/high humidity	Polygenic genes and QTLs regions
Xanthomonas spp.	Bacterial spot	30% to 100%	Warm and rainy	Various QTLs regions
Virus				
Tomato yellow leaf curl virus	*TYLC*	up to 100%	Transmitted by *Bemesia tabaci*	*Ty-1, Ty-2, Ty-3, Ty-4, Ty-5 and Ty-6*
Tomato mottle virus	*ToMoV*	up to 100%	Whitefly-transmitted viruses	*Ty-6*
Tomato mosaic virus	*ToMV*	40-100%	Whitefly-transmitted viruses	*Tm-1*, *Tm-2*, *Tm-2a*
Tomato spotted wilt virus	*TSWV*	up to 100%	Transmitted by trips	Several *Sw* genes

1.3 Tomato abiotic stress

In light of the shrinkage of natural resources, reduced cultivable areas, and increasing population, world food security in the coming years largely depends on the enhancement of agricultural practices and the use of highly resistant and productive plants. To a large extent, climatic stress causes plant yield reductions. Abiotic stresses include specific climatic conditions such as extreme temperatures, lack of water, saline soils, UV radiations, and oxidative stress. Abiotic stresses are considered the most limiting factor for plant productivity, inducing up to 70% production losses (Acquaah, 2009). Hence, identifying sources of resistance to abiotic stress is mandatory for plant breeding improvements programs. However, tolerance to abiotic stress is intricate and relies on several plant-responsive mechanisms. For example, drought stress affects plants' transpiration rates, causing dehydration and consequently osmotic stress. In response, plants induce ROS production, which accumulation could be harmful to plant metabolism and structures (Huang et al., 2019; Rai et al., 2013; Laloi et al., 2004).

Similarly, plants increase their transpiration rate during heat stress, trigging several transcriptomic changes, mainly involving Heat Shock Proteins (HSPs) and ROS enzymes. Hence, plants induce the stomatal closure with consequences on photosynthesis levels (Zhang et al., 2018). Furthermore, prolonged exposure to heat stress causes protein denaturation, damaging of the cell membrane and cytoskeleton, and decreasing of pollen viability (Parankusam et al., 2017; Xu et al., 2017). A recurrent problem in arid and semiarid regions is caused by salt stress, which is often originated by highly saline soils, poor rains, high evaporation levels, and poor quality water used for plant irrigation (Singh et al., 2020). Salt stress induces osmotic stress in the root system, leading to disorders in ions homeostasis, therefore causing decreases in water uptake. Plants respond to salt stress, promoting ABA, ROS, and Ca^{2+} synthesis to restore ion balance (Julkowska and Testerink, 2015).

Hence, plant response to abiotic stress involves inter-linked complex mechanisms, which are complicated to modify through the traditional breeding techniques (Krishna et al., 2019). Tomato wild relatives (*Solanum pennellii, Solanum pimpinellifolium, Solanum chilense*, and *Solanum habrochaites*) showed good levels of abiotic stress tolerance and have been widely explored through genetic analysis able to identify different QTLs traits (Bai et al., 2018; Böndel et al., 2018; Liu et al., 2018; Bolger et al., 2014). Furthermore, in recent years the advances in omics sciences and

the introduction of new selection methods such as Genomic selection (GS) and genome-wide association studies (GWAS) substantially contributed to the development of new lines tolerant to abiotic stress (Chaudhary et al., 2019).

1.4 A brief story of tomato breeding

Tomato (*Solanum lycopersicum L.*) presumably originated in the Andes regions (South America) and was imported in Europe during the sixteenth century by the Spanish and Portuguese soldiers (Bauchet and Causse, 2012). However, during that century, tomatoes were only cultivated as ornamental plants due to the presence of toxic compounds (Kulus, 2021). Tomato domestication took place years later, during the seventeenth century, when Italy and Spain started its cultivation for nutritional purposes (Mazzucato et al., 2010; Ercolano et al., 2020). After that, the tomato became a prevalent food for millions of people worldwide. It is rich in bioactive compounds such as vitamins, minerals, proteins, amino acids, fibers, and other molecules with numerous benefits for human health (Ramos-Bueno et al., 2017; Elbadrawy et al., 2016).

Initially, South America's tomatoes carried exposed stigma favoring plant cross-pollination. Therefore, the domestication process relied on selecting tomatoes with inserted stigma (changing the reproduction system from cross-pollination to self-pollination), with a higher number of fruits and a lower overall height (Cheema and Dhaliwal, 2005; Rick, 1976). Thus, for their economic importance and nutritional values, tomatoes have been subjected to intensive studies and selection processes, started more than two hundred years ago (Foolad, 2007).

Over the years, improvements in breeding techniques and new genetics findings increased tomatoes productions. However, the creation of new genetic variability represented a key factor in selecting desired traits. Therefore, compatible wild tomato species were crossed with cultivated tomato plants at the beginning of the twentieth century, producing segregating generations (F1) with enhanced characteristics (Afzal and Jindal, 2016). These findings paved the way for modern breeding techniques.

Indeed, various cycles of selection and self-pollination of improved F1 plants led to the generation of pure lines with stable desired traits (Bai and Lindhout, 2007). In particular, the high vigor obtained crossing two pure lines (hybrid F1 plants) led to significant production increases, completely transforming the breeding science (Godwin et al., 2019).

Furthermore, since the middle of the last century, researchers have introduced new methods to increase tomato genetic variability, using EMS, gamma-rays, fast neutron,

and the development of the tissue cultures (Chaudhary et al., 2019; Godwin et al., 2019). In recent years, tremendous progress has been made in biotechnology and comprehension of genetic mechanisms, leading to new methods that increased plant selection efficiency, simultaneously reducing time and costs.

These methods, such as marker-assisted selection (MAS), high-throughput phenotyping, GWAS, and (GS), are now widely used in plant breeding programs (Cappetta et al., 2020; Larkin et al., 2019; He et al., 2014). However, further efforts are necessary to deal with the increasing food demand in the coming years. In this respect, recent findings in genetic engineering could provide the required tools to improve plant cultivars with reasonable costs and times. Genome editing technologies, and in particular the CRISPR/Cas system is proposed as a revolutionary tool for plant genome manipulation, allowing the insertion and deletion of specific DNA fragments, the editing of single nucleotides, the transcriptional activation or suppression of particular genes, and other genomic modification (Hanna and Doench, 2020). However, the use of CRISPR/Cas edited plants for commercial purposes is not well regulated and is not yet allowed by many countries, although countries such as Japan, the USA, Argentina, and Brazil recently passed laws that allowed the cultivation of edited crops (Kumar et al., 2021).

1.5 Phenotypic, molecular, and genomic characterization for tomato breeding

The major challenge for breeders is the generation of plants with improved characteristics, decreased production costs and high yields, even in limiting conditions. To this scope, access to biodiversity is essential to provide the pool of genes necessary to improve crops with undesirable agricultural traits (Swarup et al., 2021).

Researchers and breeders may rely on different strategies to identify phenotypic, molecular, and genetic differences. The exploration of plant phenotypic traits related to plant growth habits or color, firmness, flavor, and morphology of fruits and flowers is a requisite to assess important plant characteristics related to plant heterogeneity, breeding values, and potential yield (Salim et al., 2020). However, phenotypic selection can be very tedious for complex traits. To this purpose, several high-throughput phenotyping platforms have been developed (Li et al., 2014). Breeding programs strictly depend on the precision of measurements related to phenotyped traits. Digital instruments can improve the precision of phenotyping, reducing the requirement of human data annotation (Cappetta et al., 2020; Daniel et al., 2017; Panthee and Gardner, 2013). Evaluating complex plant phenotypic traits such as plant growth, yields, morphology, and physiological state under biotic and abiotic stresses could be difficult and is often assisted by phenomic tools and associated with molecular and genetic analysis (Song et al., 2021).

Many plants' phenotypical and molecular differences rely on environmental effects and genetic background. Hence, the variability of a given trait should be explored in different conditions to identify its genetic basis correctly properly. To this end, various technologies were developed profoundly changing plant breeding science in the past fifty years. Several molecular genetic studies have been conducted to develop tomato varieties with improved agronomic and quality traits. Indeed, molecular markers, QTLs identification (Ercolano et al., 2012) and detection of small variants on large-scale for GS provided new possibilities to speed up plant selection processes (Cappetta et al., 2020).

Since 2012, the whole tomato genomic sequence has been available (Sato et al., 2012), and several genetic studies have been performed. Furthermore, the release of the complete genome sequence of wild tomato relatives (*S. pimpinellifolium*, *S. pennellii,* and *S. chilense*) (Wang et al., 2020; Stam et al., 2019; Bolger et al., 2014) allowed the comparison and the identification of several SNPs and InDels (Insertion/Deletions)

between resistant and susceptible genotypes (Pachner et al., 2015). For example, phenotypic evaluations have been combined with genotypic analyses to associate single nucleotide polymorphisms (SNPs) to specific phenotypical traits in one-hundred and sixty-three tomato accessions (Mata-Nicolás et al., 2020).

In the last few years, RNA-seq technology enabled the identification of transcriptomic responses of plants exposed to different conditions, quantifying and classifying the single RNA sequences, and leading to the identification of critical genes involved in responsive pathways (Padmanabhan et al., 2019; Zhao et al., 2019). In general, RNA-seq technology performs better than other technologies such as microarrays, offering full genome coverages (Finotello and Di Camillo, 2015) and could be carried out either using a genome reference or performing a de *novo* assembling (Ghosh and Chan, 2016). Hence, several RNA-seq experiments have been performed in recent years, allowing the identification of important genes involved in plant development or stress response (Liu et al., 2020; Zhao et al., 2020). In addition, various versions of the tomato genome have been generated and deposited in public databases, providing the opportunity to expand the tomato genetic investigations. The release of several tomato RNA-seq data (Shi and Panthee, 2020; Yang et al., 2017; Du et al., 2015) contributed to exploring various genomic responses during different perturbations. Figure 2 shows a schematic representation of genome reference-based-RNA-seq analysis for identifying differentially expressed genes (DEGs). This technology offers new solutions to make observations and interpretations of biological data and novel discoveries. Indeed, gene expression data represent an ideal source to identify key genes differentially regulated during plant-stress interaction. Cross investigations can help to recovery information for single genes or gene sets.

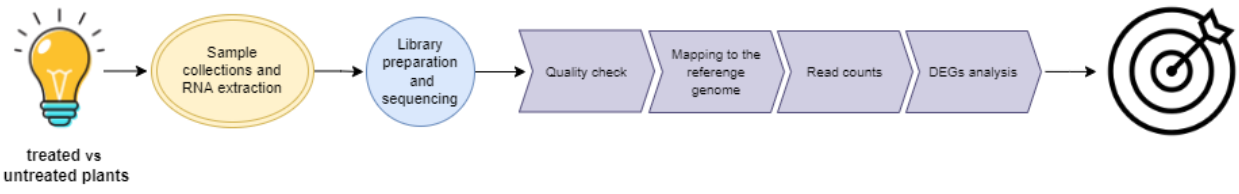


Figure 2. Workflow of RNA-seq data analysis based on a reference genome.

1.6 Biotechnological approaches for tomato improvement programs

The above-described methodologies can aid breeders in selecting varieties with improved characteristics. However, the global population is constantly growing (Sharma et al., 2019), and alternative technologies are needed to ensure food security worldwide. To this purpose, technologies developed in the last years could potentially revolutionize agriculture and breeding worldwide (Yin et al., 2017). In 1983, the first transgenic tobacco plant was generated (Fraley et al., 1983). From then, plant genetic engineering was subjected to massive improvements. In particular, tools developed for plant genome editing allowed researchers to generate precise mutations in target sequences, with implications on specific phenotypic traits (Malzahn et al., 2017). Genome editing is performed using particular engineered endonucleases such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), capable of generating double-strand breaks (DSBs) in targeted DNA regions (Sakuma and Yamamoto, 2017). In most cases, DSBs are repaired through non-homologous end joining (NHEJ), leading to random mutations (InDels) with consequent loss of function of the target gene (Chandrasegaran and Carroll, 2016). Alternatively, a donor DNA could be used to induce a homology-directed repair (HDR) but with low efficiency (Ahmad et al., 2018).

However, the application of ZFNs and TALENs was limited by their cost-effectiveness and the high level of specialization. Recently, Clustered Regularly Interspaced Short Palindromic Repeats associated-Cas protein (CRISPR/Cas) system has been engineered to work as a multifunction genome-editing tool (Belhaj et al., 2015). This technology relies on an endonuclease driven by a designed guide RNA (gRNA) of around 20nt on a target sequence (Figure 3). In particular, CRISPR/Cas system has been widely used in several tomato biology studies due to its precision, rapidity, and cost-effectiveness compared to the previous genome editing methods (Bhatta and Malla, 2020). A CRISPR/Cas experiment in plants requires four main steps: 1) gRNAs design; this step is carried out *in silico* and is based on the previous knowledge of the target sequence. 2) Assembly of gRNAs and transcriptional units (TUs) for CRISPR/Cas vector construction. 3) Engineered *Agrobacterium* lines are transformed with assembled vectors. 4) *Agrobacterium* transformed cells are used for plant cells infections, generating mutated plants. Then, transformed plants are

characterized through polymerase chain reaction (PCR) and sequencing to assess the mutations at target loci.

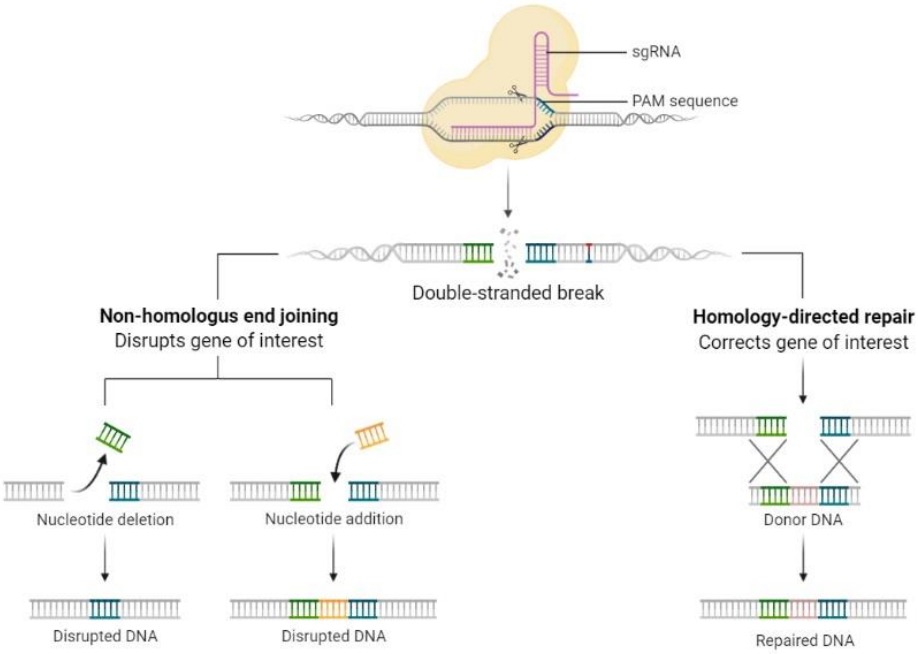


Figure 3. Schematic representation of CRISPR–Cas9 system. This scheme represents the Streptococcus pyogenes SpCas9 driven by an RNA (sgRNA) sequence (of 20nt) on a complementary target DNA portion. DNA cutting occurs 4-5bp upstream the protospacer-associated motif (PAM), resulting in DNA double-strand break (DSB). dsDNA breaks induce the activation of DNA repair mechanisms, leading to random InDels (NHEJ) or precise exogenous DNA introduction into the target region (HDR). Image created with Biorender.com

CRISPR/Cas technology has been extensively used in different crops to provide resistance against biotic and abiotic stress (Saikia et al., 2020; Zhang et al., 2018). In particular, several susceptibility genes (S-genes) have been targeted and silenced through CRISPR/Cas system.

S-genes are involved in stress susceptibility through different mechanisms (van Schie and Takken, 2014), and their knockout could increase plant resistance against various stress (Zaidi et al., 2018). However, in tomatoes, the identification of regulatory pathways to multiple stress responses is still poorly understood. A multiplexing approach has been used to simultaneously target different tomato fruit characteristics (Li et al., 2018), for *de novo* domestication of wild tomato species (Zsögön et al., 2018) and for targeting various metabolic processes (Li et al., 2018; Li et al., 2018). CRISPR/Cas9 system was also used to enhance tomato resistance to *P. infestans*, targeting two micro RNAs and increasing the expression of specific nucleotide-binding site genes (NBS) (Hong et al., 2020). Hence, multiplexing CRISPR/Cas systems

could be used to simultaneously knockout S-genes involved in both biotic and abiotic stress susceptibility, increasing tomato resistance to multiple stressors.

1.7 Objectives

The thesis's main goal was to exploit tomato knowledge and genetic resources available in a breeding company to increase resistance against biotic and abiotic stress. To this purpose, we used innovative technologies such as RNA-seq investigation for target genes identification and CRISPR/Cas9 for gene knockout. This work was carried out at the University of Naples "Federico II," in collaboration with the seed company "La Semiorto Sementi" and the CSIC, Spain.

- In chapter II, ten commercial tomato varieties were evaluated for their phenotypical traits, the presence of genetic markers associated with resistance loci, and the *in vitro* regeneration ability to select suitable genotypes for following gene editing approaches.
- In chapter III, RNA-seq datasets were analyzed and compared to find genes involved in multiple stress responses to provide a list of genes that could be implicated in response to multiple stress and that could be further investigated with biotechnological approaches.
- In chapter IV, two genes (*WATI* and *HyPRPI*) were edited singularly and in combination by CRISPR/CAS9 technology in SanMarzano2 and MoneyMaker genotypes to test their involvement in stress response.

CHAPTER II

PHENOTYPICAL, MOLECULAR, AND IN VITRO CHARACTERIZATION OF TOMATO CULTIVARS

2.1 Introduction

Tomato (Solanum lycopersicum) is one of the most important horticultural crops with high economic and scientific importance. The yield and quality of tomato fruits can be affected by several stresses (Kissoudis et al., 2015). In particular, biotic stresses such as *Xanthomonas campestris*, *Pseudomonas syringae*, *Cladosporium fulvum*, *Phytophthora infestans*, *Verticillium dahliae*, *Fusarium oxysporum*, *Tobacco mosaic virus* (*TMV*), *Tomato spotted wilt virus* (*TSWV*) and, *Tomato yellow leaf curl virus* (*TYLCV*) challenge tomato crops in Mediterranean area (Mândru et al., 2018; Thompson and Tepfer, 2010). Moreover, climate changes are increasing the CO_2 levels in the atmosphere, resulting in temperature rising and frequent droughts periods (Dai, 2013). An indirect consequence of drought and temperature increase is soil salinization, an emerging problem, especially in arid and semi-arid areas (Corwin, 2021). Other than soil desertification, higher temperatures can result in pathogen geographic expansion, directly influencing the rise of more virulent strains (Garrett et al., 2006; Harvell et al., 2002). Generally, the optimum growth temperature for tomatoes ranges from 21C° to 30C° during the day and 15C° to 18C° during the night (Hazra et al., 2007). Biotic and abiotic stress can often interact additively in the field, leading to several yield losses. In tomatoes, increasing soil-salt concentration or drought periods leads to plants' susceptibility to soil-borne pathogens (Triky-Dotan et al., 2005). Plant responses to combined or individual stress are variable (Bai et al., 2018; Zhang and Sonnewald, 2017). The reaction to combined stresses (e.g., plant-pathogen interactions under abiotic stress) depends on different factors, including plant phenotype and genotypes (Bostock et al., 2014). For example, tomato plants subjected to drought and salt stress increased ABA (abscisic acid) synthesis, stimulating pathogen-defense pathways that suppress the tomato resistance to pathogens such as *O.neolycopersici* and *B. cinerea* (Achuo et al., 2006). Developing multiple stress-resistant tomatoes can support food

security during uncertain periods due to climate fluctuations. To date, several resistance genes (R-genes) have been identified in wild tomato relatives, as they represent the primary source of resistance for the cultivated tomato (Stam et al., 2019; Ercolano et al., 2012). Breeders worldwide are constantly developing new tomato varieties with superior agronomic traits. In breeding programs, seed companies score their tomato lines for phenotypic traits such as growth, plant length, presence of abscission axis at the pedicel level, greenback, fruit shape, consistency and weight, in different environments (Tardieu, 2013). In addition, it is also necessary to include resistance genes against biotic or abiotic stresses to improve cultivars' performance under adverse conditions. However, introducing resistance genes in a superior cultivar is time-consuming and involves many selection rounds. In this work, we aimed to speed up the production of new tomato cultivars with increased resistance to biotic and abiotic stress making use of breeding strategies based on genomic analysis and genome editing technologies. To this scope, we first assessed in field 10 tomato lines developed from the seed company "La Semiorto Sementi," observing their phenotypic characteristics. Then we collected genetic material to evaluate the presence or the absence of eight among the most common resistance genes against tomato pathogens through the support of molecular markers. Finally, we performed an *in vitro* regeneration experiment to assess the regeneration rate of these varieties in preparation for future genome editing approaches mediated by *Agrobacterium tumefaciens* transformations.

2.2 Results

2.2.1 Analysis of phenotypic traits in ten selected tomato lines

In this study, the following tomato genotypes: 1027, 1014, 1009, 1081, 1064, 1006, 1043, Principe Borghese, San Marzano 2 and, Sorent, have been phenotypically assessed using UPOV (International Union for the Protection of New Varieties of Plants) descriptors. In our analysis, we evaluated the most important plants and fruits phenotypic traits such as plant length, growth (determinate or indeterminate genotypes), presence or absence of abscission axis at the flower pedicel level, the presence or absence and the intensity of the tomato greenback, the number of locular cavities, the fruits consistency and, the fruit size, shape and weight. Table 2 shows some of the phenotypical traits analyzed.

Table 2. Evaluation of the most important phenotypic traits of ten tomato lines produced from "La Semiorto Sementi." From left to right columns: Det= determinate, Ind= indeterminate; S.= short, M.= medium, T.= tall; N.P.= not present, P.= present; Med.Hig.= medium-high, Med.Low.= medium-low, Int= intermediate, Tou.= Tough, Sof.= soft.

Genotype	Growth	length	Abscission axis	Greenback	Locular cavities	firmness	Average weight (g)
1027	Det	S.	N.P.	N.P.	2 or 3	Med.Hig.	100
1014	Det	S.	N.P.	N.P.	2 or 3	Int.	100
1009	Det	S.	P.	N.P.	2 or 3	Med.Hig.	90
1081	Ind	M.	N.P.	N.P.	6+	Int.	300
1064	Ind	T.	P.	P.	2	Int.	15
1006	Ind	T.	N.P.	N.P.	2 or 3	Tou.	110
SanMarzano2	Ind	S.	N.P.	P.	2	Sof.	70
Principe Borghese	Ind	T.	P.	P.	2	Med.Low.	30
Sorent	Ind	M.	N.P.	P.	6+	Int.	400
1043	Ind	T.	P.	P.	2	Med.Low.	20

Phenotypic evaluations showed that most genotypes had an indeterminate habitus without the abscission axis at the pedicel level. In addition, in most cases, fruits were firm and varied in size and weight since we tested varieties for both fresh markets and processing industries. The only exception was given by SanMarzano2, which had

soft fruits. Differences in fruit weights were highly uneven, ranging from 15g/fruit of line 1064 to 400g/fruit of Sorent (Table 2).

A high fruit shape variation was observed in lines used in our study. They were classified as flattened, circular, rectangular, cylindrical, heart-shaped, obovate, ovate and, pear-shaped (www.upov.int). They also differed for fruit blossom end which resulted flat or pointed; this trait can be particularly relevant, especially for industrial tomato, due to the possible damages that can occur during harvest and transport caused by fruit rubbing. Figure 4 shows the fruit ripening stages, shapes, and longitudinal sections of evaluated lines.

Figure 4. Fruit's phenotypical characterization of ten tomato lines

Line 1006 and SanMarzano2 had cylindrical fruits, Sorent and 1081 were rectangular and circular respectively, 1064 had elliptic fruits, while fruits of Principe Borghese, 1027, 1014 1009, and 1043 were obovate (Figure 4). To perform an extensive characterization of our lines, we also evaluated other phenotypical traits such as leaf blade division, Inflorescence type (from 2nd and 3rd trusses), the intensity of the green color of the fruit shoulder, fruit size and shape, the ribbing at peduncle end, the depression at peduncle end, the shape at the blossom end, and the color at maturity stage and others (Supplementary Table 1).

2.2.2 Molecular detection of resistance loci in ten selected tomato lines

Several tomato diseases have been identified, mapped, and characterized to date. In our study, we analyzed markers linked to genetic traits conferring resistance to six among the major tomato pathogens: *FOL* (*Fusarium oxysporum f.sp. lycopersici*), *FORL* (*Fusarium oxysporum f.sp. Radicis-lycopersici*), *Ve* (*Verticillium dahlie*), *Mi* (*Meloidogyne incognita*), *TSWV* (*Tomato Spotted Wilt Virus*) and *ToMV* (*Tomato Mosaic Virus*). Table 3 shows the result obtained for each molecular-marker assessed in our work.

Table 3. Molecular analysis of ten tomato lines to test the presence of resistance loci against *FOR* (*Fusarium oxisporum f sp. Radicis Lycopersici*), *FOL* (*Fusarium oxisporum f sp. Lycopersici*), *Vd* (*Verticillium dahlie*), *Mi* (*Meloidogyne incognita), TSWV (Tomato Spotted Wilt Virus), and ToMV (Tomato mosaic virus).* X= the molecular-marker was present; - = the molecular-marker was absent.

Genotype	Frl	I	I2	Ve	Mi	Sw5	Tm2a
1027	-	X	X	X	-	-	-
1014	-	X	-	X	-	-	-
1009	-	X	-	X	-	-	-
1064	-	-	-	-	-	-	-
Principe Borghese	-	-	-	-	-	-	-
SanMarzano2	-	-	-	-	-	-	-
Sorent	-	-	-	X	-	-	-
1081	-	X	-	X	-	-	-
1043	-	-	-	-	-	-	-
1006	X	X	X	X	X	-	X

As for the phenotypic characteristics, resistance traits tested in tomato lines showed significant variability regarding the presence or absence of resistance loci. In particular, line 1006 displayed 6 loci to *FOL* (race 1 and 2), *FORL*, *Vd*, *Mi*, *TSWV*, and *ToMV*. On the other hand, SanMarzano2, Principe Borghese, 1064, and 1014 did not have any resistance locus. Lines such as 1081, 1009, 1014, and 1027 only showed molecular-markers associated with *FOL* and *Ve* (Table 3).

2.2.3 Evaluation of tomato lines for the "*in vitro*" regeneration ability

The ability of ten tomato lines to regenerate new shoots starting from cotyledonary explants was assessed. To this purpose, a tomato regeneration protocol based on Murashige and Skoog (MS) media, including vitamins and hormones, supplemented with four different levels of BAP (6-benzylaminopurine), was used. The number of callus and shoots was evaluated after twenty-eight and forty-two days. Figure 5 shows the total number of shoots produced from all tomato lines at forty-two days.

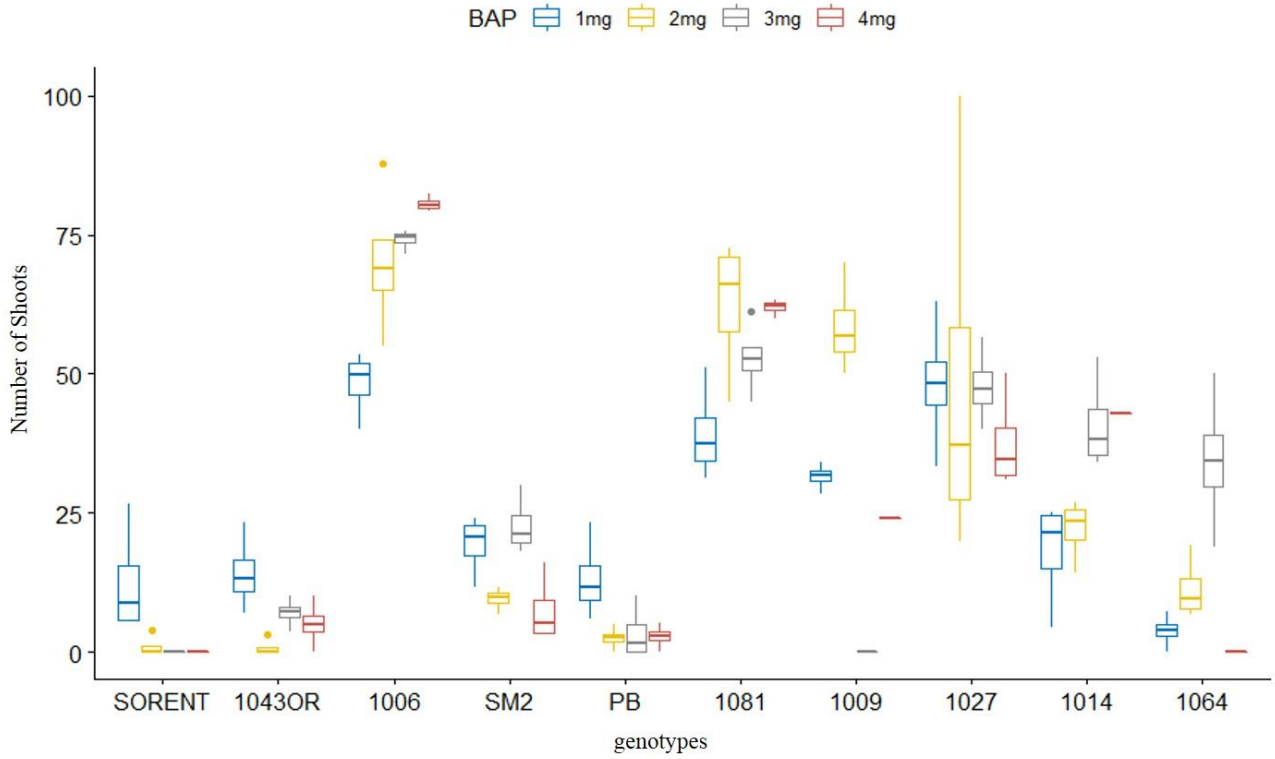


Figure 5. The number of shoots produced by ten tomato lines using four different levels of BAP. In blue: 1mg/l of BAP, in yellow: 2mg/l of BAP, in grey: 3mg/l of BAP, and in red: 4mg/l of BAP. The number of shoots was evaluated at forty-two days.

The plant regeneration ability was not uniform among tested cultivars. Our analysis showed that except for 1006 and 1014, most of the analyzed genotypes produced a high number of shoots using low BAP concentrations (1 or 2mg/l of BAP), whilst lines 1006 and 1014 performed better using 4mg/l of BAP and SanMarzano2 and 1027 developed their maximum number of shoots under 1 or 3mg/l of BAP. A good number of regenerated shoots was observed in 1081, 1009, and 1027 using 2mg/l of BAP, while Sorent, 1043, and Principe Borghese emitted few shoots under all the BAP levels tested (Figure 5).

ANOVA (Analysis of Variance) test was used to analyze the main effects of the genotype and BAP and the interaction between these two factors. A significant effect of the "Genotype" factor on plant regeneration rates was found (Table 4). In addition, the interaction between genotype and BAP was also significant (p-value≤ 0.001), indicating that the observed differences in regeneration ability were also due to the combination between the genotype and the different levels of BAP (Table 4).

Hence, the pair-wise Tukey-test was applied considering the number of shoots produced after forty-two days to determine which genotypes were significantly different one each other. Most of the pair-wise comparisons showed significant differences (Table 5). In particular, this test indicated that the regeneration ability of 1064 and 1043 was similar to Principe Borghese, San Marzano 2 and Sorent genotypes. Furthermore, 1081, 1027, 1014, and 1009 did not show significant regeneration rate differences, while shoots produced by these genotypes resulted significantly different from Principe Borghese, San Marzano 2, and Sorent. In addition, the most significant differences appeared comparing 1043 and 1027, Sorent and 1027, and Sorent and 1081 (Table 5).

We also applied the pair-wise Tukey-test for evaluating the differences in the number of shoots produced considering the combined effect of genotype and BAP level (Supplementary Table 2). However, due to the high number of comparisons, we only reported the most interesting for our purposes. In particular, we focused on the comparisons between SanMarzano2 (with 1 and 3 mg/l of BAP) and the other genotypes. Significant differences emerged comparing SanMarzano2 with 1006, 1081, 1009, and 1027.

Table 4. ANOVA (Analysis of variance) test of genotype and BAP factors for plant shoots production. Df: Degrees of freedom. Sum sq: Sum of squares. Mean sq: mean square.

Data	Df	Sum sq	Mean sq	F value	Pr (>F)	Significance
Genotype	9	73651	8183	113.795	<2e-16	***
BAP	3	397	132	1.842	0.143	
Genotype:BAP	27	17226	638	8.872	<2e-16	***
Residuals	120	8630	72			

Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 5. Tukey test (HSD) comparing the number of shoots emitted at 42 days by ten analyzed genotypes. Diff= differences between means of the groups; lwr and Upr= the lower and the upper-end point of the confidence interval at 95%; p adj= p-value after adjustment for the multiple comparisons.

Genotypes	Diff	lwr	Upr	p adj	Sign
1009-1006	-39.81	-49.48	-30.14	2.45E-14	***
1014-1006	-37.35	-47.01	-27.68	2.95E-14	***
1027-1006	-22.76	-32.43	-13.10	3.39E-10	***
1043OR-1006	-61.56	-71.22	-51.89	2.41E-14	***
1064-1006	-55.96	-65.62	-46.29	2.41E-14	***
1081-1006	-14.19	-23.86	-4.53	2.55E-04	***
PB-1006	-62.89	-72.56	-53.22	2.41E-14	***
SM2-1006	-53.56	-63.23	-43.89	2.41E-14	***
SORENT-1006	-64.95	-74.62	-55.29	2.41E-14	***
1014-1009	2.46	-7.20	12.13	9.98E-01	N.S.
1027-1009	17.05	7.38	26.72	4.09E-06	***
1043OR-1009	-21.75	-31.41	-12.08	1.95E-09	***
1064-1009	-16.15	-25.81	-6.48	1.59E-05	***
1081-1009	25.62	15.95	35.29	2.29E-12	***
PB-1009	-23.08	-32.75	-13.41	1.95E-10	***
SM2-1009	-13.75	-23.42	-4.08	4.61E-04	***
SORENT-1009	-25.14	-34.81	-15.48	5.19E-12	***
1027-1014	14.59	4.92	24.25	1.49E-04	***
1043OR-1014	-24.21	-33.87	-14.54	2.69E-12	***
1064-1014	-18.61	-28.28	-8.94	3.56E-07	***
1081-1014	23.16	13.49	32.82	1.70E-10	***
PB-1014	-25.54	-35.21	-15.88	2.60E-12	***
SM2-1014	-16.21	-25.88	-6.55	1.44E-05	***
SORENT-1014	-27.61	-37.27	-17.94	1.79E-14	***
1043OR-1027	-38.79	-48.46	-29.13	2.54E-15	***
1064-1027	-33.20	-42.86	-23.53	7.54E-14	***
1081-1027	8.57	-1.10	18.24	1.29E-01	N.S.
PB-1027	-40.13	-49.80	-30.46	2.44E-14	***
SM2-1027	-30.80	-40.47	-21.13	1.09E-13	***
SORENT-1027	-42.19	-51.86	-32.53	2.41E-15	***
1064-1043OR	5.60	-4.07	15.27	6.91E-01	N.S.
1081-1043OR	47.36	37.70	57.03	2.41E-14	***
PB-1043OR	-1.33	-11.00	8.33	1.00E+00	N.S.
SM2-1043OR	8.00	-1.67	17.66	1.99E-01	N.S.
SORENT-1043OR	-3.40	-13.06	6.27	9.80E-01	N.S.
1081-1064	41.77	32.10	51.43	2.41E-14	***
PB-1064	-6.93	-16.60	2.73	3.89E-01	N.S.
SM2-1064	2.40	-7.27	12.06	9.98E-01	N.S.
SORENT-1064	-9.00	-18.66	0.67	9.10E-02	N.S.
PB-1081	-48.70	-58.37	-39.03	2.41E-14	***
SM2-1081	-39.37	-49.04	-29.70	2.49E-14	***
SORENT-1081	-50.76	-60.43	-41.10	2.41E-15	***
SM2-PB	9.33	-0.34	19.00	6.80E-02	N.S.

SORENT-PB	-2.06	-11.73	7.60	1.00E+00	N.S.
SORENT-SM2	-11.39	-21.06	-1.73	8.37E-03	**
Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' N.S. 'Not significant'					

Moreover, supplementary figure 1 shows the number of calli and shoots produced by all tested genotypes at twenty-eight and forty-two days.

Finally, the best performing genotypes in terms of shoots developed were 1006, 1081, 1009, 1014, and 1027.

2.3 Discussion

In this study, ten tomato lines: 1027, 1081, 1014, 1009, 1064, 1006, 1043, Principe Borghese, San Marzano 2 and, Sorent, kindly provided by "La Semiorto Sementi" company, were assessed for their phenotypical traits, the presence or absence of resistance loci, and their ability to regenerate new shoots *in vitro* conditions. The final scope was to select a suitable line for carrying a CRISPR/Cas9 experiment to improve plant resistance against both abiotic and biotic stresses. The phenotypic evaluation highlighted several differences among analyzed lines. All the tomato plants had an important tomato niche market in the south of Italy. In particular, SanMarzano (SM) was one of the most famous Italian tomato cultivars and could be used for dual purposes, fresh consumption and processing. SM started to be popular at the beginning of the twentieth century in the Agro Sarnese Nocerino (province of Naples, Italy) and got the Protected Designation of Origin (DOP) label for its cultivation (Monti et al., 2004).

To date, SM has been investigated in several studies, including biochemical and sensorial characterization (D'Esposito et al., 2017; Ercolano et al., 2008), and its genome was sequenced (Ercolano et al., 2014). Our analysis confirmed that SM2 accession showed indeterminate growth habitus with elongated fruits of about 60–80 g, with a vivid green shoulder (Ercolano et al., 2008; Monti et al., 2004). Besides its importance, SM lacks resistance loci against biotic stresses. Therefore, editing for empowering tolerance to pathogen attacks could be desirable.

Traditional tomato varieties are often infected by diseases caused by bacterial, fungal, and viral pathogens that reduce yields, fruit quality, and shelf life. In some cases, the heavy production losses forced farmers to give up their cultivation. An effective control strategy is introducing new resistance genes in tomato cultivars (Hanson et al., 2016). To date, several resistance genes have been mapped in tomatoes, and molecular markers linked to these genes have been developed for MAS. The availability of resistant cultivars could help farmers avoid the use of chemicals in disease control that are dangerous to farmers' and consumers' health and have a substantial economic impact on production (Wilson and Tisdell, 2001). The employment of resistant cultivars remains the cheapest, simplest, and most environmentally safe way to limit disease spread.

In our study, we tested the presence of resistance loci among six critical tomato pathogens: *FOL* (*Fusarium oxysporum f.sp. lycopersici*), *FORL* (*Fusarium oxysporum f.sp. Radicis-lycopersici*), *Ve* (*Verticillium dahlie*), *Mi* (*Meloidogyne incognita*), *TSWV* (*Tomato Spotted Wilt Virus*) and *ToMV* (*Tomato Mosaic Virus*). Line 1006 carried almost all the resistant loci tested, while Principe Borghese, SanMarzano2, 1043, and 1064 did not carry any resistant locus. The seed company was very interested in introducing resistance to soil-borne pathogens, which remain major limiting factors for tomato production in the greenhouses and fields (McGovern, 2015). *FOL* and *FORL* are two of the broadest spread vascular pathogens in horticultural plants as well as *Verticillium* spp.

Extensively yield losses (from 45% up to 95% of productions) due to *FORL* infections have been recorded in Canada, Tunisia, and India (Ramyabharathi et al., 2012; Hibar et al., 2007). The *I2* gene introgressed from *L. peruvianum* confers resistance to *FOL* race1 and 2 (Neha et al., 2016), and the Frl locus to *FORL* (Devran et al., 2018). Furthermore, the *Ve* locus contains two genes encoding for leucine-rich repeat receptor-like proteins conferring resistance against race 1 isolates of *Verticillium dahliae* (*Vd1*) and *V. albo-atrum* (*Vaa1*) (Van Ooijin et al., 2007).

One of the major problems during the introduction of resistance genes in new tomato lines is that some essential phenotypical traits may be lost due to the genetic recombination, which implies several years of backcrossing to reintroduce the required traits. By contrast, chemical treatments and soil solarisation in fields and greenhouses usually fail to control the vascular wilt fungus. Genetic engineering offers different solutions for modifying selected target genes to improve plant resistances without compromising other required plant traits.

For this reason, our attention moved on lines appreciated for their quality but completely lacked in resistance traits. Moreover, to choose a line for further biotechnological experiments, we investigated the plant regeneration protocols suitable for selected tomato lines.

In modern plant improvement programs, *in vitro* techniques are fundamental tools to develop new tomato cultivars (Taji et al., 2002). The tomato regeneration process is a necessary step for producing genomic-edited tomatoes. Indeed, after initial infections with *Agrobacterium tumefaciens,* to include a T-DNA into transcriptionally active chromosomic regions, infected cells start to emit (under particular conditions)

new shoots and seedlings. Several regeneration protocols have been tested in tomatoes using different genotypes and hormone levels to date. These factors can strongly affect the number of calli and shoots produced, being more or less efficient for a particular genotype (Mamidala et al., 2011).

In our study, plant regeneration rates using the BAP as a growth regulator for cotyledonary leaf explants were recorded. Different concentrations of growth regulators were added to "MS media including vitamins" to observe the number of shoot initiation. In particular, 1006, 1081, 1009, 1014, and 1027 lines gave the best regeneration rates, but also SM2 showed a good number of regenerated shoots.

The phenotypic and genotypic characterization and the regeneration ability of ten tomato lines in this work were finalized to choose the most suitable lines for further CRISPR/Cas9 experiments. Among them, we choose the SanMarzano2 for its economic importance, the lack of genetic resistance, and the good regeneration rate showed.

2.4 Materials and methods

2.4.1 Material and plant grown

The tomato lines: 1027, 1081, 1014, 1009, 1064, 1006, 1043, Principe Borghese, San Marzano 2 and, Sorent were kindly provided by "La Semiorto Sementi" (Sarno, Italy). 14°38'33.4068"). Fifty seeds for each line were sown in plateau under plastic-house. Plants were sowed on 7 of March 2019 and transplanted in the open field on the 15 of April 2019 (Sarno, Campania; 40°47'47.6808"). Tomato plants were cultivated in rows of 50 plants, following the standard cultural practices of the company, and temperature and climatic data were collected using the local weather station (Figure 6). Tomatoes harvest was made from June to July.

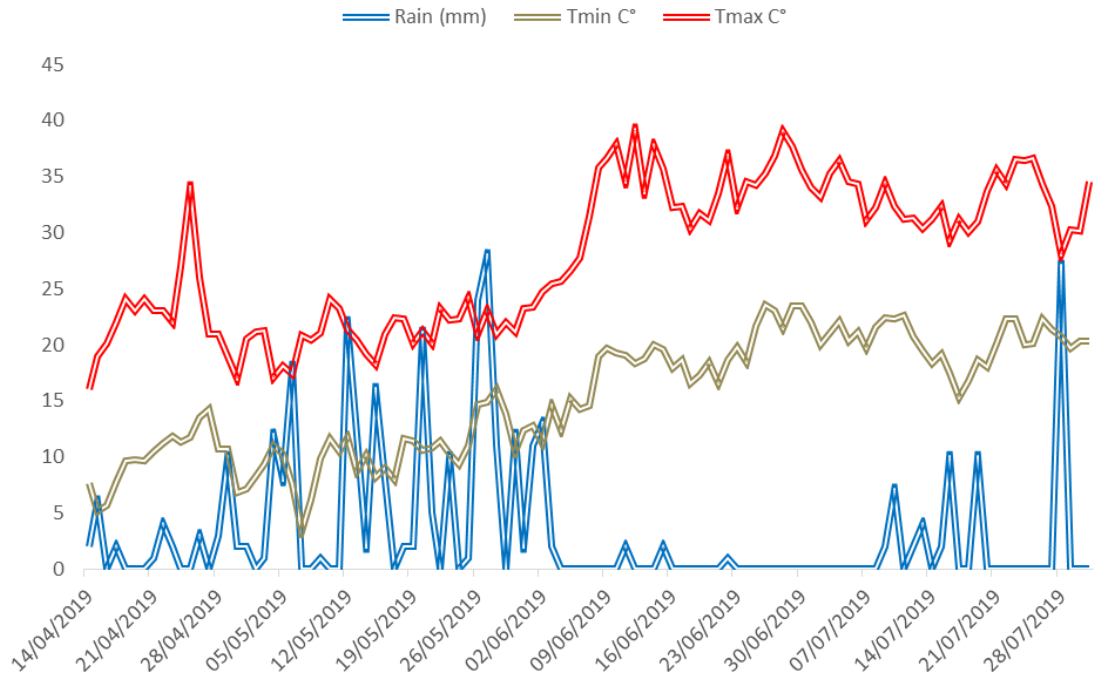


Figure 6. Meteorological data of rainfall (blue) and daily temperatures (minimum in grey and maximum in red) during tomato cultivation.

2.4.2 Phenotypical traits evaluation

Plants were characterized for their phenotypical traits during vegetative growth, flowering, and fructification. Analyzed plant traits have been: growth type (determinate or indeterminate), length, and leaf blade division. Type of inflorescence (at 2nd and 3rd trusses) and the presence or absence of abscission layer at peduncle level were also observed. Finally, fruit characteristics such as presence or absence of green shoulder (before the stage of maturity), the color intensity of the green shoulder, the size and shape in longitudinal section, the ribbing at peduncle end, the depression at peduncle end, the shape at the blossom end, the number of locules, the fruit color at maturity stage and the fruit firmness at the harvest stage were evaluated. Phenotypical characterization was made following the UPOV factsheet (International Union for the Protection of New Varieties of Plants).

2.4.3 Genetic screening to identify plant resistance genes

Tomato lines were assessed for the presence or absence of six resistance loci by analyzing linked molecular markers. In particular, we evaluated the presence of seven crucial tomato resistance loci: *I- I2* (*Fusarium oxysporum f.sp. lycopersici*), *FORL*

(*Fusarium oxysporum f.sp. Radicis-lycopersici*), *Ve* (*Verticillium dahlie*), *Mi* (*Meloidogyne incognita*), *Sw5* (*Tomato Spotted Wilt Virus*) and *Tm2* (*Tomato Mosaic Virus*). The primers and the PCR cycle used in this study are reported in Table 6 and Table 7.

Table 6. List of primer used in this study for plant genetic screening

Pathogen	Forward (Fw) sequence	Reverse (Rv) Sequence	Locus	Wild species	Genetic marker	Annealing (C°)
F. oxysporum f. sp. Lycopersici (Fol:1)	ATTTGAAAGCGTGGTATTGC	CTTAAACTCACCATTAAATC	*I2*	*solanum pimpinellifolium*	Z1063	58
F. oxysporum f. sp. Lycopersici (Fol:0)	CGAATCTGTATATTACATCCGTCGT	GGTGAATACCGATCATAGTCGAG	*I*	*solanum pimpinellifolium*	At2	63
Tomato Mosaic Virus (ToMV:0,1,2)	GGGTATACTGGGAGTGTCCAATTC; CTCATCAAGCTTACTCTAGCCTACTTTAGT	CCGTGCACGTTACTTCAGACAA; CTGCCAGTATATAACGGTCTACCG	*Tm2²* *And* *Tm2*	*solanum peruvianum*	ARMS SNP2493/2494	65
Verticillium albo-atrum and dahliae race 1 (Va:0 e Vd:0)	GGATCTTAGCTCACTTTATGTTTTGAAC; GGATCTTAGCTCACTTTATGTTTTGAAC	GGTGCTGGTTTCAACTCTGAAGT; GGATCTCCCCGGACAGGTGGATTC	*Ve*	-	ARMS Ve2	64
F. oxysporum f.sp.radicis-lycopersici (For)	TGGACATTAAGTGCTAACAATAG	ACTAGGCCCAAGAATGAGTTTG	*Frl*	-	CAPS frl	59
Meloidogyne incognita (Mi)	TGGAAAAATGTTGAATTTCTTTTG	GCATACTATATGGCTTGTTTACCC	*Mi*	-	SCAR Mi-1-23	58
Tomato Spotted Wilt Virus (TSWV)	AATTAGGTTCTTGAAGCCCATCT	TTCCGCATCAGCCAATAGTGT	*Sw5*	-	SCAR Sw5	63

In order to perform DNA extraction, leaf material was collected from young seedlings. Fresh tissues were immediately frozen, leaving the samples at -20C° for 2 hours. Once the tissues were frozen, the TissueLyser LT (Qiagen) was used for tissue grinding. Then, extraction was made using the protocol described in Fulton et al. (2005). After DNA extraction, primer pairs were tested using the PCR condition described in Table 7.

Table 7. PCR conditions used in the molecular-marker analysis

STEP	**TEMPERATURE (C°)**	**TIME**	**CYCLES**
Initial denaturation	95	4 minutes	1 X
Denaturation	95	30 seconds	30 X
Annealing	58 up to 64	30 - 45 seconds	
Extension	72	45 seconds	
Final Extension	72	8 minutes	1 X
End temperature	10	∞	-

2.4.4 In vitro regeneration rate evaluation and statistical analysis

To evaluate the ability of ten tomato lines to produce new shoots *in vitro*, we started from cotyledonary explants. The experiment was set up using the conditions reported in Table 8.

Table 8. Protocol used in tomato in vitro regeneration experiment.

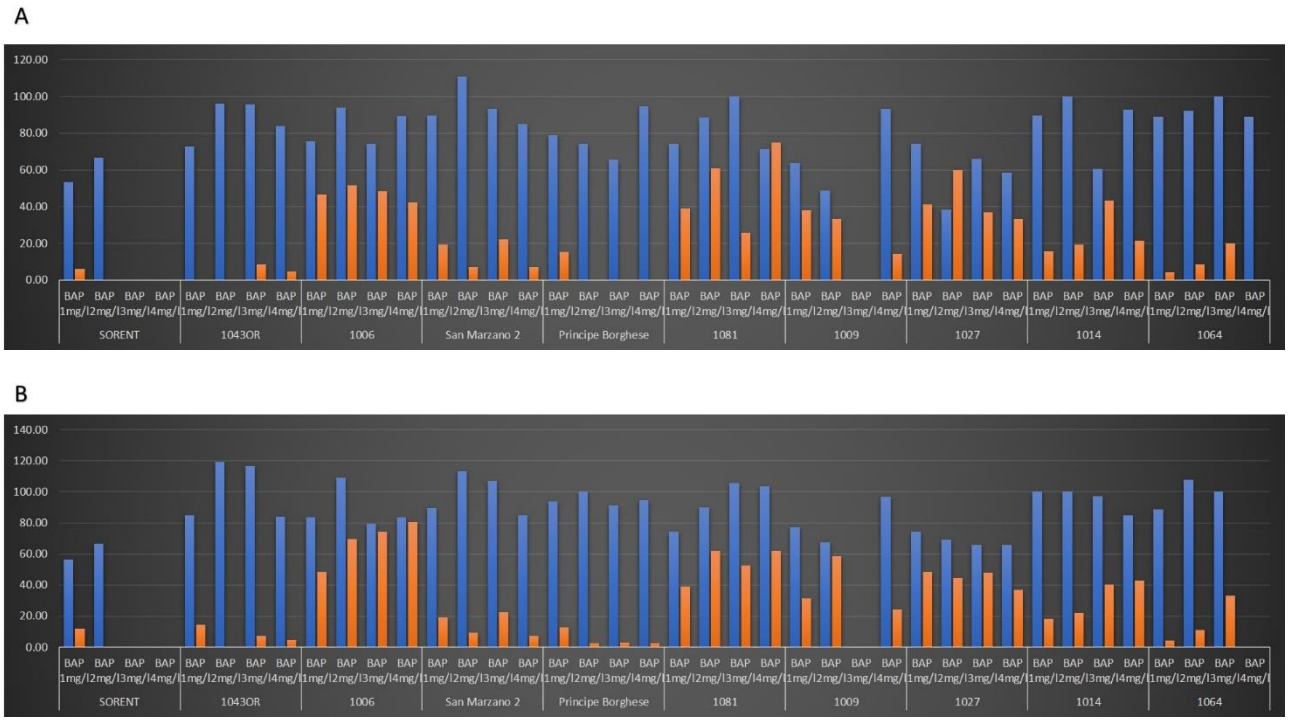
Step 1	**Product**	**Quantity**	**Time**
Seeds sterilization:	ethanol (70%)	-	1 (x 1 minute)
	sodium hypochlorite (2,5%)	-	1 (x 10 minutes)
	H_2O milliQ	-	3 (x 0.5 minutes)
Step 2	**Product**	**Quantity (g/l)**	**Time**
Sowing media (pH: 5.8):	MS including vitamins	1,101	14 days
	Sucrose	30	
	Plant agar	8	
Step 3	**Product**	**Quantity**	**Time**
Regeneration media (pH: 5.8)	MS including vitamins	1,101g/l	Place explants on fresh media each 2 weeks
	Sucrose	30g/l	
	Plant agar	8g/l	
	BAP	1 – 2 – 3 – 4 (mg/l)	

Seeds were sowed in June 2019. When seedlings were fourteen days old, cotyledons were cut and were placed on regeneration media. Two-hundreds Cotyledons from one hundred seedlings of fourteen days old were cut in half and placed on different regeneration media, containing four different levels of BAP as described in Table 8. Every fourteen days, explants were passed on new (fresh) regeneration media. The calli and shoots produced were assessed at twenty-eight and forty-two days after cutting.

2.4.4.1 Statistical analysis

Analysis of variance (ANOVA), the boxplot, and Tukey HDS tests, were processed in the statistical software R (R Core Team, 2020).

2.5 Supplementary materials



Supplementary Figure 1. Evaluation of regeneration rates of ten tomato lines. The total number of calli (in blue) and shoots (in orange). A) Twenty-eight days; B) Forty-two days after explants cut.

Supplementary Table 1. Phenotypical characterization of ten tomato lines.

Line	Trait	Comments
1027	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	-
	Fruit: size	Medium
	Fruit: shape in longitudinal section	Obovate
	Fruit: ribbing at peduncle end	Absent or very weak
	Fruit: depression at peduncle end	Absent or very weak
	Fruit: shape at the blossom end	Pointed
	Fruit: color at maturity	Red

Line	Trait	Comments
San marzano 2	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	Medium
	Fruit: size	Medium
	Fruit: shape in longitudinal section	Cylindrical
	Fruit: ribbing at peduncle end	Absent or very weak
	Fruit: depression at peduncle end	Absent or very weak
	Fruit: shape at the blossom end	Pointed
	Fruit: color at maturity	Red

Line	Trait	Comments
Principe Borghese	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	Dark
	Fruit: size	Small
	Fruit: shape in longitudinal section	Obovate
	Fruit: ribbing at peduncle end	Absent or very weak
	Fruit: depression at peduncle end	Weak
	Fruit: shape at the blossom end	Pointed
	Fruit: color at maturity	Red

Line	Trait	Comments
1006	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	-
	Fruit: size	Medium
	Fruit: shape in longitudinal section	Cylindrical
	Fruit: ribbing at peduncle end	Absent or very weak
	Fruit: depression at peduncle end	Absent or very weak
	Fruit: shape at the blossom end	Flat
	Fruit: color at maturity	Red

Line	Trait	Comments
Sorent	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	Dark
	Fruit: size	Very large
	Fruit: shape in longitudinal section	Rectangular
	Fruit: ribbing at peduncle end	Weak
	Fruit: depression at peduncle end	Weak
	Fruit: shape at the blossom end	Flat
	Fruit: color at maturity	Pink

Line	Trait	Comments
1081	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	-
	Fruit: size	Large
	Fruit: shape in longitudinal section	Circular
	Fruit: ribbing at peduncle end	Weak
	Fruit: depression at peduncle end	Medium
	Fruit: shape at the blossom end	Flat to pointed
	Fruit: color at maturity	Red

Line	Trait	Comments
1064	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	Medium
	Fruit: size	Very small
	Fruit: shape in longitudinal section	Elliptic
	Fruit: ribbing at peduncle end	Absent or very weak
	Fruit: depression at peduncle end	Absent or very weak
	Fruit: shape at the blossom end	Flat to pointed
	Fruit: color at maturity	Yellow

Line	Trait	Comments
1009	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	-
	Fruit: size	Medium
	Fruit: shape in longitudinal section	Obovate
	Fruit: ribbing at peduncle end	Absent or very weak
	Fruit: depression at peduncle end	Absent or very weak
	Fruit: shape at the blossom end	Flat to pointed
	Fruit: color at maturity	Red

Line	Trait	Comments
1043	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	Medium
	Fruit: size	Small
	Fruit: shape in longitudinal section	Obovate
	Fruit: ribbing at peduncle end	Absent or very weak
	Fruit: depression at peduncle end	Absent or very weak
	Fruit: shape at the blossom end	Pointed
	Fruit: color at maturity	Red

Line	Trait	Comments
1014	Leaf: division of blade	Bipennate
	Inflorescence: type (2nd and 3rd truss)	Mainly multiparous
	Fruit: intensity of the green color of the shoulder	-
	Fruit: size	Medium
	Fruit: shape in longitudinal section	Obovate
	Fruit: ribbing at peduncle end	Absent or very weak
	Fruit: depression at peduncle end	Absent or very weak
	Fruit: shape at the blossom end	Flat to pointed
	Fruit: color at maturity	Orange

Supplementary Table 2. Comparisons of shoots produced by SanMarzano2 (with 1 and 3mg/l of BAP) and the other genotypes. In this table, comparisons without significant differences are not reported. 1= 1mg/l of BAP, 3= 3mg/l of BAP; Diff= differences between means of the groups; lwr and Upr= the lower and the upper end point of the confidence interval at 95%; p adj= p-value after adjustment for the multiple comparisons.

Genotype and level of BAP	diff	Lwr	upr	p adj	Sign
SM2:1-1006:1	-29.065	-52.9943	-5.1357	0.002345	**
1006:2-SM2:1	50.9775	27.0482	74.9068	4.76E-11	***
1006:3-SM2:1	54.85	30.9207	78.7793	1.83E-12	***
1006:4-SM2:1	61.3325	37.4032	85.2618	3.75E-13	***
SM2:1-1027:1	-29.015	-52.9443	-5.0857	0.002424	**
1027:2-SM2:1	29.365	5.435703	53.2943	0.001921	**
1027:3-SM2:1	28.4925	4.563203	52.4218	0.003411	**
1081:2-SM2:1	43.185	19.2557	67.1143	4.34E-08	***
1081:3-SM2:1	33.53	9.600703	57.4593	0.000101	***
1081:4-SM2:1	42.7275	18.7982	66.6568	6.4E-08	***
1009:2-SM2:1	39.2025	15.2732	63.1318	1.2E-06	***
SM2:3-1006:1	-25.62	-49.5493	-1.6907	0.019813	*
SM2:3-1006:2	-47.5325	-71.4618	-23.6032	1.01E-09	***
SM2:3-1006:3	-51.405	-75.3343	-27.4757	3.26E-11	***
SM2:3-1027:1	-25.57	-49.4993	-1.6407	0.020389	*
SM2:3-1027:2	-25.92	-49.8493	-1.9907	0.016659	*
SM2:3-1027:3	-25.0475	-48.9768	-1.1182	0.027382	*
SM2:3-1081:2	-39.74	-63.6693	-15.8107	7.74E-07	***
SM2:3-1081:3	-30.085	-54.0143	-6.1557	0.001182	**
SM2:3-1009:2	-35.7575	-59.6868	-11.8282	1.86E-05	***

CHAPTER III

COMPARATIVE-ANALYSIS OF TOMATOES TREATED WITH DIFFERENT BIOTIC AND ABIOTIC STRESSES SHOWED SEVERAL GENES INVOLVED IN MULTIPLE STRESS-RESPONSE

3.1 Introduction

Plants are sessile living organisms, which developed many strategies for quickly adapting to environmental changes. Despite this, the occurrence of adverse environmental factors (known as abiotic stress), such as heatwaves, drought, salinity, or low temperatures, can negatively affect plant growth and productions (Cappetta et al., 2020). In addition, abiotic stress can often concur with plant pathogen or pest attacks (from now on called biotic stress). Simultaneous exposition to biotic and abiotic stress can induce tremendous crop yield losses. Therefore, the resistance mechanisms to various tomato stresses have been under investigation for a long time. Until a few years ago, most of the stress-related studies were focused on the single stress response mechanism. Recently, more emphasis has been given to studies investigating the plant response to combinations of multiple stresses. An increasing number of studies have been conducted to identify new forms of resistance to multiple stress and several genes that recognize both biotic and abiotic stress have been found (Saijo and Loo, 2020; Ku et al., 2018).

In tomato, different genes for signaling, perception, hormone balancing and transcription modulation are involved in various biotic and abiotic stress responses (Krishna et al., 2019; Bai et al., 2018; Bouzroud et al., 2018; Zhu et al., 2018; Li et al., 2016; Sun et al., 2014).

The modulation of these pathways leads to the activation or repression of several responsive proteins such as pathogen-related proteins (PR), peroxidases, chitinases, Heat shock proteins (HSPs), Pectin methylesterases (PMEs), Glutathione s-transferases (GSTFs), Reactive oxygen species (ROS), and defensive compounds such as phenols, anthocyanins, salicylates, and glucosinolates. In this context, genetic

engineering became a fundamental tool for creating new plants that quickly adapt to biotic and abiotic stress without compromising plants' phenotypical traits and yields (Mackelprang and Lemaux, 2020). To this purpose, the study of transcriptomic alterations in plants subjected to various stress could aid the identification of principal genes that participate in resistance or susceptibility processes.

Gene expression profiles under different conditions can be measured using several methods (Costa-Silva et al., 2017). In particular, high-throughput sequencing RNA-seq technology allows quantifying the different gene expression levels with high accuracy (McDermaid et al., 2019). The application of this technology is based on six main steps: 1) mRNA extraction and reverse transcription in complementary DNA (cDNA); 2) fragmentation of the cDNA, library preparation and sequencing; 3) mapping of reads to a reference genome; 4) evaluation of mapped reads for the estimation of gene expressions; 5) normalization of mapped data and the use of statistical analysis for differentially expressed genes (DEGs) identification; 6) examination of the biological relevance of identified DEGs (Costa-Silva et al., 2017). Thus, identifying DEGs involved in various tomato stress could facilitate the discovery of specific genes conferring resistance or susceptibility to multiple stresses. In 2018, Ashrafi-Dehkordi et al. (2018) published a work of a tomato meta-analysis in which DEGs participating in biotic and abiotic stress were described. That work was performed using the microarray gene expression technique and allowed the identification of 1,862 and 835 genes responding to biotic and abiotic stress, respectively.

However, RNA-seq technology offers full-genome coverage. It also allows detecting the expression level of transcripts showing higher sensitivity for genes expression than microarray technology (Finotello and di Camillo, 2015). So far, Illumina technology is the most used sequencing platform for RNA samples due to its accuracy, rapidity, and reduced prices (Rasheed, 2020).

In our work, large-scale bibliographical research was conducted for downloading RNA-seq raw data related to tomato stressed samples. Out of a total of 30 retrieved studies, only 11 RNA-seq experiments analyzing tomato response to 12 different stresses (eight biotic and four abiotic) were considered further. Analyzed studies were chosen considering at least three biological replicates per treatment and raw RNA-Seq data obtained with Illumina platforms.

Experiments were re-analyzed using a standard pipeline, and DEGs from different experiments were compared for identifying genes responsive to various stress. In particular, genes involved in cell wall metabolism, membrane receptors, transcription factors (TFs), and PR proteins involved in biotic and abiotic stress response were investigated in detail. The final goal of our analysis was to prioritize a list of responsive genes to multiple stress, potentially candidate to be employed in genetic engineering programs. The research for genes involved in response to multiple stress is useful for the development of new tomato resistant-cultivars subject to climate fluctuations during cultivation. Hence, genes resulting from our comparative analysis could be further characterized through biotechnological approaches to investigate their role in tomato response to multiple stresses.

3.2 Results

We downloaded and analyzed publicly available RNA-Seq raw data from studies of tomato plants exposed to different abiotic and biotic stresses to investigate tomato response. In particular, we selected eight studies, including some of the most important tomato-biotic stressors and four among the most common abiotic stress; in some cases, interactions were evaluated at different time points (Table 9). Biotic stressors challenging tomato were: *Cladosporium fulvum* infecting tomato, at 7 and 20 dpi (days post-infection), *Phytophthora infestans* at 40 dpi, *Pseudomonas syringae* and *Ralstonia solanacearum* at 1 and 2 dpi, *Sclerotinia sclerotiorum* and *Southern tomato virus* (*STV*) at 30dpi, *Tomato spotted wilt virus* (*TSWV*) at 4, 7, 14, 21 and 35 dpi and *Tuta absoluta* at 40dpi. Response to abiotic stress was assessed by analyzing four tomatoes RNA-seq studies of plants treated with drought (from two different studies), salt, cold and oxidative stress (Table 9). A comparative analysis of transcriptomic data of twelve among the most common tomato stress interactions was conducted with the purpose of investigating plant responses under different biotic and abiotic stresses. From our analysis, several genes taking part in tomato response to multiple stress were identified.

Table 9. List of the studies used in this comparative analysis. Dpi = Days post-inoculation.

Stress Type	Time	Genotype		SRA code	References
	RNA-extraction	Resistant	Susceptible		
ABIOTIC					
Drought	After 10 days of stress	IL9-1	M82	SRP100604	(Liu et al., 2017)
Salt	After 6 hours of stress	-	MicroTom	SRP150651	(Keshishian et al., 2018)
Oxidative stress	After 6 hours of stress	-	MicroTom	SRP150651	(Keshishian et al., 2018)
Drought	After 5 days of stress	-	Jinlingmeyu	SRP156535	(Zhou et al., 2019)
Low temperature	After 2 days of stress	-	Jinlingmeyu	SRP156535	(Zhou et al., 2019)
BIOTIC					
C. fulvum	7, 20 dpi	CGN18423	MoneyMaker	SRP157120	(Zhao et al., 2019)
P. infestans	40 dpi	Resistants transgenic lines	M82	SRP168458	(Canto-Pastor et al., 2019)
P. syringae	2 dpi	RL-Light treatment	Ailsa Craig	SRP051074	(Yang et al., 2015)
R. solanacearum	1, 2 dpi	Hawaii 7996	West Virginia 700	SRP078159	(French et al., 2018)
S. sclerotiorum	30dpi	-	Heinz	SRP124841	(Badet et al., 2017)
STV	30 dpi	-	M82	SRP221436	(Fukuhara et al., 2020)
TSWV	4, 7, 14, 21, 35 dpi	Fla8059.Sw7	Fla8059	SRP119544	(Padmanabhanet al., 2019)
Tuta absoluta	40 dpi	BR221	PS650	SRP286525	(D'esposito et al., 2021)

3.2.1 RNA-Seq Data Processing and DEGs analysis

Raw RNA-Seq data retrieved from the studies reported in Table 9 were uniformly processed and re-analyzed, as described in Materials and Methods. A standard pipeline for consistently processing all raw sequencing data files was used that included the following steps: removal of low-quality reads, alignment to the reference genome, count of reads, and normalization; a diagram of the workflow used is shown in Figure 7.

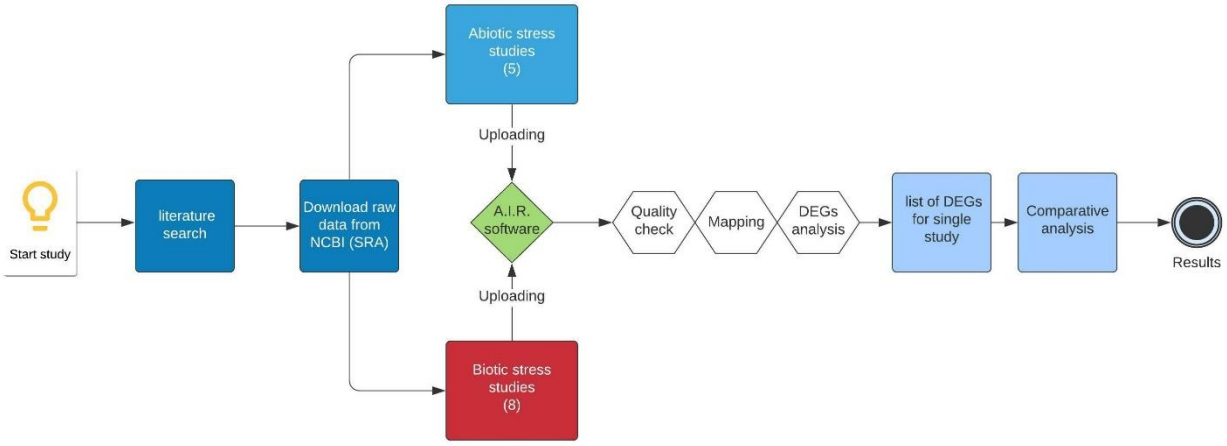


Figure 7. Schematic diagram of the workflow used in our comparative study.

A differential gene expression analysis was conducted separately for each individual study. DEGs for the different comparisons within each study are reported in Table 10.

Table 10. Number of DEGs resulting from the analysis of each experiment.

Stress type	Genotypes compared	Dpi	Up	Down	Total DEGs
C. fulvum	Si. Vs. Sni.	7 vs 0	4698	4848	9546
	Si. Vs. Sni.	20 vs 0	2718	2487	5205
	Ri. Vs. Rni.	7 vs 0	3562	3721	7283
	Ri. Vs. Rni.	20 vs 0	4335	3904	8239
P. infestans	Ri_{mi482} Vs. Rni_{mi482}	40	5986	5667	11635
	Ri_{mi2118} Vs. Rni_{mi2128}		7150	6768	13918
	Si. Vs. Sni.		6914	6416	13330
P. syringae	Si. Vs. mock	2	5500	5543	11043
	S. (treated) Vs. mock		5684	5881	11565
R. solanacearum	Si. Vs. Sni.	1 vs 0	523	655	1178
	Si. Vs. Sni.	2 vs 0	1955	2426	4381
	Ri. Vs. Rni.	1 vs 0	673	907	1580
	Ri. Vs. Rni.	2 vs 0	1135	1247	2382
S. sclerotiorum	Si. Vs. Sni	30	6065	6059	12124
STV	Si. Vs. Sni	30	0	5	5
TSWV	Si. Vs. Ri	4	18	76	94
	Si. Vs. Ri	7	18	11	29
	Si. Vs. Ri	14	617	499	1116
	Si. Vs. Ri	21	722	768	1490
	Si. Vs. Ri	35	485	726	1211
Tuta Absoluta	Si. Vs. Sni	40	3287	2576	5863
	Ri. Vs. Rni		5176	4940	10116
Drought	Si. Vs. Sni	10	1283	2242	3525
	Ri. Vs. Rni		682	1290	1972
Drought	Si. Vs. Sni	2	2725	3374	6099
Low temperature	Si. Vs. Sni	2	6323	6513	12836
Salt	Si. Vs. Sni	0.25	3600	2965	6565
Oxidation	Si. Vs. Sni	0.25	2849	2475	5324

Time points among single studies differed considerably, especially in biotic stress datasets. RNA-seq data were collected at different times for tomatoes exposed to *C. fulvum, R. solanacearum,* and *TSWV*, which gave us the chance to explore various time points (early and late response). A variable number of tomato DEGs in analyzed

experiments was found (Table 10). Indeed, *P. infestans, P. syringae*, *S. sclerotiorum,* and tolerant genotype to *Tuta Absoluta* induced the differential expression of a high number of tomato genes (higher than 10.000 DEGs) *Southern Tomato Virus* (*STV*), which is known to be asymptomatic, did not show any up-regulated gene and only five down-regulated genes. For this reason, we excluded it by further analysis. Concerning experiments with multiple time points, *TSWV* induced a peak of DEGs at 21dpi (1490 DEGs), while *R. solanacearum* and resistant genotype to *C. fulvum* displayed an increasing number of DEGs at 2dpi and 20dpi, respectively. In contrast, the susceptible genotype to *C. fulvum* showed a decreasing DEGs number from 7 to 20dpi.

Among abiotic stress analyzed, DEGs ranged from 2 to 6000, except for the low-temperature experiment (12.836 DEGs).

To better understand which DEGs responded to different stresses, we first analyzed biotic and abiotic stress separately, searching for DEGs in more than a single experiment. Then, we extended the comparison of the results for detecting genes involved in both biotic and abiotic responsive pathways.

3.2.2 Tomato core biotic stress response

Datasets of susceptible genotypes were compared to identify tomato biotic stress-responsive genes. We divided biotic stress into three main groups: 1) Fungi, including *C. fulvum*, *P. infestans,* and *S. sclerotiorum* pathogens; 2) Bacteria, containing *P. syringae* and *S. sclerotiorum,* and 3) Virus and Pest, including *TSWV* and *Tuta absoluta* diseases (Figure 8).

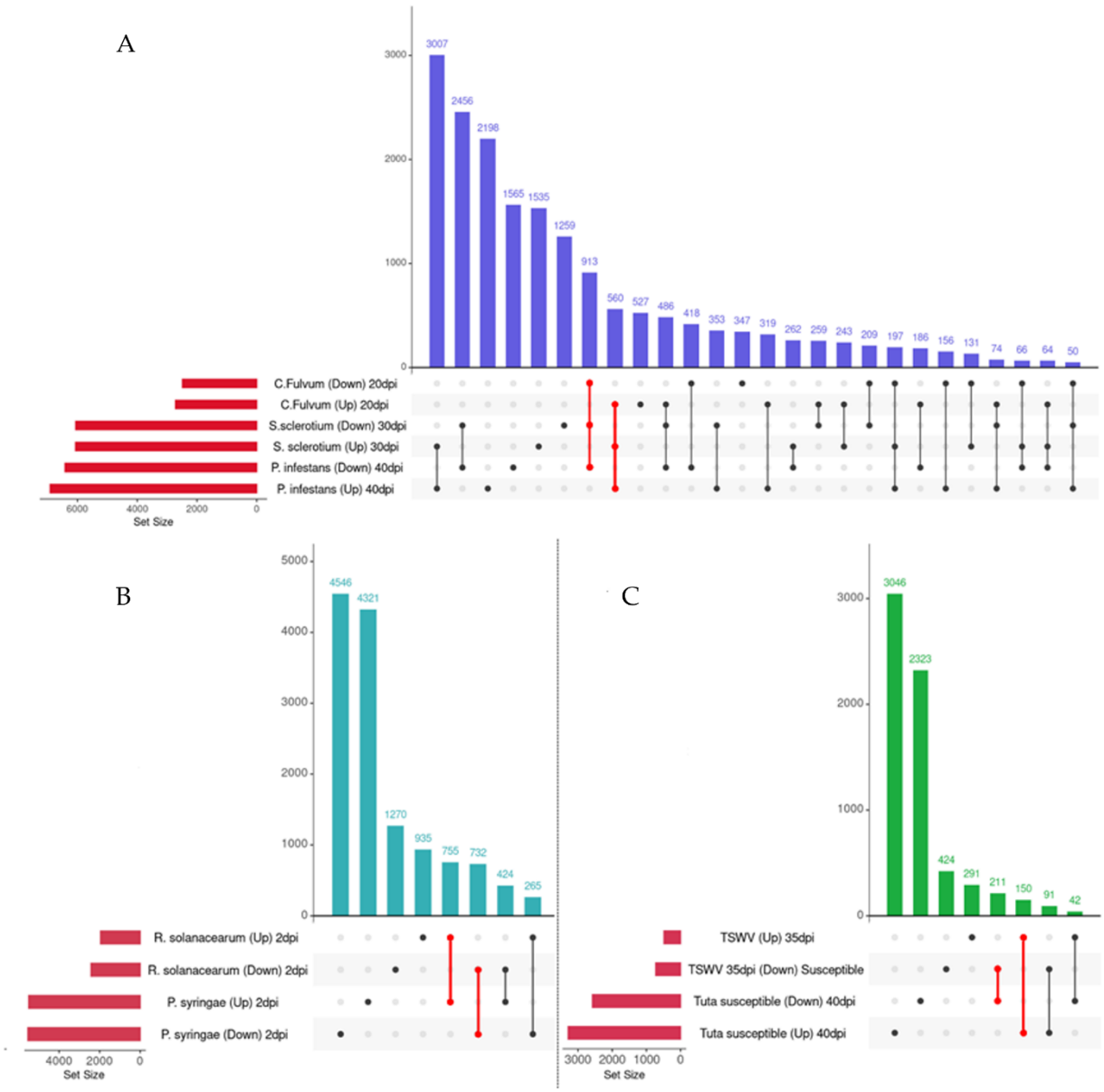


Figure 8. Upset plot. Comparison of datasets of DEGs induced in susceptible genotypes infected with different tomato pathogens. A) Up and Down DEGs induced genotypes infected with tomato fungi; B) Up and down DEGs induced genotypes during tomato bacterial infections; C) Up and down DEGs induced in genotypes during *TSWV* and *Tuta absoluta* infestations. In red, up and down DEGs are shared among datasets.

Several genes were differentially regulated during tomato-pathogen interaction. The number of shared genes among tomato biotic stress is shown in Figure 8.

We focused on genes with the same expression trend (up or down-regulated) in all datasets. Thus, we found that 913 down and 560 up-regulated genes were shared among tomatoes stressed with fungi. Also, for bacteria and virus-pest groups, we

found common activated and repressed genes. In particular, genotypes infected with *R. solanacearum* and *P syringae* (bacteria) shared 755 up and 732 down-regulated genes, respectively. At the same time, *TSWV* and *Tuta absoluta* (Virus and pest) induced 150 common-up and 211 common-down regulated genes.

However, we also found common DEGs with opposite regulations among various tomato stress, but due to the high number of possible comparisons, we focused on DEGs with the same expression trend among datasets.

Finally, we found nine genes with a functional role in biotic stress response, induced in all stressed-genotypes with fungi, bacteria, the virus, and the pest (Table 11).

Table 11. Common DEGs induced by all the analyzed biotic stress.

Gene ID	Regulation	Function
Solyc01g097240	Up	Pathogenesis-related protein PR-4
Solyc07g005100	Up	Chitinase/lysozyme
Solyc10g083690	Up	Cytochrome P450
Solyc03g078490	Up	Glycosyltransferase
Solyc04g040180	Up	S-adenosylmethionine-dependent methyltransferase
Solyc10g084410	Up	Protein phosphatase 2C family protein
Solyc05g051570	Up	Ras-related protein (Rab6A)
Solyc07g042550	Up	Sucrose synthase (SUS3)
Solyc02g080810	Down	Aminomethyltransferase

3.2.3 Regulation of genes involved in tomato-pathogen interaction

To detect differences in pathways induced by different tomato pathogens, we performed a Mapman analysis using datasets of DEGs common activated or repressed by each group of stress.

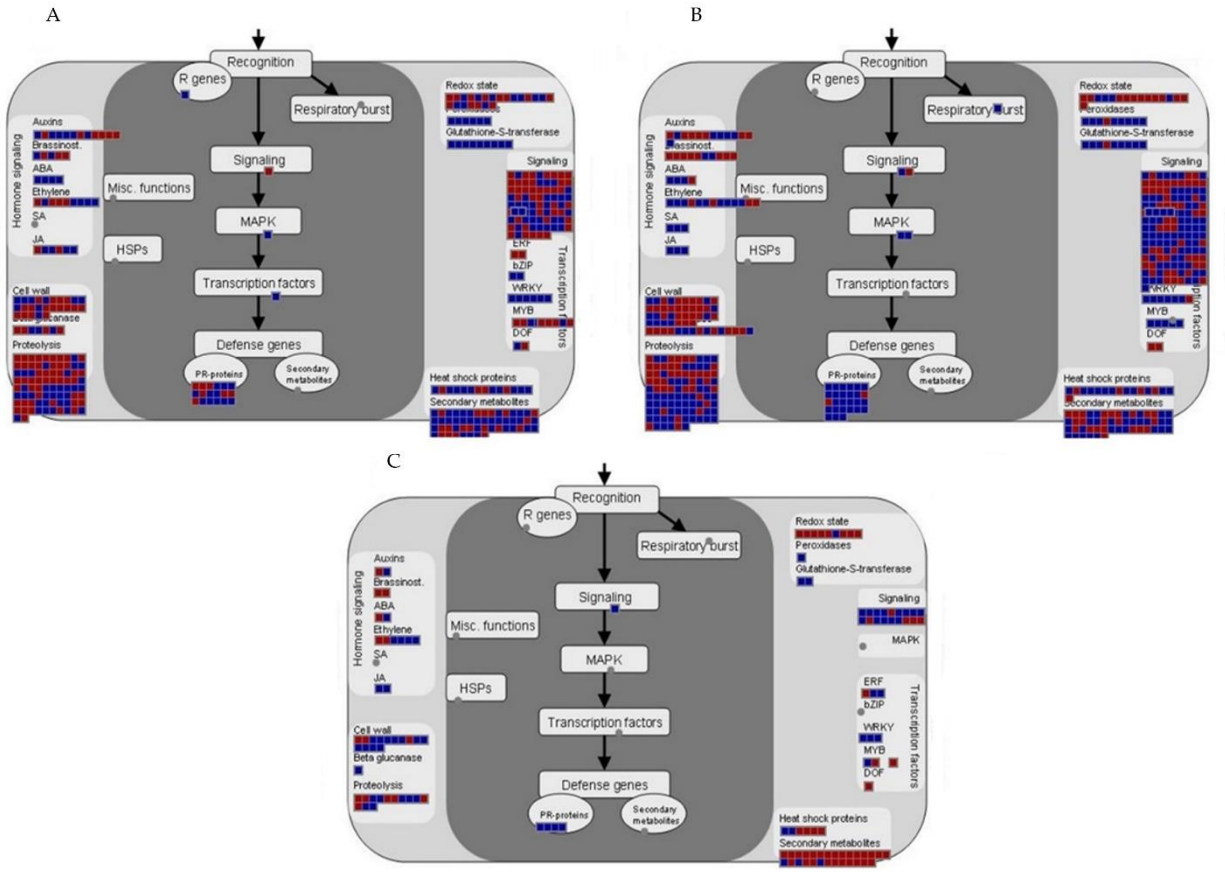


Figure 9. Genes related to pathways activated or repressed during biotic-stress response: A) DEGs induced by Fungi B) DEGs induced by Bacteria; C) DEGs induced by Virus and Pest infections. In blue: up-regulated genes, in red: down-regulated genes.

As can be observed in Figure 9, stressed tomatoes induced or repressed different genes under fungi, bacteria, and the virus and the pest. Differences in induced or repressed pathways mainly relied on hormone signaling, transcription factors (TFs), PR-proteins (pathogen-related proteins), cell wall biosynthesis, and plant secondary metabolism.

Unlike fungi and virus-pest groups, salicylic and jasmonic acid were common up-regulation during bacterial infections. On the other hand, the virus and pest induced the down-regulation of the secondary metabolism pathway. Susceptible

genotypes to bacterial infections showed the up-regulation of ERF and MYB transcription factors, which were down-regulated during fungi infections. WRKYs appeared generally up-regulated during all tomato biotic stress, while DOFs were generally down-regulated. Metabolism related to cell wall was affected during all three stress groups but with different intensities. Interestingly, a resistance-gene (Solyc07g052780, TIR-NBS-LRR) was up-regulated in susceptible genotypes during fungi infections.

Considering the importance of plant recognition and signaling in plant-pathogen interactions, we focused on receptor-like kinases (RLKs) that play a crucial role in plant-stress interaction.

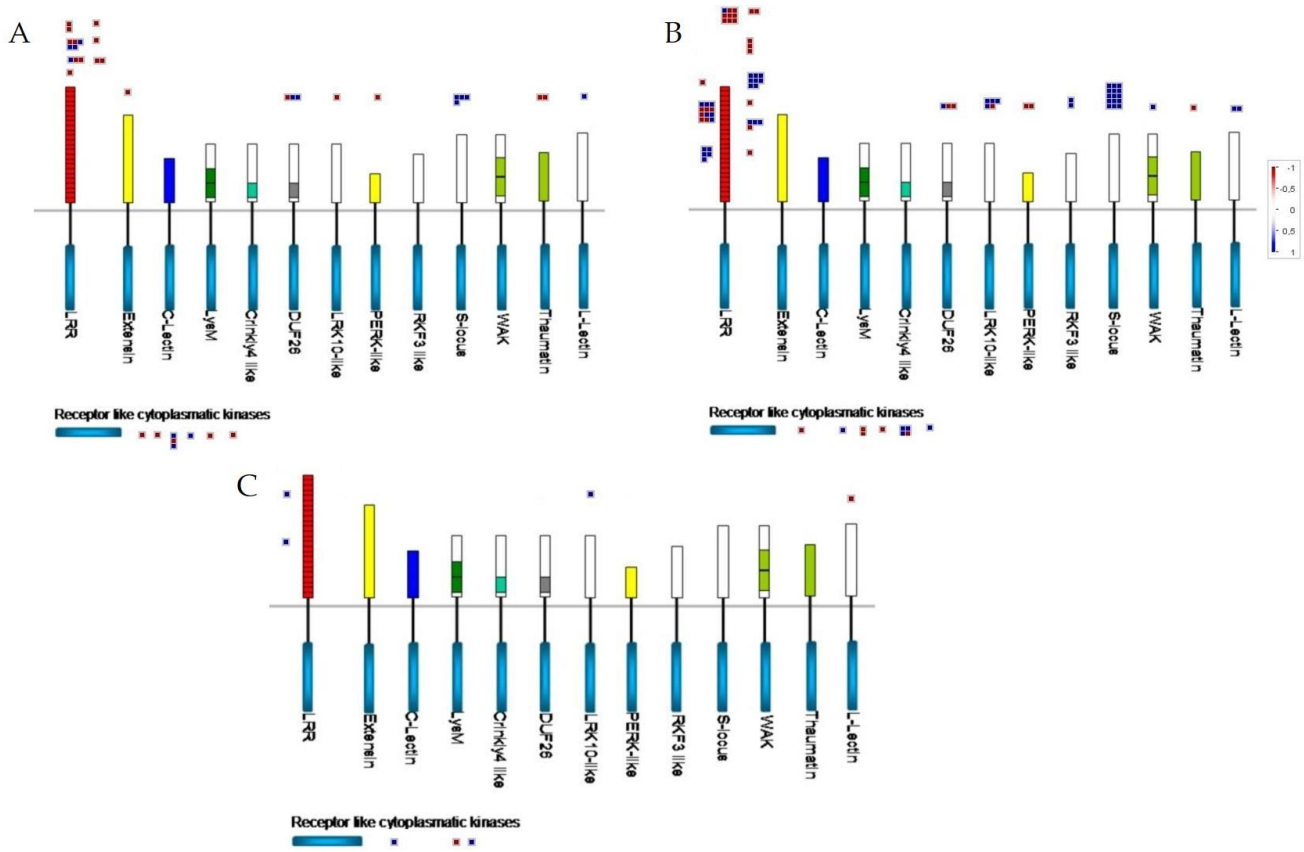


Figure 10. Differential expression of RLKs during A) Fungi; B) Bacteria; C) Virus and pest interactions. In blue: up-regulated genes, in red: down-regulated genes.

Several differences arose during fungi, bacteria, and virus and pest infections. Fungi-susceptible infected genotypes showed the down-regulation of an Extensin like-protein while bacteria induced the up-regulation of a WAK receptor (Figure 10A, B).

However, several RLK genes were differentially regulated during bacterial diseases (Figure 10B). In particular, numerous receptor type S-locus resulted up-regulated.

Virus and pest showed few differentially regulated genes encoding for RLK proteins compared with fungi and bacteria. Two Leucine reach proteins (LRR) and an LRK10-like protein resulted up-regulated, while an L-lectin receptor was down-regulated (Figure 4C).

Cell wall represents the first barrier to ensure plant life, providing a mechanical defense against biotic and abiotic stresses. Therefore, we studied those DEGs involved in cell wall biosynthesis.

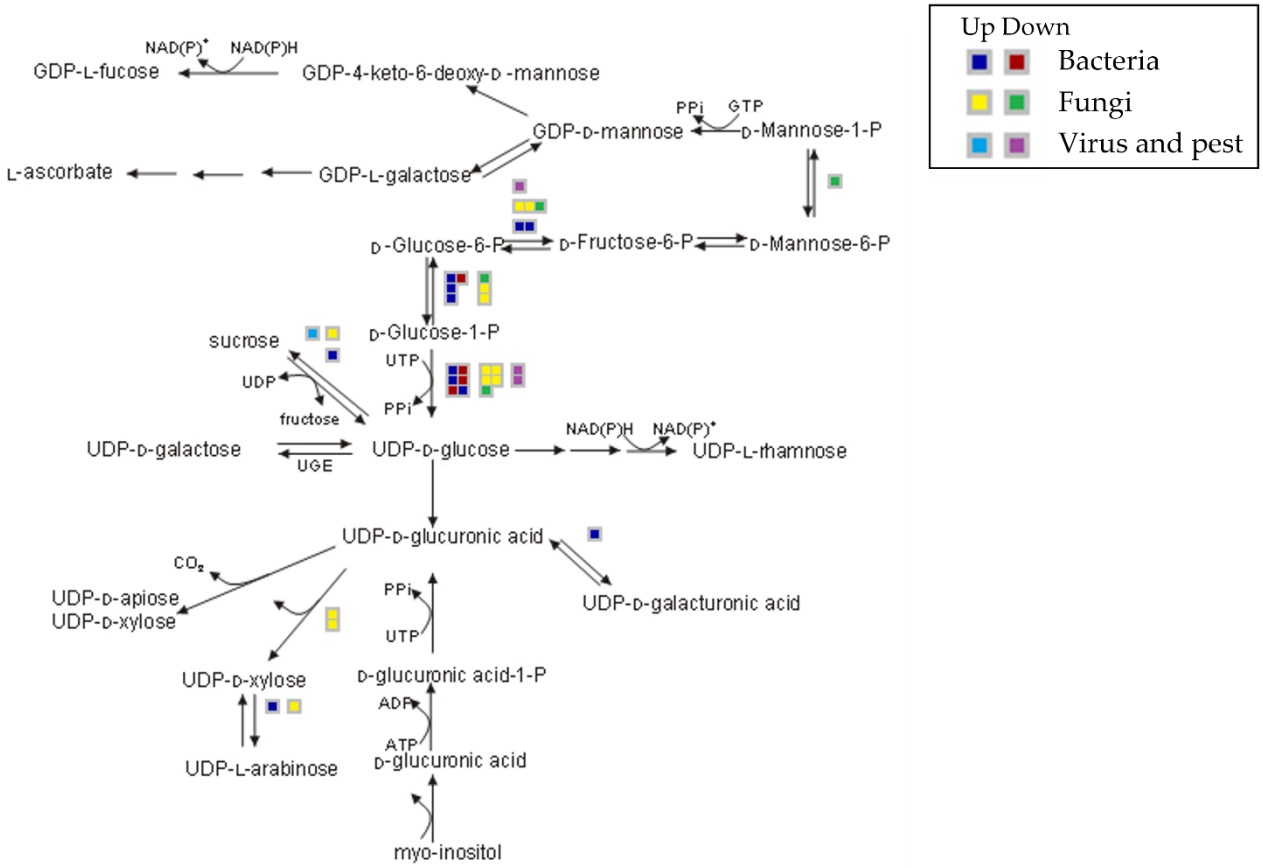


Figure 11. Precursors compounds of cell wall biosynthesis activated or repressed under various tomato biotic stress. Colors indicate up and down-regulated genes for each group of analysis.

As can be observed in Figure 11, differences were found in the synthesis of the precursors of the cell wall components. Susceptible genotypes infected by fungi showed the up-regulation of genes involved in the UDP-D-xylose production (Solyc09g075120, Solyc11g066150); which is a precursor of xylan and xyloglucan, some

of the most abundant primary-wall hemicellulose compounds in plants. By contrast, bacterial infections induced the up-regulation of a gene for the UDP-D-galacturonic acid synthesis (Solyc08g079440, belonging to the GAEs family), an indispensable precursor of pectin biosynthesis. Disease caused by the virus and pest group did not appear to affect the cell wall biosynthesis process; few genes were differentially regulated. In particular, there was a down-regulation of genes involved in the production of D-fructose-6-P and UDP-D-glucose.

3.2.4 Comparison of resistant genotypes

We compared DEGs of stressed-resistant genotypes to understand the basal mechanisms involved in plant resistance to biotic stress. Our datasets were composed of tomatoes resistant to *P. infestans*, *R. solanacerarum*, *Tuta absoluta*, *C. fulvum,* and *TSWV*.

However, mechanisms of resistance differed among genotypes. In particular, resistances to *C. fulvum* and *TSWV* were conferred by two R-genes (*Cf-19* and *Sw-7*), while resistance to *R. solanacearum,* as reported by French et al. (2018), was based on gene expression changes in roots. Resistant genotypes to *P. infestans* were obtained by overexpressing short tandem target mimic (STTM) RNAs, targeting miR482 and miR2118, thus enhancing the NLR proteins expression. Finally, tomato-specific plant volatile compounds and phenotypical traits were identified in genotypes tolerant to *Tuta absoluta*. (D'Esposito et al., 2021; Canto-Pastor et al., 2019; Padmanabhan et al., 2019; Zhao et al., 2019b).

Therefore, the great variability in resistance mechanisms among analyzed genotypes allowed us to investigate not the unique responsive mechanism but rather the common basal response activated by resistant genotypes. To this purpose, we identified common DEGs between resistant genotypes. In particular, we had two transcriptomic datasets of resistant tomatoes infected with *P. infestans*. Therefore, we first identified common DEGs induced by *P.infestans,* and then we compared this unique dataset with the DEGs induced in the remaining stressed-resistant genotypes. Figure 6 reported the number of common DEGs among biotic stress-resistant genotypes.

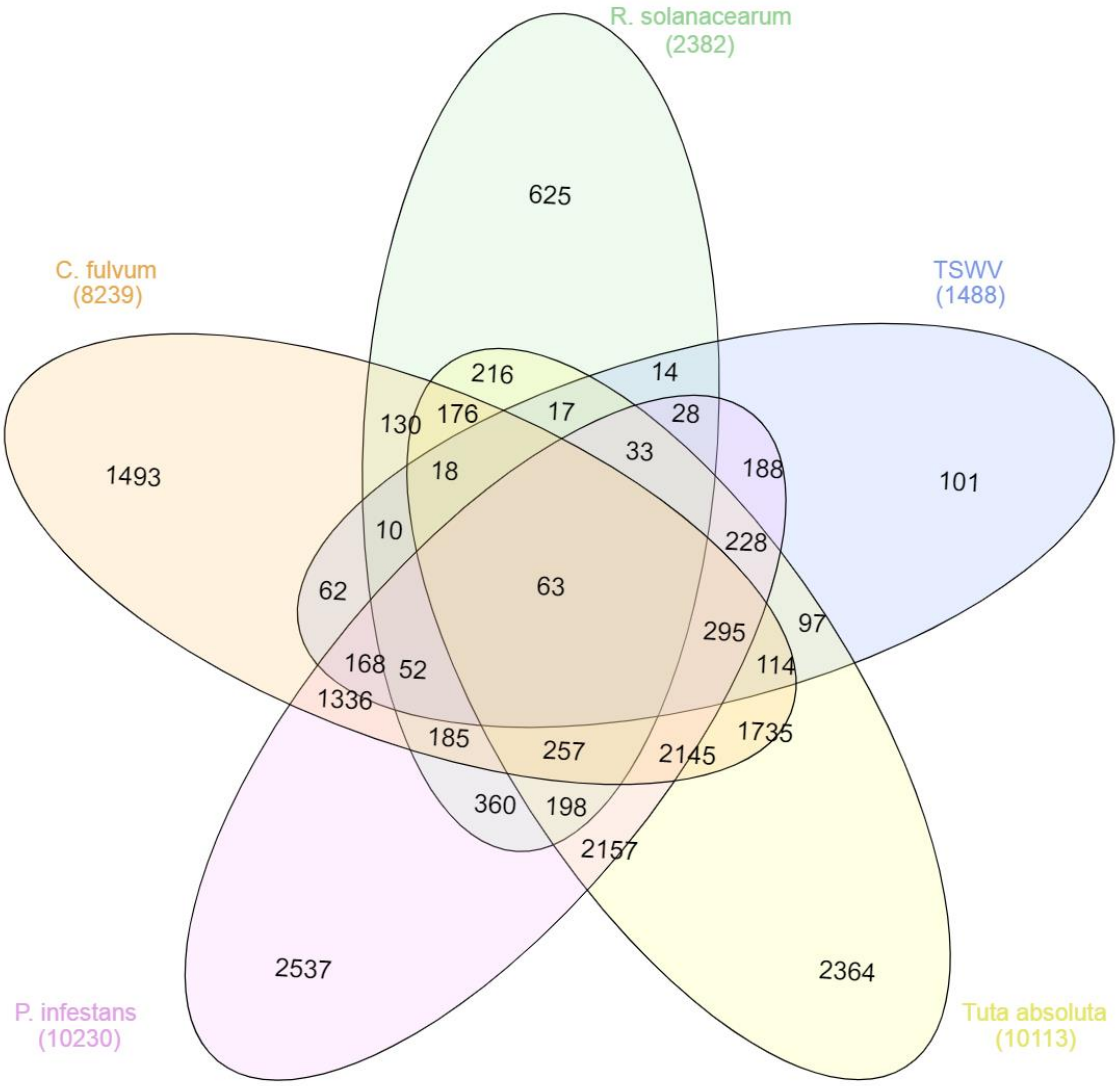


Figure 12. Number of DEGs resulting from multiple comparisons among biotic stress-resistant genotypes.

As a result of our analysis, after exposure to pathogens, 63 genes were differentially regulated in all resistant genotypes (Figure 12). However, these genes had not the same trend in all datasets. For example, Solyc05g041200 (HPPD) was down-regulated during *Tuta absoluta* attacks and up-regulated during all the other treatments. At the same time, Solyc06g076570 (HSP) resulted down-regulated during *TSWV*, *Tuta absoluta,* and *P. infestans* infections and up-regulated during *R. solanacearum* and *C. fulvum* interactions. Comparing *C. fulvum, R. solanacearum, TSWV, Tuta absoluta,* and *P.infestans* resistant genotypes, we identified 3 common up-regulated genes (Table 12).

Table 12. Common DEGs among resistant genotypes to biotic stress.

Gene ID	Regulation	Function
Solyc02g071475	Up	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase
Solyc01g097270	Up	pathogen-induced protein
Solyc12g096570	Up	ARGOS

To better understand the resistant reprogramming process during the pathogen and pest attacks, we evaluated the list of 63 common DEGs (Figure 12) involved in biotic stress response. Transcriptomes of resistant genotypes exposed to biotic stress infections resulted in differentially regulation of genes involved in four important cellular pathways: secondary metabolism, hormone metabolism, stress response, and signaling (Figure 13).

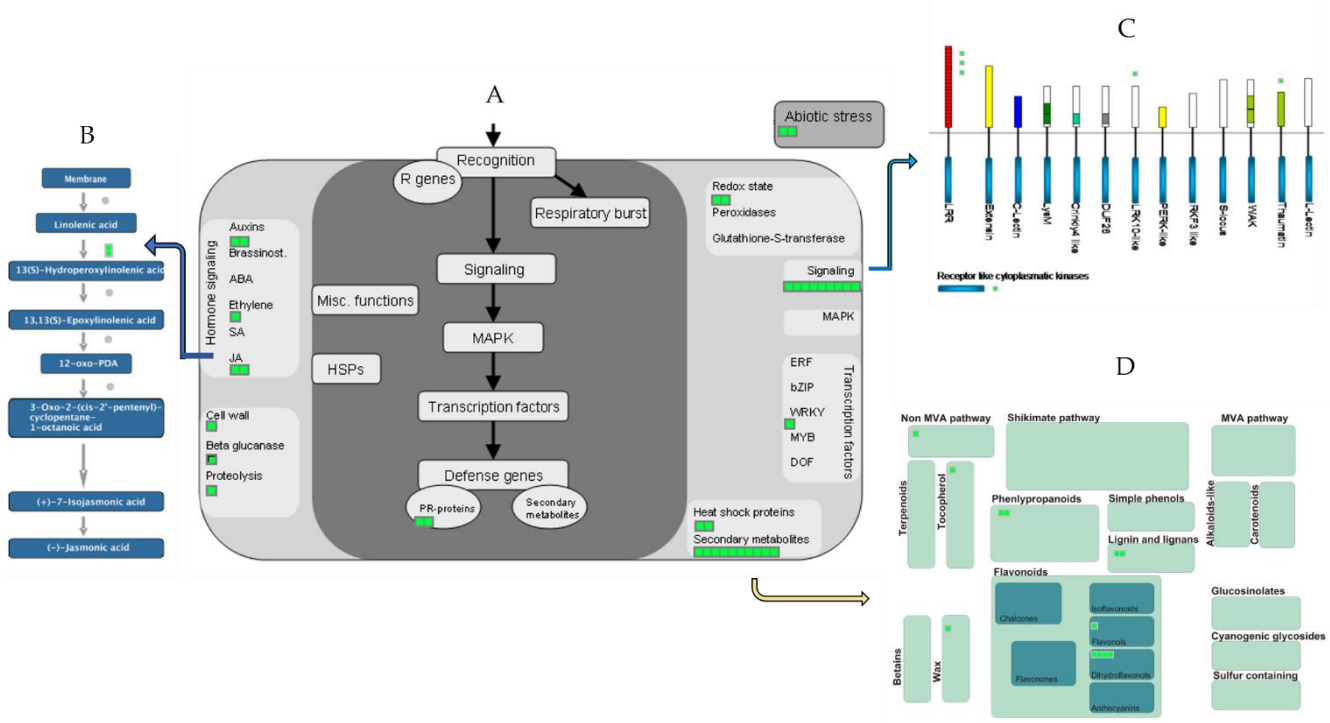


Figure 13. Pathways differentially regulated in biotic stress-resistant genotypes. A) Process activated during biotic stress response; B) Jasmonic acid synthesis pathway; C) Receptors like kinases; D) Cell secondary metabolism. In green are reported differentially expressed genes.

Secondary metabolism (Figure 13D) was particularly involved in resistance to biotic stress. A number of DEGs for flavonoid regulation (Solyc04g071780,

Solyc12g042480, Solyc12g045020, Solyc02g071475, Solyc02g071475), WAX (Solyc12g100270), Tocopherol (Solyc05g041200), Phenylpropanoids, Lignin (Solyc12g042480, Solyc12g045020), and non-MVA (Solyc03g115980) biosynthesis were differentially expressed under biotic stress. The signaling pathway also appeared particularly challenged in infected resistant genotypes. Concerning RLK genes, we found that three genes encoding for leucine-reach repeat proteins (LRR) were differentially regulated (Solyc11g006040, Solyc07g006480, Solyc08g061560) as well as a Thaumatin and an LRK10-like gene (Solyc04g007380 and Solyc04g007380) (Figure 13C).

Interestingly, two genes encoding for receptor-like proteins (RLPs) (Solyc01g102850 and Solyc07g008620) were differentially regulated in all genotypes along with a cell wall-pectin esterase (Solyc02g080220). We found that Solyc07g008620 was down-regulated during *TSWV* and up-regulated in *R. solanacearum*, *P. infestans*, *C. fulvum,* and *Tuta absoluta* infested genotypes. Furthermore, both genes Solyc07g008620 and Solyc01g102850 were DE during *TSWV* infection after 14dpi. We also found that Solyc01g102850 was down-regulated in susceptible genotypes to fungi while Solyc07g008620 was up-regulated in susceptible genotypes to bacteria and the virus and pest. On the other hand, Solyc01g102850 encoding for a TIR-NBS-LRR resistance protein resulted up-regulated in *TSWV* and *R. solanacearum* infections and down-regulated during *C. fulvum, P. infestans,* and *Tuta abosulta* stresses.

Concerning the hormone metabolism, here we found that two genes involved in the linoleic acid pathway, for jasmonic acid synthesis (Figure 13B), were differentially regulated (Solyc01g009680 and Solyc08g029000). In addition, the hormonal response to biotic stress included genes involved in ethylene and auxin biosynthesis (Solyc01g100370, Solyc10g011660, and Solyc10g018340).

3.2.5 Tomato core abiotic stress response

Our study analyzed the tomato transcriptomic response to four among the most common tomato abiotic stress: drought, salinity, low temperatures, and oxidative stress. We first compared stressed-tomato transcriptomes to find common responsive genes under all the abiotic stress. To this scope, a unique dataset for drought (503 up and 1046 down-regulated genes) was generated, composed of common DEGs between

the two drought stress studies included in the complete analysis (Table 10). Based on those data, we compared the different stresses as shown in Figure 14.

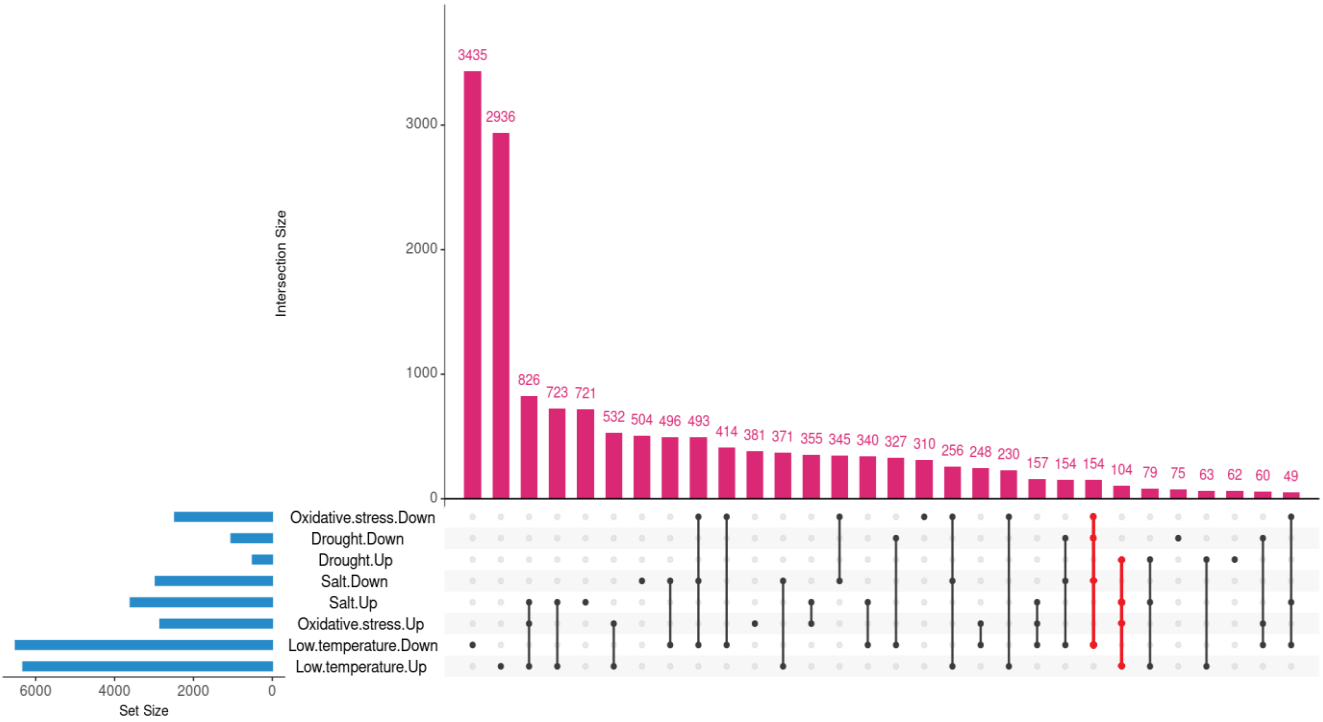


Figure 14. Upset plot. Comparison of DEGs dataset of susceptible genotypes subjected to abiotic stress. In red, up and down DEGs are shared among datasets.

Here, we found that susceptible tomatoes treated with abiotic stress shared 104 up and 154 down-regulated genes (highlighted in red in Figure 14). To further investigate the different responses of susceptible genotypes to abiotic stress, we grouped datasets in two sub-categories: drought and salt stresses and low temperature and oxidative stresses groups (Figure 15). This approach was used to identify common responsive genes involved in specific abiotic stress groups.

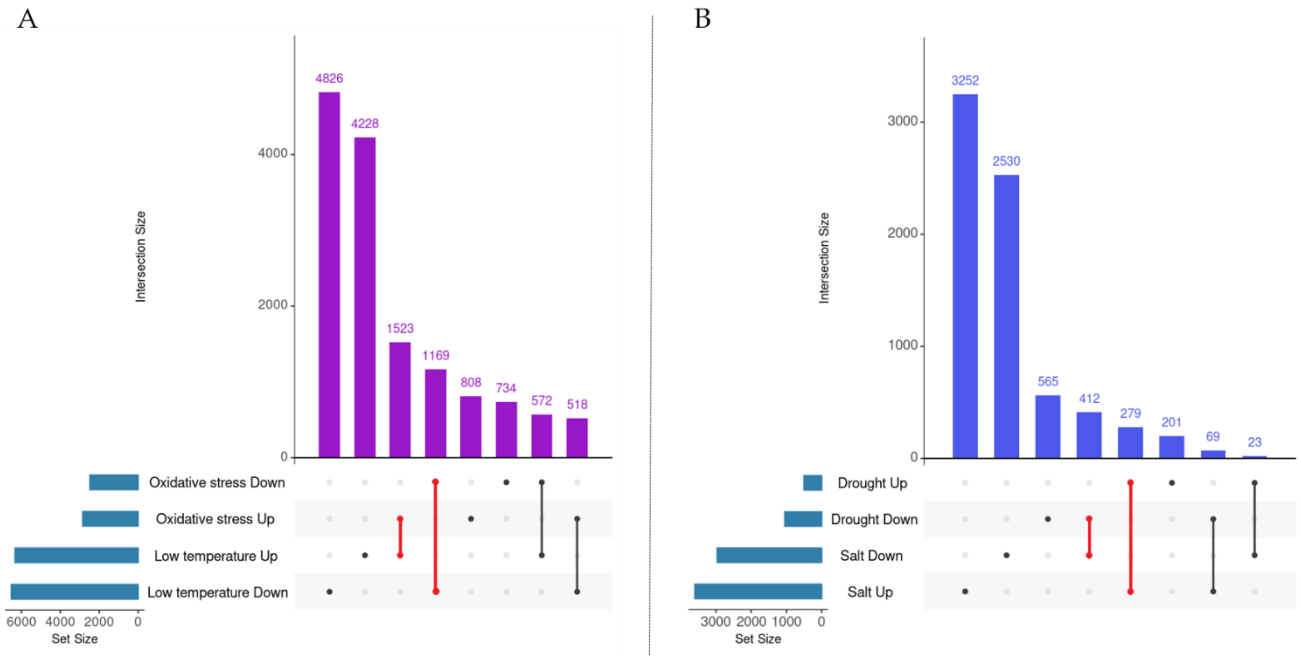


Figure 15. Upset plot. A) Comparison of stressed genotypes susceptible to oxidative and low temperature; B) Comparison of stressed genotypes susceptible to drought and salt stresses. In red, up and down shared DEGs.

It is worth noting that oxidative stress and low temperature resulted in 1523 up-regulated and 1169 down-regulated genes (Figure 15). On the other hand, drought and salt stress shared 279 up and 412 down-regulated genes, respectively. Therefore, according to the procedure used for biotic stress studies, we investigated common DEGs implicated in stress's cellular response (Figure 16).

3.2.6 Stress-responsive pathways regulation during tomato-abiotic stress interaction

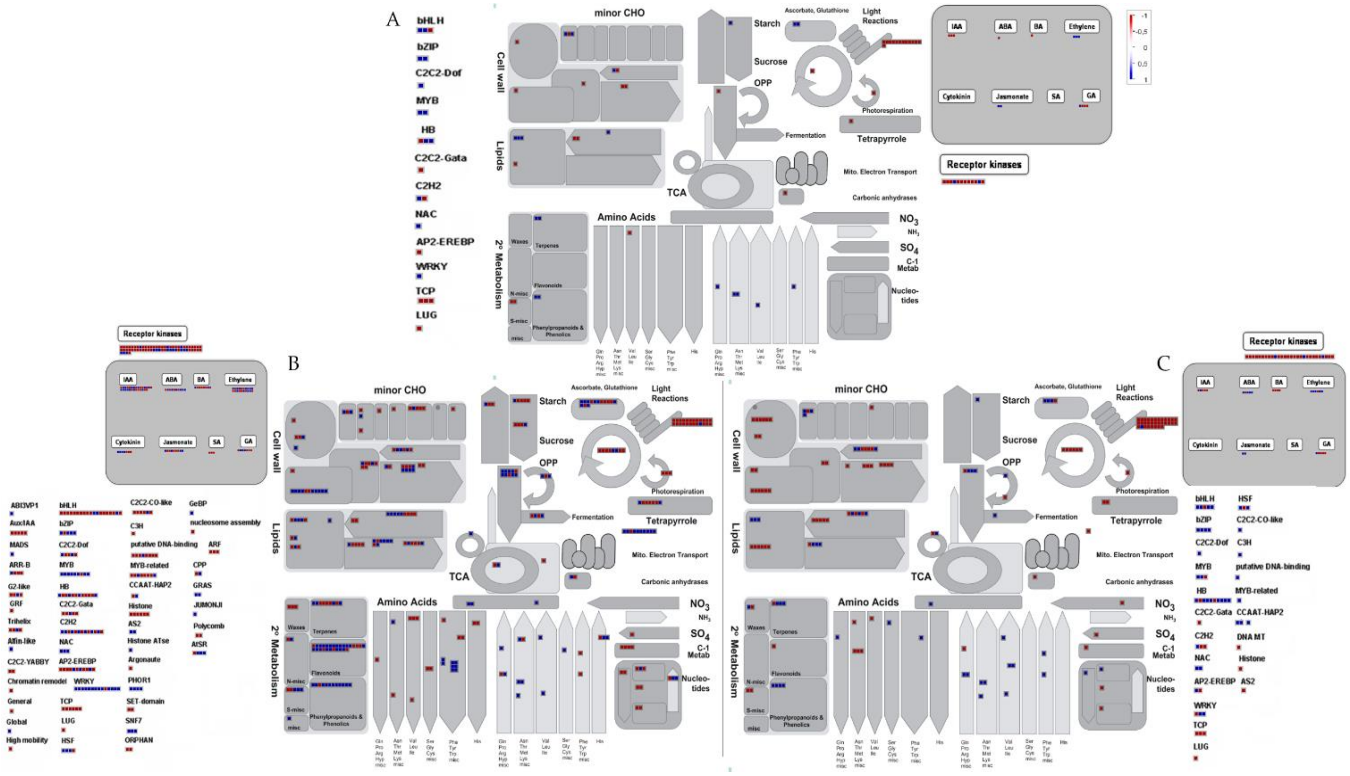


Figure 16. DEGs are involved in abiotic stress response. A) Common DEGs among all the abiotic stress; B) Common DEGs between cold and oxidative stress; C) Common DEGs between drought and salt stress. In blue = up-regulated; in red = down-regulated genes.

Figure 16 allowed us to observe pathways differentially regulated during abiotic stress. In particular, during all abiotic stresses (Figure 16A), plant hormone signaling was balanced in favor of ethylene and jasmonic acid biosynthesis with the abscisic acid (ABA), brassinosteroids, and auxin down-regulation. Genes responsible for cell wall building were largely down-regulated during abiotic stress, along with beta glucanases. Transcription factors such as bZIP, WRKY, MYB, DOF, and NAC resulted up-regulated while ERFs were down-regulated (Figure 16A). Two sub-categories, hormone trends, and transcription regulation, were similar in abiotic stress and drought stress. Dataset related to low temperatures and oxidative stress exposure showed slight differences in hormone signaling regulation, with the down-regulation of genes involved in the salicylic acid pathway and the variable regulation of the other hormonal compounds. Furthermore, low temperatures and oxidative stress groups

induced a higher number of up-regulated genes for glutathione s-transferase proteins (Figure 16B, C).

Once we identified the main differences in cellular response during abiotic stress, we focused on signaling and cell wall processes (Figure 17).

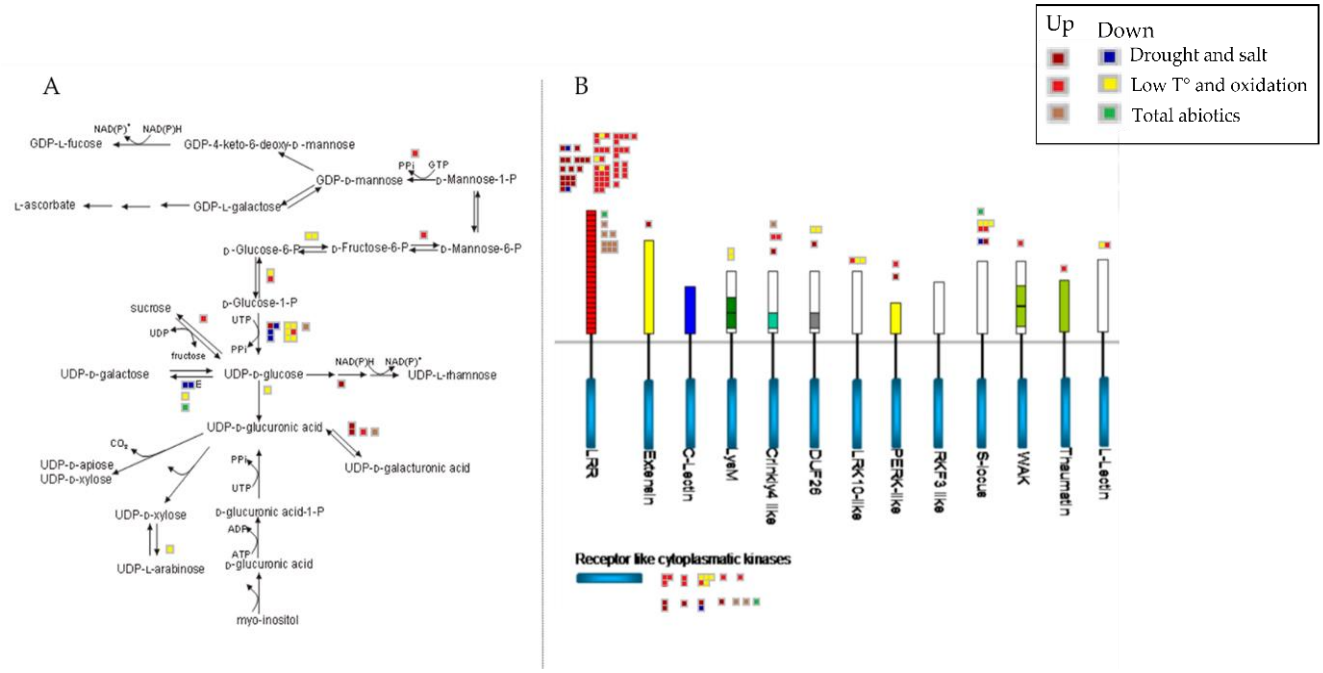


Figure 17. DEGs in A) cell wall precursors, and B) receptor-like kinases involved in abiotic stress response.

Several DEGs shared among abiotic stress were involved in the cell wall and signaling processes. In particular, during abiotic stress, DEGs involved in cell wall biosynthesis resulted generally down-regulated (Figure 17A). Low temperature and oxidative stress dataset induced the synthesis of the UDP-L-arabinose thanks to the up-regulation of a gene (Solyc02g069580) encoding a type-II membrane protein that catalyzes 4-epimerization of UDP-D-Xylose to UDP-L-Arabinose and it is known as *MUR4*.

Moreover, the low temperature and oxidative stress induced the up-regulation of a UGD (Solyc02g088690) and two aldolase genes (Solyc09g009260 and Solyc10g083570). By contrast, both stresses down-regulated a GDP-D-mannose pyrophosphorylase 2 (Solyc06g051270) and Solyc02g086095, involved in Ascorbic acid (AsA) biosynthesis. On the other hand, drought and salinity induced the down-regulation of two UDP-D-glucuronate 4-epimerase (GAEs) proteins (*GAE3* and *GAE6*), involved in galacturonic acid synthesis (Solyc05g050990 and Solyc09g092330), and the

up-regulation of two genes for D-galactose production (Solyc08g080570 and Solyc08g082440). The DEGs resulting from low temperature and oxidative stress showed a common regulation of cell wall precursors synthesis similar to that induced by drought and salt stresses. In general, abiotic stress induced a down-regulation of genes coding for RLKs (Figure 17B), while during all abiotic stress, only an S-locus (Solyc03g078360) and an LRR (Solyc03g006500) resulted up-regulated. Two genes for lysine motif proteins (Solyc02g089920 and Solyc07g049180) and two genes encoding for DUF proteins (Solyc01g007960 and Solyc04g007880) showed an opposite regulation, resulting up-regulated during the low temperatures and oxidative stresses and down-regulated during drought and salt stresses. Furthermore, low temperature and oxidation stresses induced the down-regulation of a WAK and a Thaumatin protein (Solyc09g014720 and Solyc02g081485), while drought and salt stress produced the down-regulation of an Extensin like-protein (Solyc01g098740).

3.2.7 Comparison of biotic and abiotic datasets allowed identifying a core-stress response

We compared transcriptomic datasets of both susceptible and resistant stressed genotypes to identify common responsive genes to biotic and abiotic stresses (Table 13).

Table 13. Comparisons used in this study. S= susceptible and R= resistant genotypes, TOT= total.

Dataset comparisons	UP	DOWN
Biotic stress		
TOT Biotics (S)	8	1
Fungi (S)	560	913
Bacteria (S)	755	732
Virus and Pest (S)	150	211
TOT Biotics (R)	8	1
Abiotic stress		
Abiotics (S)	104	154
Drought and Salt (S)	279	412
Low T and Oxidative (S)	972	1169
Biotic and Abiotic stress combination		
TOT Biotics (R) and Drought (R)	0	0
P. infestans and Drought (R)	314	669
C. fulvum and Drought (R)	345	460
TSWV and Drought (R)	7	14
R. solanacearum and Drought (R)	47	134
TOT Biotics (S) and TOT abiotic (S)	0	0
Fungi (S) and TOT Abiotics (S)	32	28
Bacteria (S) and TOT Abiotics (S)	6	24
Virus and Pest (S) and TOT Abiotics (S)	3	1

As shown in Table 13, datasets comparison did not allow the identification of common responsive genes among all susceptible genotypes to biotic and abiotic stress (TOT Biotics S and TOT abiotic S). However, comparing each category for separately, we found that susceptible genotypes infested with fungi shared 32 up and 28 down-regulated genes with plants stressed by all abiotic stresses (Fungi S and TOT Abiotics S). Bacteria and all abiotic stress caused the up-regulation of 6 genes and the down-regulation of 24 genes (Bacteria S and TOT Abiotics S). When compared with all abiotic stress, the virus and pest group showed few common DEGs (3 up and 1 down-regulated). However, due to the extremely high number of potential comparisons, in this study, we focused on genes having the same expression patterns.

To this aim, DEGs of drought-resistant genotype (the only abiotic-stress resistant genotype) were used for comparisons against resistant genotypes to biotic stresses (*P. infestans, C. fulvum, TSWV*, and *R. solanacearum*). Results showed that the

fungi group shared several activated and repressed genes with drought stress. The highest number of shared DEGs emerged from *P. infestans* and drought-resistant genotypes comparison, with 314 up and 669 down-regulated genes. However, *C. fulvum* and drought-resistant genotypes also shared many DEGs (345 up and 460 down-regulated genes). On the other hand, tomato genotypes resistant to *R. solanacearum* and drought showed a lower number of common DEGs (47 up and 134 down), while genotypes resistant to *TSWV* and drought only share 7 up and 14 down-regulated genes.

3.2.7 Cellular response during both biotic and abiotic stress

As a first step, we looked at changes in metabolic regulation in plants exposed to biotic or abiotic stresses (Figure 18).

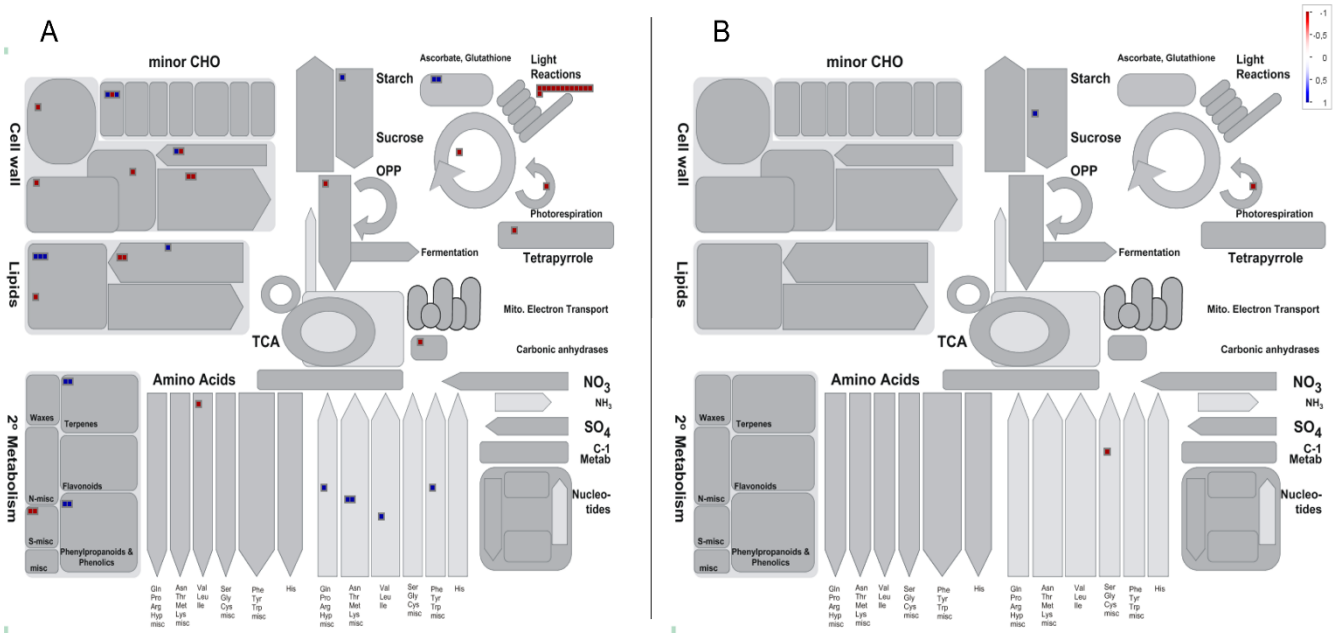


Figure 18. DEGs of stressed-susceptible genotypes involved in cellular metabolism: A) abiotic stress; B) biotic stress. Red= down-regulated genes, Blue= up-regulated genes.

As expected, plants showed a general photosynthetic activity and photorespiration down-regulation, with the abiotic stresses showing a higher number of DEGs (Figure 18A).

Moreover, abiotic stresses induced the down-regulation of cell wall biosynthesis processes. By contrast, several phenolic compounds and genes for synthesizing terpenes and amino acids were up-regulated (Solyc04g015100, Solyc03g007960). DEGs shared by biotic stress showed the down-regulation of an amino methyltransferase (Solyc02g080810) involved in glycine breakdown that takes part in the photorespiration process (Figure 18B).

An exploration of the most important metabolic processes involved in photosynthesis and chloroplast functions was conducted to explore further the cellular differences between biotic and abiotic stress responses (Figure 19).

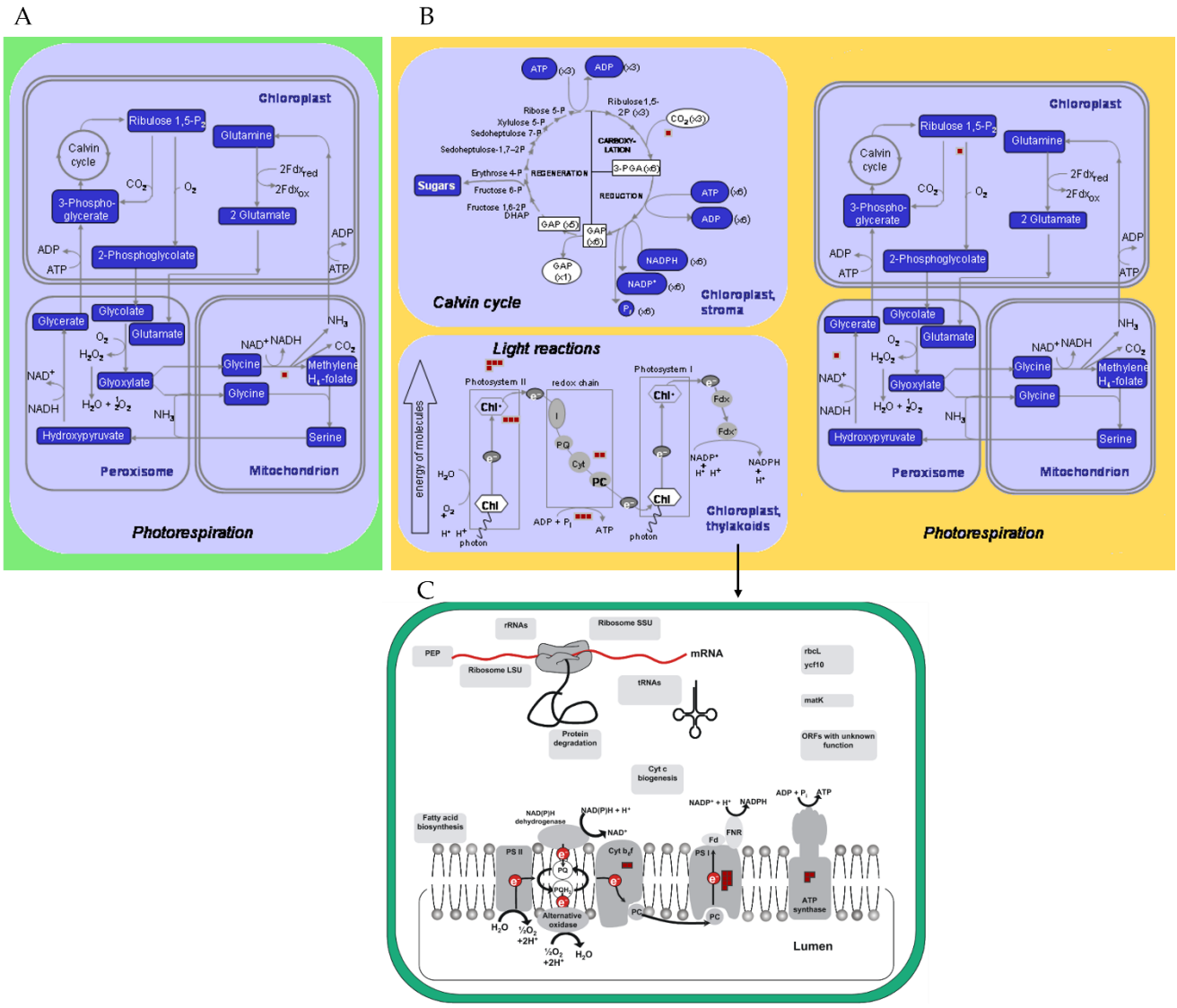


Figure 19. Photosynthesis and respiration pathways. A) Biotic stress; B) Abiotic stress; C) Detail of chloroplast DEGs during abiotic stress. Red= down-regulated genes.

A general photosynthetic and photorespiratory down-regulation was observed in plants challenged by abiotic stress (Figure 19B). Several genes related to photosynthesis light reaction and energy production, such as the two chloroplastic RuBisCO (Solyc02g085950 and Solyc02g085950), resulted down-regulated in susceptible genotypes during abiotic stress (Figure 19B,C).

3.2.8 Differentially regulated pathways during biotic and abiotic stress: plant signaling and cell wall modifications

Variation in expression values of stress recognition and signaling genes is crucial in both biotic and abiotic stress responses. RLKs and genes involved in cell wall biosynthesis expressed in the three biotic stress subgroups for susceptible genotypes (fungi, bacteria and, virus and pest) were compared against datasets of common genes expressed in abiotic stresses (Table 13). We found three common down-regulated genes between bacteria and abiotic stress, taking part in RLKs regulation during the susceptible response (Solyc03g043770, Solyc04g050170, and Solyc04g081590).

By contrast, focusing on DEGs expressed in resistant genotypes to biotic stress (resistant to: *C.fulvum*, *TSWV*, *P.infestans*, *Tuta absoluta,* and *R.solanacearum*) and abiotic stress (resistant to drought), several common DEGs were found (Figure 20, and Table 13). S-locus proteins were up-regulated during drought and *C. fulvum, P. infestans,* and *R. solanacearum* infections. In particular, an S-locus gene (Solyc03g078360) was DE in all the datasets, while Solyc02g079710 resulted up-regulated during the combination of drought and *C. fulvum* and drought and *P. infestans* (both of them belonging to fungi's group). Another S-locus (Solyc08g076050) was up-regulated during *R. solanacearum* and drought stress. Two genes encoding for an Exstensin-protein and a DUF26 receptor (Solyc01g098740 and Solyc09g090680) were down-regulated during *P. infestans* and *C. fulvum* and drought stresses (Figure 20B).

As shown in Figure 20B, several genes encoding for LRR proteins were down-regulated during drought and biotic stress combination, except for Solyc06g006020 (encoding for *FLS2*). Thus, this gene could have a crucial role in *P.infestans* stress resistance and could also be involved in drought response.

Our study also highlighted important differences in cell wall biosynthesis (Figure 20A). The up-regulation of solyc08g082440 (UDP-glucose 4-epimerase) interconverting UDP-glucose and UDP-galactose and the down-regulation of solyc09g092330 in galacturonic acid synthesis were observed in drought, and *P.infestans* stresses, along with the down-regulation of Solyc03g096730, involved in cell wall carbohydrate biosynthesis and protein glycosylation. On the other hand, during *R. solanacearum* and drought stress, there was a down-regulation of a gene participating in D-xylose synthesis (Solyc05g054590, *UXS6*). A gene for Myo-inositol oxygenase (Solyc06g062430) was up-regulated during drought, and *T. absoluta* and

drought and *P. infestans* stresses, while Solyc10g005400 was down-regulated during *P. infestans*, *C. fulvum*, and drought stresses. Finally, *TSWV* and drought-resistant genotypes did not show any common DEGs involved in cell wall biosynthesis, while *Tuta absoluta* and drought did not share common responsive genes encoding for cell wall precursors or RLKs.

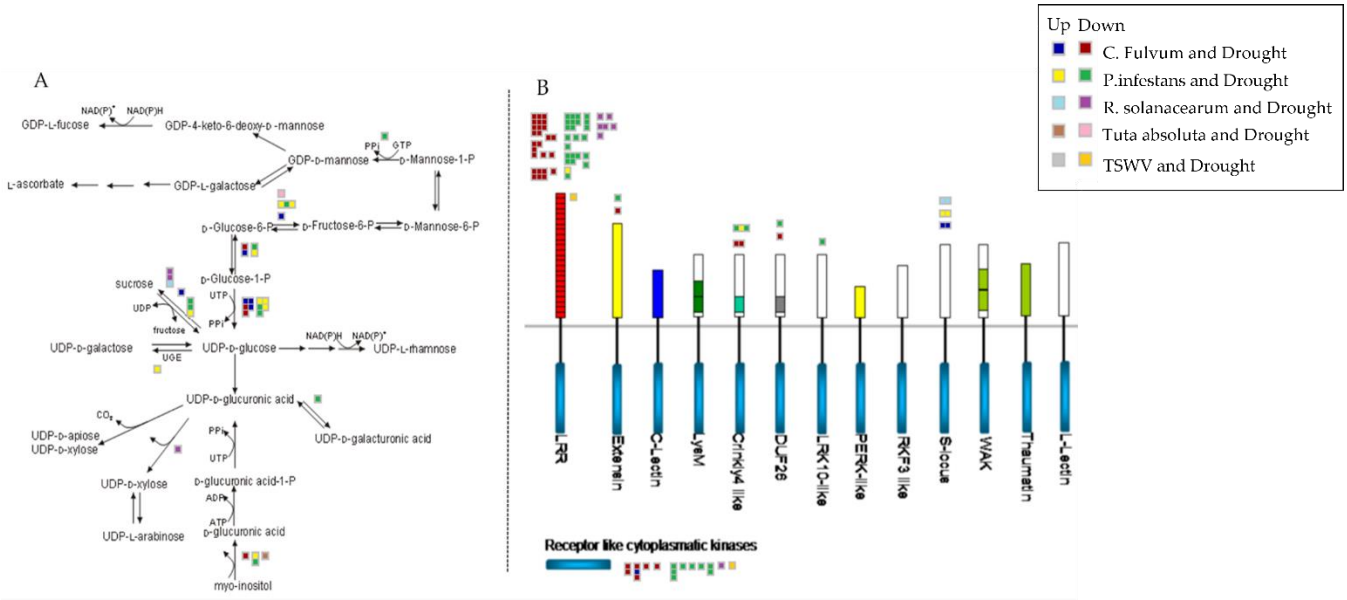


Figure 20. Common DEGs induced by drought and biotic stress in resistant genotypes; A) Cell wall precursors pathway; B) Receptor-like kinases

3.2.9 Identification of DEGs involved in response to multiple stress: a focus on genes involved in drought, salt, and pathogens response

In this study, we looked for genes involved in responsive pathways against different tomato stresses. To simplify the identification of key genes involved in response to multiple stress, we evaluated datasets of shared DEGs induced in susceptible and resistant genotypes during various stress (comparing 13 different groups), as reported in Table 14.

Table 14. List of compared datasets and their relative number of DEGs. S= Susceptible genotypes and R= Resistant genotypes.

Group of stress	Number of DEGs
Fungi (S)	1473
Bacteria (S)	1487
Virus and Pest (S)	361
Total Biotics (S)	9
Low temperature and Oxidation (S)	2692
Drought and Salt (S)	691
Total abiotics (s)	258
C fulvum and Drought (R)	805
TSWV and Drought (R)	21
R solanacearum and Drought (R)	181
Tuta absoluta and Drought (R)	131
P infestans and Drought (R)	983
Total Biotics (R)	9

Firstly, the attention was focused on susceptible genotypes to fungi, bacteria, virus, pest, drought, and salt stresses. As shown in Figure 22, 118 common DEGs were identified as induced by fungi, drought, and salt stress (Figure 22A). Bacteria, drought, and salt stresses induced 98 common DEGs in susceptible genotypes (Figure 22B). Virus, pest, drought, and Salt stress shared 28 DEGs (Figure 23). In some cases, different genes were DE during several stresses (Figure 22, 23, 24, 25).

Later, we compared DEGs induced by resistant-stressed genotypes (Figure 24A, B). We used datasets of common DEGs induced by resistant genotypes to *R. solanacearum*, *P. infestans,* and *C. fulvum* and compared them separately with the dataset of the drought-resistant genotype. The resistant datasets comparison allowed the identification of 106 DEGs involved in *R. solanacearum*, *P. infestans* and, drought

response. In addition, we found that 47 DEGs were commonly induced in all datasets (*R. solanacearum*, *P. infestans*, *C fulvum*, and drought). Finally, comparing DEGs induced in resistant genotypes during fungi infections with those induced by all abiotic stress, we identified 88 common DEGs (Figure 25).

With the purpose to select and characterize in detail the most interesting genes involved in response to multiple stress, we applied two different filtering criteria:

1) We selected genes included in at least 6 datasets. For example, Solyc12g009650 (*HyPRPI*) was down-regulated during fungi, total abiotic, low temperatures, oxidation, drought, salt stresses, and in resistant genotypes to *C. fulvum*, *P.infestans*, and drought.
2) A second and more stringent selection was made to identify those genes DE in both resistant and susceptible genotypes. To this scope, we first compared the resistant genotypes to bacteria and fungi (*R. solanacearum* and drought dataset with the *C. fulvum* and drought or the *P.infestans* and drought datasets). Then, the resulting genes were compared to datasets of biotic stressed-susceptible genotypes (fungi, bacteria, virus, and pest). This approach was used to select genes DE during biotic or abiotic stress in both resistant and susceptible genotypes. A graphical scheme of the second selection criteria is reported in Figure 21.

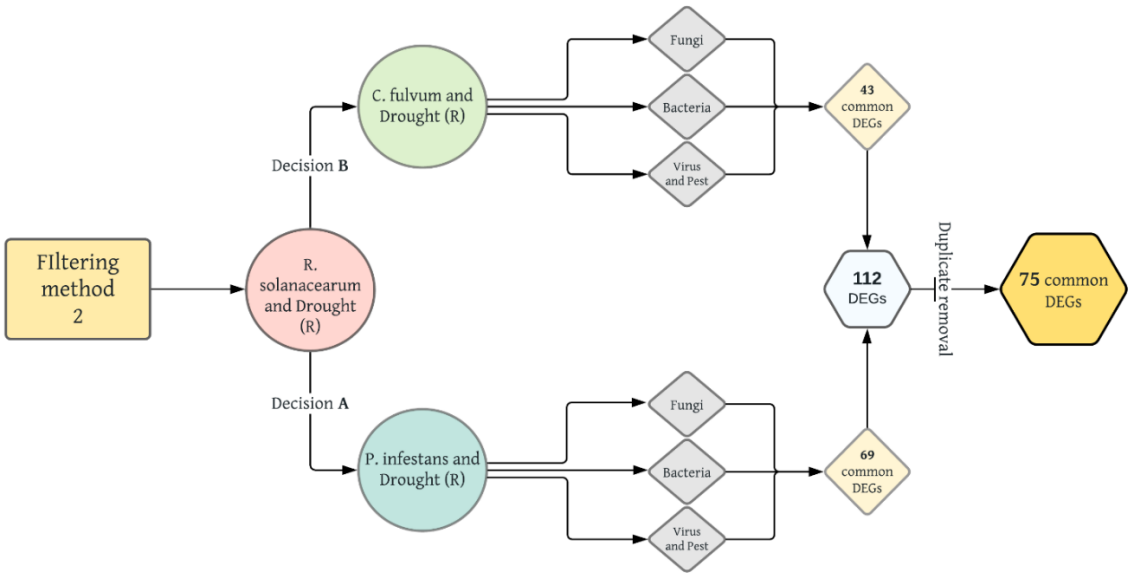


Figure 21. Flowchart of the second screening method for the identification of DEGs involved in multiple stress response

We identified 66 (method 1) and 75 (method 2) DEGs through this approach. Table 15 shows genes deriving from the two selection criteria, with their respective function and role in plant stress response.

Intending to identify genes involved in multiple stress resistance or susceptibility, we studied in detail the function of each gene and, eventually, their previous characterization in other tomato-stressed related studies.

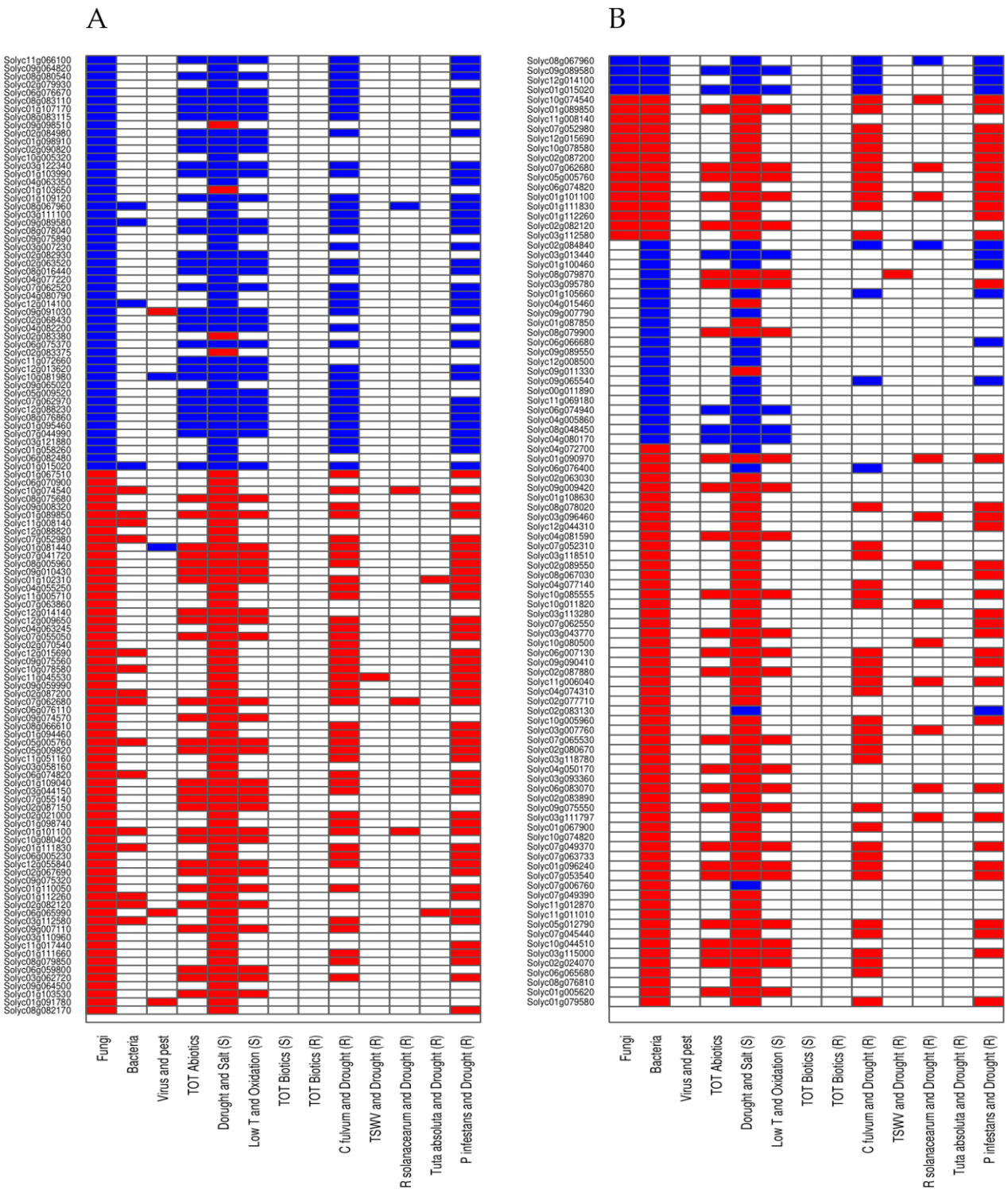


Figure 22. HeatMap of DEGs involved in response to multiple stress A) filtering for common DEGs in fungi and drought and salt datasets; B) filtering for common DEGs in bacteria and drought and salt datasets. Blue= up-regulated and Red= down-regulated genes

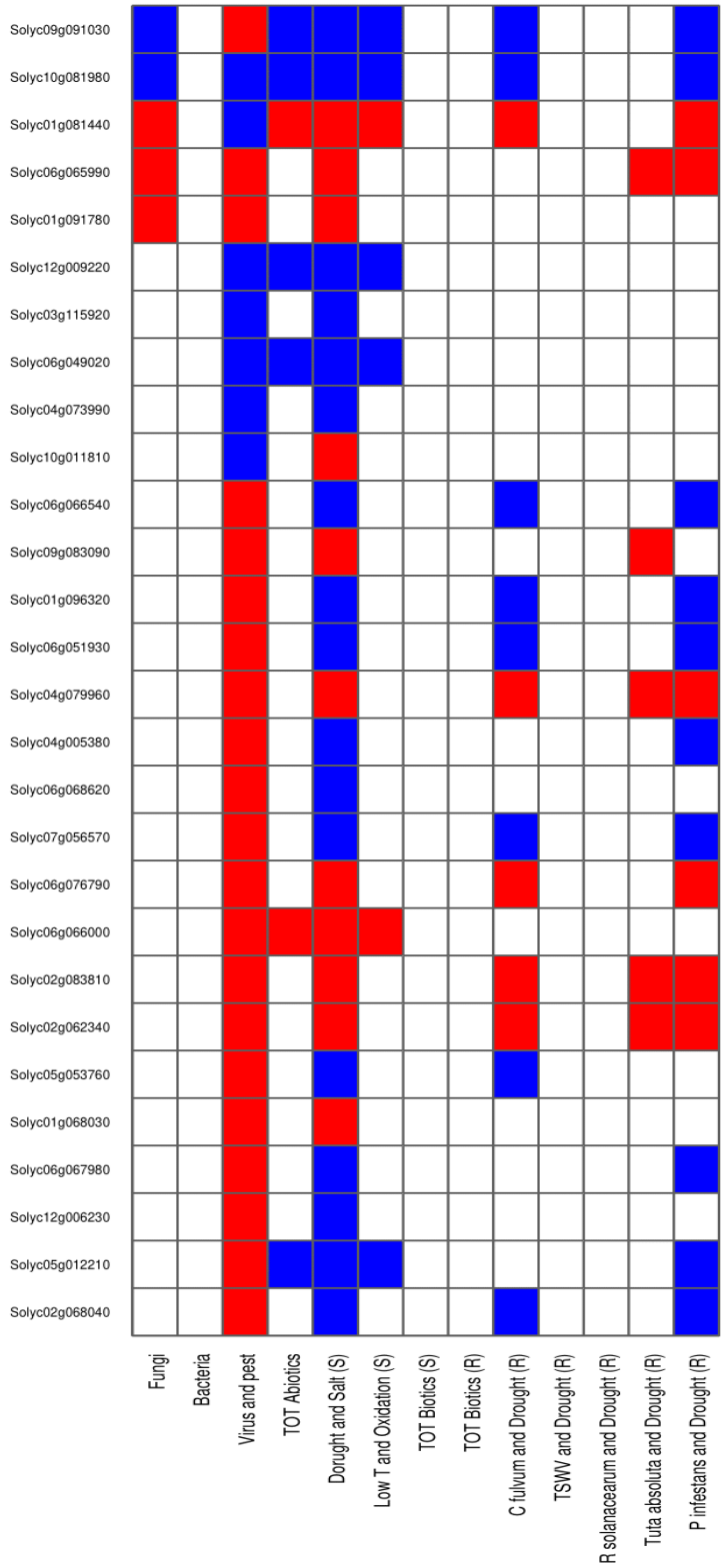


Figure 23. HeatMap of DEGs involved in response to multiple stressors. A filter was applied to find common genes between virus and pest and drought and salt stress datasets. Blue= up-regulated and Red= down-regulated genes

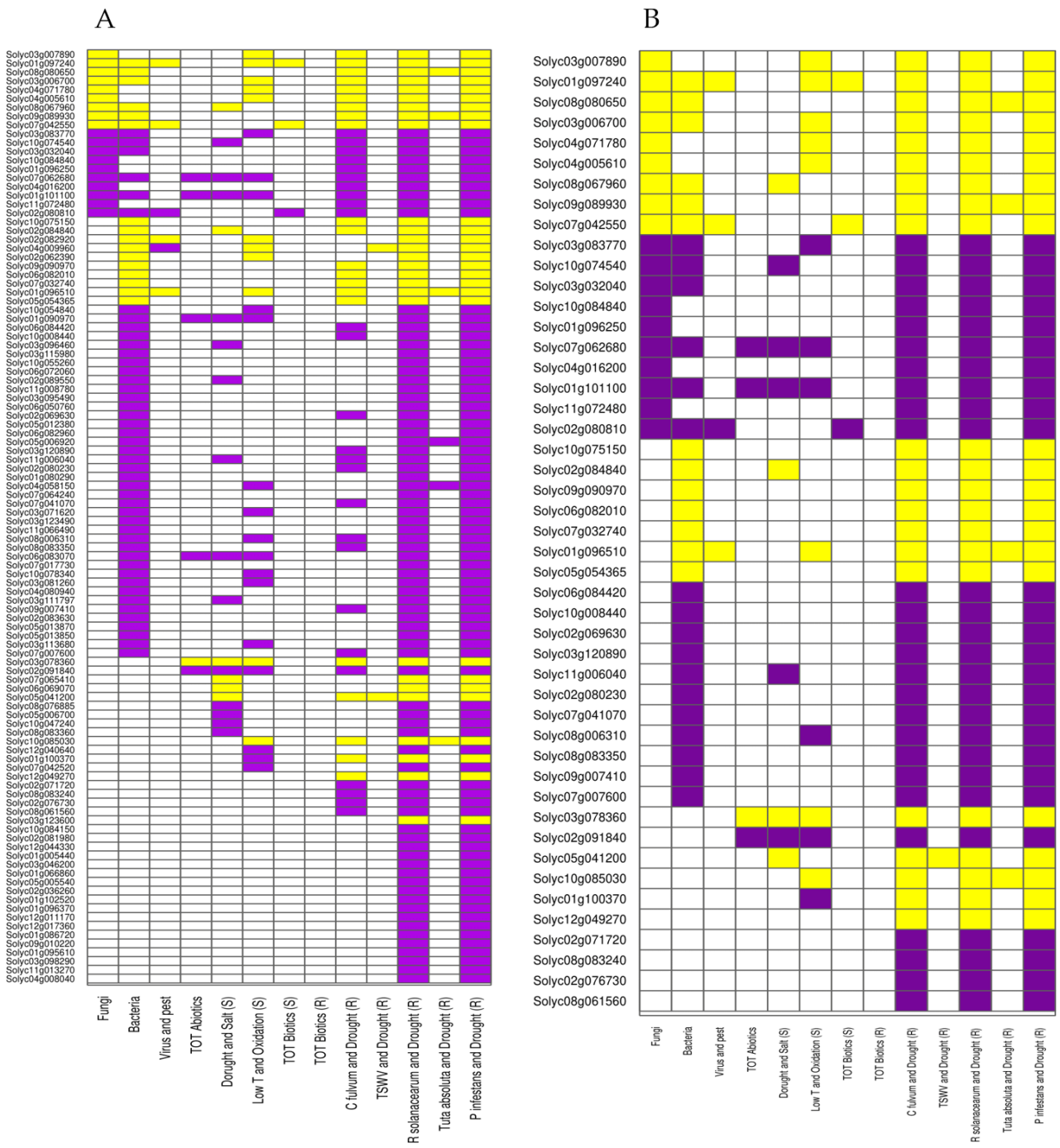


Figure 24. HeatMap of DEGs involved in response to multiple stressors. A) DEGs were filtered to find *R. solanacearum* and Drought (R) and *P. infestans* and Drought (R) common genes. B) DEGs were filtered to find *C. fulvum* and drought (R) and *P. infestans* and drought (R) common genes. Yellow= up-regulated and purple= down-regulated genes

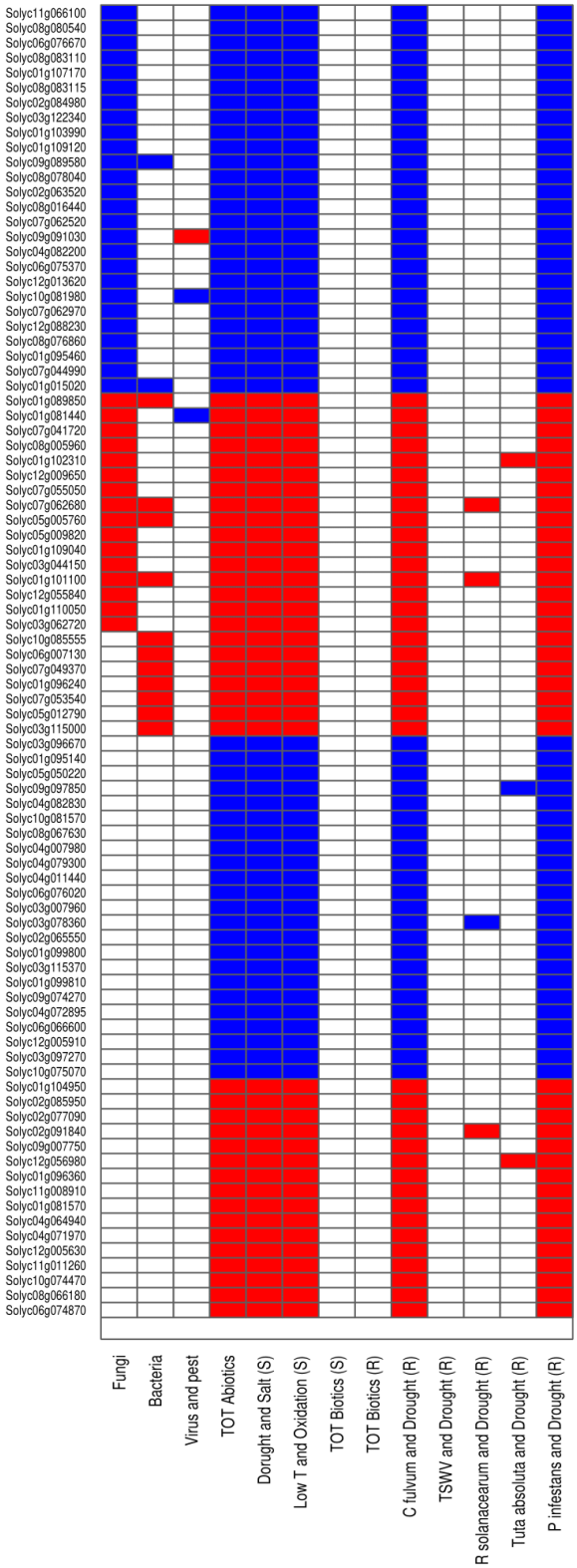


Figure 25. HeatMap of DEGs involved in response to multiple stressors. A filter was applied to show common DEGs among *C. fulvum* and Drought resistant genotypes, *P. infestans,* and drought-resistant genotypes and DEGs in the abiotic stress datasets. Blue= up-regulated and Red= down-regulated genes

Table 15. List of DEGs involved in multiple stress responses resulting from "method 1" and "method 2" screenings (described above). N.F.: not fund in other tomato-stressed studies.

Solyc ID	Function	Found in	Stress response
1st screening method			
Solyc11g066100	heat shock protein 70	(Bineau et al., 2021)	Heat response
Solyc08g080540	HsfB2b	(Cruz-Mendívil et al., 2015)	Chilling tolerance
Solyc06g076670	Serine/Arginine-Rich Protein (Sl-SR46a)	(Rosenkranz et al., 2021)	Heat response
Solyc08g083110	Methionine gamma-synthase	(Jiang et al., 2019)	P. infestans response
Solyc01g107170	Zinc finger protein	(Hu et al., 2019)	Heat response
Solyc08g083115	Cystathionine gamma-synthase		N.F.
Solyc02g084980	Hexosyltransferase	(Abdelkareem et al., 2019)	Induced by JRE3 (JRE3 is induced by salt stress)
Solyc03g122340	lipoxygenase D	(Cervantes-Gámez et al., 2016)	X. campestris response
Solyc01g103990	T-complex protein 11		N.F.
Solyc01g109120	Transducin/WD40	(Outchkourov et al., 2018)	Anthocyanin Biosynthesis
Solyc08g067960	RING finger and CHY zinc finger	(Kulshrestha et al., 2020)	Nematode resistance
Solyc09g089580	2-oxoglutarate and Fe(II) oxygenase (ethylene pathway)	(Cruz-Mendívil et al., 2015)	Chilling response
Solyc08g078040	FAD/NAD(P)-binding oxidoreductase		N.F.
Solyc02g063520	Homeobox-leucine zipper	(P. Gong et al., 2010)	Drought responsive
Solyc08g016440	Polynucleotidyl transferase, ribonuclease H-like		N.F.
Solyc07g062520	Cytochrome P450		N.F.
Solyc09g091030	Beta-amylase 1	(Batista et al., 2020)	Seeds treated with heat
Solyc04g082200	Dehydrin	(Święcicka et al., 2017)and (Gonzalez et al., 2019)	Nematode and chilling response
Solyc06g075370	Dof zinc finger		N.F.
Solyc12g013620	jasmonic acid 2	(Al-Abdallat et al., 2015)	Drought and Salt tolerance
Solyc10g081980	Late embryogenesis abundant (LEA) hydroxyproline-rich		N.F.

Solyc07g062970	protein phosphatase 2C		N.F.
Solyc12g088230	mitochondrial malate dehydrogenase	(H. J. Lee and Seo 2021)	Ca2+ Channels putatively acting as stress Sensors
Solyc08g076860	PLATZ transcription factor		N.F.
Solyc01g095460	TAF-2		N.F.
Solyc07g044990	Transmembrane protein 56		N.F.
Solyc01g015020	PRLI-interacting factor		N.F.
Solyc10g074540	Phosphate-responsive 1		N.F.
Solyc01g089850	cyclinU4_1		N.F.
Solyc01g081440	nuclear factor 1 A-type (DUF1005)		N.F.
Solyc07g041720	Germin-like protein	(Moon et al., 2018)	Drought tolerance
Solyc08g005960	inhibitor/lipid-transfer protein/seed storage 2S albumin		N.F.
Solyc01g102310	GRIP and coiled-coil domain-containing protein	(Ding et al., 2018)	Heat tolerance
Solyc12g009650	Sl proline-rich protein	(Tran et al., 2021; Li et al., 2016; Yeom et al., 2012)	Salt and other Abiotic stress susceptibilities
Solyc07g055050	ATP synthase protein I		N.F.
Solyc07g062680	Lanceolate		N.F.
Solyc05g005760	NHL repeat		N.F.
Solyc05g009820	Hexosyltransferase		N.F.
Solyc01g109040	Cytochrome b6-f complex subunit 7	(Zouari et al., 2014)	Mycorrhizal fungi
Solyc03g044150	Subtilisin-like protease		N.F.
Solyc01g101100	Kinase family protein		N.F.
Solyc12g055840	Glucan endo-1,3-beta-glucosidase		N.F.
Solyc01g110050	Purple acid phosphatase	(Srivastava et al., 2020)	Phosphorus deficiency
Solyc03g062720	Photosynthetic NDH subcomplex B2		N.F.
Solyc01g090970	14 kDa proline-rich protein DC2.15		N.F.
Solyc10g085555	Enolase	(Li et al., 2022)	Response to N deficiency
Solyc06g007130	omega-3 fatty acid desaturase-3	(Zhang et al., 2019)	Tolerance to herbivores *Spodoptera littoralis* and *Heliothis peltigera*
Solyc06g083070	Fimbrin		N.F.

Solyc07g049370	Glucan endo-1,3-beta-glucosidase	(Gong et al., 2020)	salt stress tolerance
Solyc01g096240	plant/protein (AHRD V3.3 *** AT3G61870.1)		N.F.
Solyc07g053540	Fasciclin-like arabinogalactan protein		N.F.
Solyc05g012790	S-acyltransferase		N.F.
Solyc03g115000	Longifolia protein	(Lee et al., 2018)	Putatively role in Cell wall xyloglucan modulation
Solyc01g097240	Pathogenesis-related protein PR-4	(Basim et al., 2021; M. Z. Zhang et al., 2020; Naveed and Ali 2018; Manzo et al., 2016)	*C. michiganensis, B. cinerea, F. oxysporum, and P. parasitica* response
Solyc08g080650	Thaumatin, pathogenesis-related	(Singh et al., 2021; Safavi-Rizi et al., 2020)	Induced by *Chaetomium globosum and* hypoxia
Solyc03g006700	Peroxidase	(Du et al., 2015)	*X. perforans* interaction
Solyc09g089930	ethylene-responsive factor E.2	(Xue et al., 2021)	Phytophthora infestans
Solyc07g042550	Sucrose synthase	(de Ollas et al., 2021)	Soil flooding/ hypoxia
Solyc03g083770	Plant invertase/pectin methylesterase inhibitor	(Singh et al., 2021)	Induced by *Chaetomium globosum*
Solyc02g080810	Aminomethyltransferase		N.F.
Solyc04g009960	L-allo-threonine aldolase	(D'Angelo et al., 2019)	Changing in threonine values
Solyc01g096510	Sigma Factor binding protein 1 (SIB1)	(Aamir et al., 2018)	F. oxysporum
Solyc03g078360	Serine/threonine-protein kinase		N.F.
Solyc02g091840	Serine/threonine-protein kinase		N.F.
Solyc09g097850	Cysteine proteinase inhibitor		N.F.
Solyc12g056980	Ethylene-responsive element		N.F.
2nd screening method			
Solyc03g007890	class 2 small heat shock protein Le-HSP17.6	(Gonzalo et al., 2021)	Heat response
Solyc04g071780	Cytochrome P450	(Manzo 2016)	Tuta absoluta
Solyc04g005610	NAC2	(Al-Abdallat et al., 2015; Balyan et al., 2020)	drought and salinity treatments and heat stress tolerance
Solyc03g032040	Tonoplast monosaccharide transporter 1		N.F.
Solyc10g084840	Pathogenesis-related thaumatin protein		N.F.

Solyc01g096250	oxidoreductases, acting on NADH or NADPH		N.F.
Solyc04g016200	Glycosyltransferase		N.F.
Solyc11g072480	Tetraspanin		N.F.
Solyc10g075150	lipid-transfer protein	(Cruz-Mendívil et al., 2015)	Chilling tolerance
Solyc02g084840	Dhn1 protein	(Cruz-Mendívil et al., 2015; González-Morales et al., 2021)	Chilling tolerance and drought stress
Solyc02g082920	acidic extracellular 26 kD chitinase	(Mazzeo et al., 2014)	F. oxysporum
Solyc02g062390	Dehydrin	(Gonzalo et al., 2021)	Heat response
Solyc09g090970	Major allergen Pru ar 1	(Basim et al., 2021; Mazzeo et al., 2014)	*C. michiganensis and* F. oxysporum
Solyc06g082010	Zinc finger transcription factor 48		N.F.
Solyc07g032740	Aspartate aminotransferase		N.F.
Solyc05g054365	acyl-CoA dehydrogenase		N.F.
Solyc10g054840	X-intrinsic protein 1.1		N.F.
Solyc06g084420	Leucine-rich repeat receptor-like kinase		N.F.
Solyc10g008440	expansin B3		N.F.
Solyc03g096460	wound/stress protein	(Meng et al., 2020)	Salinity
Solyc03g115980	Geranylgeranyl reductase		N.F.
Solyc10g055260	SlLAX5		N.F.
Solyc06g072060	transmembrane protein, putative (DUF 3339)		N.F.
Solyc02g089550	NSP-interacting kinase 1	(Sakamoto et al., 2012)	Begomovirus infection
Solyc11g008780	Acetolactate synthase small subunit		N.F.
Solyc03g095490	Receptor-like kinase		N.F.
Solyc06g050760	Wound-induced protein 1		N.F.
Solyc02g069630	Subtilisin-like protease		N.F.
Solyc05g012380	Glucan endo-1,3-beta-glucosidase-like	(de Ollas et al., 2021)	Soil flooding/ hypoxia
Solyc06g082960	maternal effect embryo arrest protein		N.F.
Solyc05g006920	Major facilitator superfamily protein		N.F.
Solyc03g120890	GATA transcription factor		N.F.
Solyc11g006040	Receptor protein kinase	(Meng et al., 2020)	Salinity

Solyc02g080230	ROP-interactive CRIB		N.F.
Solyc01g080290	AWPM-19-like membrane family protein		N.F.
Solyc04g058150	Metallothionein-like protein	(Myers 2020)	Ralstonia solanacearum
Solyc07g064240	Early nodulin-like protein		N.F.
Solyc07g041070	hydroxyproline-rich glycoprotein		N.F.
Solyc03g071620	Histone H2B		N.F.
Solyc03g123490	Subtilisin-like protease		N.F.
Solyc11g066490	Late embryogenesis abundant (LEA) hydroxyproline-rich		N.F.
Solyc08g006310	Cellulose synthase-like protein		N.F.
Solyc08g083350	Ribosomal protein L11		N.F.
Solyc07g017730	Glucan endo-1,3-beta-glucosidase		N.F.
Solyc10g078340	Stomatal closure-related actin-binding protein 1		N.F.
Solyc03g081260	Subtilisin-like protease		N.F.
Solyc04g080940	WAT1-related protein	(Hanika et al., 2021)	Susceptibility to Vascular Wilt Fungi
Solyc03g111797	Sieve element occlusion a		N.F.
Solyc09g007410	SUN-like protein 25		N.F.
Solyc02g083630	Ascorbate peroxidase	(Landi et al., 2017)	Potentially involved in drought, heat, and cold
Solyc05g013870	Sieve element occlusion c		N.F.
Solyc05g013850	Sieve element occlusion c	(J. Singh et al., 2021)	*Chaetomium globosum*
Solyc03g113680	Microtubule-associated protein TORTIFOLIA1		N.F.
Solyc07g007600	vacuolar-type H+-pyrophosphatase	(Asins et al., 2013)	Putatively involved in salt tolerance
Solyc01g097340	GDP-mannose 3',5'-epimerase	(Li et al., 2019; Mounet-Gilbert et al., 2016)	Oxidative stress tolerance (AsA accumulation) and cell wall development
Solyc04g064760	Carboxypeptidase		N.F.
Solyc10g049580	Arabinogalactan protein	(Myers 2020)	Ralstonia solanacearum (Extensin protein)
Solyc05g054480	Actin		N.F.
Solyc10g011820	Fatty acid/sphingolipid desaturase		N.F.
Solyc03g007760	P-loop containing nucleoside triphosphate hydrolases		N.F.

We found that forty-nine differential expressed genes have been previously characterized in other tomato studies related to stress responses. Therefore, we speculated that those genes could have a role in a larger number of tomato stresses. Furthermore, we identified seventy-seven DEGs never characterized before that could have a role in stress-response. For example, several genes encoding for membrane proteins such as Solyc07g044990 (Transmembrane protein 56), two DUF proteins (Solyc01g081440 and Solyc06g072060), different Kinase protein families (Solyc01g101100, Solyc03g095490, and Solyc06g084420), two Serine/threonine-protein kinase (Solyc03g078360, Solyc02g091840) and an Expansin B3 protein Solyc10g008440 were DE during several biotic and abiotic stress and their role in tolerance or susceptibility could be further investigated. In addition, several cell wall biosynthesis-related proteins as Solyc12g088230 (mitochondrial malate dehydrogenase), Solyc03g115000 (Longifolia protein), Solyc03g083770 (Plant invertase/pectin methylesterase inhibitor), Solyc10g049580 (Arabinogalactan protein) have been found DE in other studies and could be involved in different tomato stress. These genes appeared repressed during drought and salt stress in our work and may be involved in plants' water uptake. At the same time, they resulted down-regulated in resistant genotypes during *C. fulvum* and *P. infestans* infections, but also during bacteria, low temperatures, and oxidation stresses (Figure 22B). Thus, our results provided a priority list of biotic and abiotic responsive genes, which could be further investigated with biotechnological approaches and then used in genetic engineering programs.

3.3 Discussion

3.3.1 Transcriptomic analysis find out multiple stress-responsive genes

Transcriptomics studies allowed us to take a picture of cellular activities involved in the intricate process of the plant stress response. The identification of DEGs in response to particular stress let us to better understand the complex mechanisms that underpin plant defense processes. To shed light on genes differentially expressed during various tomato stress interactions, we analyzed the response of tomato plants exposed to 12 different stresses, using publicly available data obtained with different Illumina sequencing platforms. The number of DEGs obtained in our analysis was similar to those described in the original papers (Fukuhara et al., 2019; Padmanabhan et al., 2019; French et al., 2017), except for the analysis conducted by Zhao et al. (2019) on *C. fulvum*-tomato interaction. In this case, we obtained about twice the number of DEGs resulting from the original paper, probably because a different pipeline was used. Indeed, the filtering and the DEGs detection method differed from our study. For example, DEGs identification in Zhao et al., (2019) study was conducted using the NOIseq algorithm and the noisy distribution model, while we used DESeq2 as DEGs detection method. Furthermore, in the original paper, DEGs were filtered, cutting off those genes with a log2Ratio<1, while in our work, DEGs were not filtered to avoid losses of genes with low expression values.

The analysis conducted on tomatoes exposed to abiotic stress displayed slight differences in the number of DEGs in drought and low-temperature datasets compared with those obtained by Zhou et al. (2019). A double number of DEGs was obtained using our pipeline on RNA-seq reads of tomatoes exposed to salt and oxidative stress, compared to Keshishian et al. (2018). However, such authors employed more stringent criteria to select differential expressed genes. A standard pipeline was used to analyze all downloaded transcriptomic raw data in our study. Therefore, the differences observed in the number of DEGs among different datasets highlighted activated and repressed genes in each experiment, except for differences in sequencing procedures.

3.3.2 Basis of tomato response during biotic stress

We analyzed transcriptomic data of susceptible and resistant plants exposed to seven of the most common tomato pathogens to find out shared DEGs during different tomatoes-biotic stress responses. Transcriptomic data analysis allowed us to shed light on tomato basal response to pathogens. In particular, comparing datasets of DEGs in response to biotic stress, we obtained three lists of common DEGs induced in susceptible genotypes to fungi, bacteria, and the virus and pest. Thus, we identified responsive tomato networks activated during different tomato pathogens interactions.

3.3.2.1 Common DEGs in biotic stress susceptible genotypes

Common genes responsive to fungi, bacteria, the virus, and the pest allowed us to find fundamental genes involved in the overall biotic stress response. Nine DEGs in all the susceptible genotypes to biotic stress were identified. Notably, almost all of them were known as stress-responsive markers. In particular, susceptible genotypes showed the up-regulation of two PR-genes (Solyc01g097240 and Solyc07g005100) expressed in response to several pathogens in resistant and susceptible genotypes (Basim et al., 2021; Zhang et al., 2020). Elicitors and phytohormones can induce PR-genes expression, often associated with plant SAR (systemic acquired resistance) or HR (hypersensitive response). Hormones such as ethylene and jasmonate induced the expression of PR-4 (Solyc01g097240) and Chitinase-lysozyme (Solyc07g005100) genes (Jain and Khurana, 2018). The up-regulation of the PR-4 gene (Solyc01g097240) was also found in a tomato susceptible genotype infected with *P. parasitica* (Naveed and Ali, 2018). It is interesting to note the up-regulation of a Chitinase (Solyc07g005100) in all three biotic stress groups. This enzyme catalyzed the reaction of hydrolysis of pathogens chitin and resulted strongly up-regulated in *S. habrochaites*, *S. arcanum*, and *S. lycopersicum* during nematode infections (Kulshrestha et al., 2020). Therefore, plants may activate these genes as a general basal response during biotic stress.

Susceptible plants attempted to respond using all the possible defense mechanisms. Therefore, the up-regulation of two genes encoding for a Cytochrome P450 and a Glycosyltransferase (Solyc10g083690 and Solyc03g078490) have a clear role in the cell detoxification process. In particular, cytochromes P450 could be involved O^2 reduction and in multiple stress signaling pathways (Pandian et al., 2020), while the UDP-Glycosyltransferases (UGTs) were involved in Cytokinin (CK) homeostasis, and

their up-regulation could affect the senescence processes (Marchetti et al., 2018). Solyc03g078490 also resulted up-regulated in tomato during salt and oxidative stress treatments (Keshishian et al., 2018)

The up-regulation of a protein phosphatase 2C (Solyc10g084410) could negatively affect the ABA signaling (an essential hormone in abiotic stress response) phosphorylating proximity proteins such as *SnRK2s* and reducing the MAPK activity and thus controlling the hormone balance during biotic stress (Saini et al., 2020). A *Rab6A* gene (Solyc05g051570) reported as involved in vesicle intracellular trafficking during stress response (Rosquete and Drakakaki, 2018; Akhmanova and Noordstra, 2017) was also activated. Notably, in a recent study, Huang et al. (2020) observed that *Rab6a* allowed virus movement in *Nicotiana benthamiana* infected with *Bamboo mosaic virus* (*BaMV*).

In addition, we found the up-regulation of a gene involved in sugar metabolism *SUS3* (Solyc07g042550) and a methyltransferase (Solyc04g040180). *SUS3* is necessary to transform sucrose into fructose and diphosphate glucose (UDP-G). The silencing of *SUS3* in cucumber caused a sensitive phenotype to flood (Wang et al., 2014). At the same time, Takehara et al. (2018) showed the role of *SUS3* in heat stress response in rice. These findings confirmed that *SUS3* is an essential gene involved in different stress responses. On the other hand, Solyc04g040180 (SAM-MT gene) is involved in SA metabolism and in small molecules methylation (Alseekh et al., 2020). Finally, a gene (Solyc02g080810) was found down-regulated in all biotic stress datasets. This gene resulted involved in nitrogen metabolism (Thomas et al., 2021), and its down-regulation might be related to the reduction of photosynthetic activity.

Our comparative analysis highlighted that all susceptible genotypes react to biotic stress with a general activation of defensive proteins by modulating hormone signaling in favor of ethylene and jasmonic acid. The down-regulation of ABA signaling may contribute to this process. Furthermore, susceptible genotypes also showed the up-regulation of genes involved in oxidation response, vesicle trafficking, sugar metabolism, and reduction in photosynthetic activity.

3.3.2.2 Focus on pathway regulation in biotic stressed susceptible genotypes

Plant stress recognition starts at the cell wall or membrane level, and the generated signals reach small proteins, ions, or hormone receptors, that decode and translate the signals, increasing the number of bioactive molecules near the cellular space and thus causing stress recognition. These changes activate the mitogen protein kinases (MAPKs) and the calcium-dependent protein kinases (CDPKs) cascades that can transmit the signal through the cell after phosphorylation (Cappetta et al., 2020). Once the signals reach the nucleus, TFs are activated or repressed with a subsequent differentially regulation of gene expression (Erpen et al., 2018). Hormone regulation, signaling, secondary metabolism, and PR-proteins expression are vital for these complex response mechanisms. Our analysis attempted to identify differences in susceptible genotypes in response to fungi, bacteria, the virus, and the pest. In particular, the expression of TFs was particularly stimulated by fungi and bacteria. Bacterial infections showed the up-regulation of ERF and MYB transcription factors and were down-regulated during fungi infections.

Interestingly, ERFs can bind to GCC-box in promoter regions of several PR proteins (such as PR-1, PR-2, PR-3, and PR-5) (Zarei et al., 2011). Hence, the up-regulation of ERFs could explain the higher number of up-regulated PR proteins during bacterial infections. On the other side, MYBs could be important for resistance to *Fusarium oxysporum* and *Alternaria alternata* (Liu et al., 2016) and the down-regulation of several MYBs revealed in our study, may be related to plant susceptibility. Furthermore, in our analysis, all susceptible genotypes to biotic stress down-regulated DOFs and up-regulated WRKYs.

In our datasets, genes related to SA and JA were up-regulated in response to bacteria, while during fungi infections, genes involved in SA pathways were down-regulated, and genes for ABA synthesis were up-regulated. Huang et al. (2020) found that in *Capsicum annuum* infected with *Ralstonia solanacearum*, a gene needed for resistance (*CabZIP63*) was promoted by the up-regulation of SA and the down-regulation of JA. Another study, proposed that SA enhanced *FOL* tolerance while increased ethylene (ET) levels led to plant susceptibility (Di et al., 2017). Enhancing SA content through UV-light treatments in JA deficient cultivars increased plants'

resistance to *P.syringae* (Escobar Bravo et al., 2019). In our study, susceptible genotypes to *TSWV* and *Tuta absoluta* (Virus and pest group) showed the activation of JA and suppression of the brassinosteroids pathway. According to Pan et al. (2018), brassinosteroids promote susceptibility to a rice insect (*Nilaparvata lugens*) interacting with JA and SA. Moreover, fungi showed the up-regulation of several genes encoding for glutathione-S-transferases, suggesting a role in tomato-fungi susceptibility, as reported in other experiments reviewed by Gullner et al. (2018).

The regulation of mechanisms involved in cell wall biosynthesis and membrane signaling showed differences in the expression of RLKs. These proteins carry an amino-acidic extracellular domain and a carboxyl-terminal intracellular domain. They are responsible for the first interaction between extracellular information and cell response to stimuli (Jose et al., 2020). In particular, several S-locus proteins resulted up-regulated in bacterial infection. Interestingly, our study showed that an orthologs of Arabidopsis gene *CBRLK1* (Solyc02g079540), a negative regulator of plant disease resistance (Kim et al., 2009), was up-regulated in susceptible genotypes to bacteria. Intriguingly, bacteria also showed the up-regulation of a WAK-like receptor (Solyc04g079710, *SlWAKL5*). These proteins were differentially regulated in response to pathogens and in pectin-derived fragments recognition (Rosli et al., 2013). All three of the susceptible tomato lines interacting with fungi showed the down-regulation of an Extensin like-protein (Solyc01g098740) not yet characterized, suggesting a new possible candidate involved in tomato tolerance or susceptibility to bacteria. Virus and pest showed few activated and repressed RLKs compared with the two other groups of stress, probably because of a smaller number of total DEGs. However, this group showed the down-regulation of an L-lectin gene (Solyc03g080060), while this gene class resulted up-regulated in the bacteria and fungi groups. Interestingly, the non-structural protein NSs of the *TSWV* virus needed to suppress the host RNA silencing machinery to promote disease interact with a gene strictly related to Solyc03g080060 (Zhai et al., 2021).

As discussed above, cell walls may be subjected to relevant changes during plant response to stress. In our study, genes producing xyloglucan precursors (involved in primary cell wall formation) were up-regulated during fungi infections, while bacterial infections induced genes involved in pectin production. The up-regulation of genes for xylose synthesis during fungi infections can often lead to

enhanced plant resistance (Miedes et al., 2014). Interestingly, two tomato genes, Solyc09g075120 and Solyc11g066150, similar to *Arabidopsis* UXS genes (at3g62830 and at3g46440), resulted up-regulated during fungi infections. In Arabidopsis, during *P.syringae* infections, repression of a gene belonging to the GAEs family, involved in pectin synthesis, leads to plant susceptibility (Bethke et al., 2015). However, we found the up-regulation of Solyc08g079440 (GAE family gene) in tomato susceptible genotypes during bacterial infections. Indeed, the modification of pectin levels can lead to pathogen resistance or susceptibility (Bacete et al., 2018).

3.3.2.3 Differentially regulated pathways in resistant genotypes to biotic stress

In our comparative analysis, five resistant genotypes were analyzed for their resistance response against tomato pathogens. In particular, resistance to *C. fulvum* and *TSWV* was due to the presence of two R-genes. Two transgenic genotypes resisted *P.infestans* infection because of suppressing miR482 and miR2118b, two negative regulators of NLRs. Tolerance to *R. solanacearum* was given by roots auxin and defensive pathways activation. Finally, tolerance to *Tuta abosluta* was the result of phenotypical barriers.

The scope of this analysis was to understand those mechanisms of basal response to biotic stress shared by resistant/tolerant genotypes. In total, sixty-three DEGs were shared in infected-resistant genotypes. Among them, three had the same expression trend. A Pathogen induced protein (PI-1) was up-regulated in all the resistant genotypes. This protein is known to take part in basal defense and systemic resistance induction. PI-1 is among the most induced genes in a mutant for the NLR gene *SlNRC4a* (Pizarro et al., 2020). In general, this gene seems to be up-regulated by JA, and its high expression could be involved in basal plant resistance mechanisms. A mono-oxygenase gene (Solyc02g071475), identified as a general defensive gene (López et al., 2021), was found up-regulated in all resistant genotypes. However, most shared DEGs showed different expression trends (up or down-regulated) under different stress; nevertheless, we assumed that these genes played an important role in driving plant-resistant response. Most of the DEGs were involved in hormones, secondary metabolism, and signaling pathways (Zaynab et al., 2018). In particular, we found a different regulation of several dihydro-flavonols, which dissociating in phenolate ions

can interact with proteins, changing their structures and trigging to protein inactivation.

Interestingly, most genes were up-regulated in different resistant genotypes except in response to *TSWV*. Resistant tomatoes infected with the *TSWV* showed the down-regulation of two genes involved in lignin biosynthesis (Solyc12g045020 and Solyc12g042480), which were up-regulated in all the other resistant genotypes. These genes resulted essential in anthocyanin production, and their down-regulation could affect anthocyanin and flavonol pathways during *TSWV* infections, activating complex defense mechanisms in tomato-resistant genotypes (Maruta et al., 2014).

As expected, RLKs displayed an active role in resistant genotypes, in particular, Solyc08g061560 (*ERECTA* gene), previously characterized to confer resistance to different pathogens (Zanten et al., 2009; Llorente et al., 2005; Godiard et al., 2003) were up-regulated in *TSWV* infections but down-regulated during the other pathogen-interactions. Furthermore, our analysis showed that Solyc01g102850 and Solyc07g008620 (two RLPs) were DE in stressed-resistant genotypes. In particular, Solyc01g102850 resulted up-regulated during *TSWV* and *R. solanacearum* infections and down-regulated in susceptible genotypes infected with fungi and in the other resistant tomatoes, suggesting an involvement in both *TSWV* and *R. solanacearum* resistance. In contrast, Solyc07g008620 resulted up-regulated in all the datasets except for plants infected with *TSWV*. Solyc07g008620 was also up-regulated in resistant genotypes infected with *TYLCV* and in susceptible genotypes to *P. syringae* (Kang and Yeom, 2018), suggesting a specific implication in plant virus response. Resistant plants infected with *TSWV* showed the down-regulation of two genes for JA and ET synthesis, which were up-regulated in the other resistant genotypes. However, the activation of systemic resistance during *TSWV* infections is promoted by increasing JA (Zhao et al., 2019). Divergent fine-tuned regulation of secondary metabolism, hormone compounds, and PR-proteins in resistant genotype responses was highlighted.

3.3.3 Basis of tomato response to abiotic stress

Exploring tomato response to abiotic stress may help identify cell requirements for adapting tomato plants to adverse environmental factors. Our study evaluated transcriptomic response to four abiotic stresses: drought, low temperature, salinity, and oxidation. Intending to assess the basal mechanisms activated in stressed plants

in response to abiotic stress, we searched for the common genes activated or repressed in all the experiments, finding 104 up and 154 down-regulated genes. Furthermore, to find out shared genes between groups of abiotic stress, we compared drought and salt-stressed and low temperature stress with oxidative stress. This choice was made considering that salt stress often co-occurs with drought in arid or semi-arid areas. Plants activate similar gene patterns to adapt to drought and salt (Ors et al., 2021; Li et al., 2018; Liu et al., 2011). Recently, several studies showed that combined effects of salt and drought stress increased detrimental effects given by the stress separately in spinach and *B. oleracea* (Sahin et al., 2018; Ors and Suarez, 2017). On the other side, plants react to low temperatures using plenty of mechanisms including the increase of reactive oxygen species (ROS), which are fundamental in plant stress signaling (Willems et al., 2016). Therefore, we grouped low temperature and oxidative stress datasets to find out common genes involved in stress response. Our analysis showed that salinity and drought shared 279 up and 412 down-regulated genes while low temperature and oxidative stress had 1523 up and 1169 common down-regulated genes, respectively.

3.3.3.1 Pathways involved in tomato response to abiotic stress

Datasets of common DEGs were used for identifying convergent or divergent activated pathways during abiotic stress. In particular, we focused on hormone signaling, TFs, RLKs, and cell wall-related processes. Comparing all abiotic stress datasets, we found two RLK proteins (an S-locus; Solyc03g078360, and an LRR protein; Solyc03g006500) differentially expressed, reported as involved in several abiotic stress perceptions (Ye et al., 2017), two genes involved in ABA and IBA, signaling were down-regulated while two genes for JA and three genes involved in ET pathway were up-regulated. Knowing the importance of ABA signaling in response to abiotic stress, the observed down-regulation of some ABA-responsive genes may reflect a signal attenuation mechanism rather than tolerance or susceptibility mechanisms. Susceptible genotypes showed the up-regulation of different TFs, known to be involved in the activation of ABA response: two MYB (*SlMYB102*; *SlMYB31*), two bZIP, a WRKY, a NAC (Khan et al., 2018; Lee et al., 2015). In particular, *SlMYB102* (Solyc02g079280) has been reported as involved in salt and low-temperature tolerance in tomatoes (Wang et al., 2020; Zhang et al., 2020), and *SlMYB31* (Solyc03g116100) gene

as a positive regulator of ABA signaling, interacting with ABA repressor genes (Lee et al., 2019). However, despite the up-regulation of several TFs involved in ABA response, we did not find common ABA-activated DEGs comparing all the abiotic-stressed tomato datasets. Hence, we supposed that a complex regulation system might suppress ABA hormone signaling and synthesis.

Analyzing the drought-salt stress sub-category, several common genes for ABA, ET, and JA biosynthesis resulted up-regulated, while brassinosteroids (BA) and gibberellic acid (GA) genes were down-regulated. These findings were in accordance with several studies in which modulation of ABA levels was correlated to drought and salt response (Ma et al., 2019; Hong et al., 2016). However, despite a relevant ABA up-regulation, these genotypes were susceptible to stress. Therefore, the simultaneous up-regulation of genes for JA and ET synthesis could affect plant signaling and the proper activation of defensive genes. It is worth noting that drought and salt stress showed the common down-regulation of a WRKYs (Solyc02g088345; *WRKY3*), an essential gene in salt tolerance in tomato reducing the levels of SA hormone (Hichiri et al., 2017).

Genes involved in pectin biosynthesis genes (*GAE3* and *GAE6*) were down-regulated. On the other hand, *UGE1* (Solyc08g080570) was up-regulated. This gene has been reported as induced by several abiotic stresses, and its overexpression confers resistance to salt in *Arabidopsis* (Liu et al., 2007).

In the subset including common DEGs between low temperatures and oxidative stress, SA resulted down-regulated. *Arabidopsis* plants subjected to low temperatures increased the synthesis of endogenous SA, enhancing ROS accumulation, leading to cold sensitivity (Scott et al., 2004; Miura and Tada, 2014). Some studies demonstrated that the provisional application of SA during cold stress might induce plant tolerance (Senantra et al., 2000). Interestingly, common genes between low temperature and oxidative stress showed the up-regulation of Solyc07g049180 (similar to *OsCERK1*), important in plant-pathogen interaction and salt stress (Saijo and Loo, 2019). Thus, Solyc07g049180 may have multiple roles in response to biotic and abiotic stress, including low temperature and oxidation. Another interesting gene down-regulated during low temperatures and oxidative stress was *SlWAK1* (Solyc09g014720). Mutants for this gene compromise pathogen recognition (Zhang et al., 2020) but also were more susceptible to salinity (Meco et al., 2020). Therefore, *SlWAK1* may have a role in plant resistance to both abiotic and biotic

stress. Low temperature and oxidative stress dataset also induced the up-regulation of Solyc02g069580; which encodes a type-II membrane protein that catalyzes 4-epimerization of UDP-D-Xylose to UDP-L-Arabinose, known as *MUR4*. In Arabidopsis, *MUR4* is critical for root elongation and salinity stress tolerance (Zhao et al., 2019).

3.3.4 Analysis of pathways regulation on biotic and abiotic stress

We compared the lists of abiotic and biotics responsive genes to identify a basal plant response during stress interactions. However, the dataset of susceptible genotypes to biotic stress included few genes compared with abiotic stress. Despite this, both lists showed the down-regulation of genes involved in photosynthetic and photorespiratory activities. In addition, abiotic stress showed the down-regulation of processes related to cell wall biosynthesis and the up-regulation of amino acids and secondary metabolism compounds such as phenols and terpenes. According to Silva et al. (2019), mechanisms of basal plant responses during abiotic stress may rely on the up-regulation of genes coding for amino acids. Indeed, Proline could be used as compatible osmolytes, especially during drought stress, while Trp, Phe, Tyr, Ser, and Arg can be used as precursors of secondary metabolites.

Due to the low number of shared genes among all the biotic stress datasets, we compared biotic stress sub-groups (fungi, bacteria, and virus-pest) with abiotic stresses. However, we found few common genes among biotics and abiotic datasets, such as three RLKs involved in abiotic stress and bacteria perception. In particular, an ortholog of *CLV1* (Solyc04g081590, *SlFAB*) resulted down-regulated in both bacteria and total abiotic datasets. Mutants for *CLV1* were more resistant to nematodes (*H. arabidopsidis*) and *R. solanacearum* infections (Hanemian et al., 2016; Replogle et al., 2013; 2011).

Comparing datasets of single resistant genotypes to biotic stress and drought, we obtained five datasets of common genes: *C. fulvum* and drought, *P. infestans* and drought, *TSWV* and drought, *R. solanacearum,* and drought, and *Tuta absoluta* and drought. Notably, we found a gene (Solyc03g078360) up-regulated in all the resistant genotypes infected with *R. solanacearum*, *P. infestans*, *C. fulvum,* and drought. This gene resulted down-regulated in a susceptible tomato line (NC-714) infected with *X.*

perforans (Shi and Panthee, 2020). Thus, we could speculate about its putative role in resistance to different biotic and abiotic stress. In general, it is involved in fls22 (a peptide from flagellin) recognition and PTI activation (Lu et al., 2010). *FLS2* was also reported up-regulated in other drought stress studies (Qi et al., 2018; Haider et al., 2017). In *Arabidopsis, FLS2* increased plant resistance to bacterial infection, but in concomitance with heat stress exposure, the transcription level of *FLS2* decreased, leading to subsequent bacteria susceptibility (Janda et al., 2019). Thus, *FLS2* may have a role in pathogen and drought resistance, but the interaction with other treats such as heat stress should be considered. Moreover, we did not detect the simultaneous up-regulation of its co-receptor (*BAK1*).

Process related to the cell wall in resistant genotypes, in response to biotic and drought stresses, were affected by the up-regulation of Solyc08g082440 (UDP-glucose 4-epimerase), which in *Arabidopsis* regulate the cell wall carbohydrate biosynthesis (Rösti et al., 2007). A *Myo-inositol oxygenase 1* gene (Solyc08g082440) was up-regulated in drought, *Tuta absoluta,* and *P. infestans* resistant genotypes. This gene was also up-regulated during tomato-salt stress response Zhang et al., (2017), suggesting a role in response to different stress.

3.3.4.1 Prioritization of key genes involved in biotic and abiotic stress response

The main goal of our study was the identification of genes involved in response to multiple stressors. For this purpose, we compared thirteen datasets of DEGs from different abiotic and biotic stressed plants. To point out shared DEGs, we applied two filtering methods. With the first selection method, we aimed to identify genes involved in at least 6 lists of different stresses and, the second selection method allowed identifying DEGs shared by both resistant and susceptible genotypes during tomato stresses. A total of one hundred and twenty-six DEGs were identified. Notably, forty-nine DEGs were found in transcriptomic studies related to plant stress (Table 15). This discussion is focused on those genes taking part in cell wall modification and membrane signaling during drought, salinity, fungi, and bacteria responses.

Resistant genotypes to drought, *C. fulvum,* and *R. solanacearum* showed the up-regulation of Solyc02g084980, Solyc08g083110, and Solyc03g122340. Solyc08g083110

was involved in methionine metabolism, which is at the basis of ET biosynthesis, and was suppressed in a transgenic tomato line susceptible to *P. infestans* (Jiang et al., 2019). Furthermore, Solyc02g084980 and Solyc03g122340 (LoxD family), involved in JA response (Abdelkareem et al., 2019; Upadhyay and Mattoo, 2018), were up-regulated. In particular, Solyc02g084980 was induced by JA during salt stress. Hence, these genes could be the base of the resistance process activated in response to different stress. Interestingly, these genes were also DE in susceptible genotypes to fungi and abiotic stress.

Notably, we found that several stress-responsive genes were down-regulated in stressed susceptible and resistant genotypes with different expression levels. A greater silencing level in resistant genotypes could guarantee resistance. For example, Hanika et al. (2021) silencing a susceptibility gene in tomatoes (*SlWATI*), using both RNAi and CRISPR/Cas9 methods, observed that the silencing levels obtained using RNAi were not relevant enough to increase plant resistance. In contrast, a complete gene knockout obtained using CRISPR/Cas9 led to a more robust gene silencing and a subsequent increase in plant-stress resistance. Therefore, a strongly induced gene down-regulation in resistant genotypes could enhance resistance, while susceptible genotypes could not be able to decrease gene expressions levels to the extent of completely silencing their adverse effects. We could not compare DEGs expression levels among experiments, because different experimental designs were used. However, a similar regulation trend in various stress responses was pointed out.

For example, in our dataset, Solyc08g067960 was up-regulated by several resistant plants stressed with *R. solanacearum*, *C. fulvum*, *P. infestans,* and drought but also by susceptible genotypes during fungi, bacteria, drought, and salt interactions. This gene was also identified in a stressed-resistant *S. arcanum* accession (Kulshrestha et al., 2020), supporting the hypothesis of an important role in tomato-stress interaction. Interestingly, a pectin-esterase inhibitor (Solyc03g083770) was down-regulated in susceptible genotypes to fungi, bacteria, low temperature, oxidative stress, and by resistant genotypes to *C. fulvum*, *P. infestans,* and *R. solanacearum*. Singh et al. (2021) showed that this gene was highly induced by *Chaetomium globosum,* a fungus used as a biological control agent against other pathogens, and it is required to prevent cell wall degradation during pathogen attacks.

The up-regulation of a zinc finger TF (Solyc02g063520), revealed in response to drought and biotic stress, was in agreement with Gong et al. (2010), which found it involved in drought response. The up-regulation of a NAC TF (Solyc04g005610) in susceptible genotypes in response to biotic and abiotic stress could be related to the ABA induction of leaf senescence (Asad et al., 2019). However, it was also up-regulated in resistant genotypes. Indeed, it is possible that Solyc04g005610 silencing may induce a delay in plant senescence with a subsequent higher tolerance in plant multiple stress interaction. We also found that Solyc04g082200, involved in ABA metabolism and conferring tolerance to low temperature and drought (Blanchard-Gros et al., 2021; Swięcicka et al., 2017), was up-regulated in tomato resistant genotypes to *C. fulvum*, *P. infestans,* and drought and in susceptible genotypes during abiotic stress and in fungi infections. Furthermore, a NAC TF (*JA2*) resulted up-regulated in both susceptible and resistant genotypes to biotic and abiotic stress. This gene was reported as involved in abiotic stress tolerance by activating the ABA signaling and stomatal closure (Al-Abdallat et al., 2015). Interestingly, a gene coding for a Ca^{2+} channel (Solyc12g088230) resulted up-regulated in both abiotic and biotic stress and, according to different studies, Ca^{2+} signaling can be involved in the activation of plant-stress response (Lee and Seo, 2021).

Solyc12g009650 (*HyPRPI*), a proline-rich protein, was down-regulated in resistant and susceptible genotypes under biotic and abiotic stresses. *HyPRPI* has been described as a susceptibility gene during several abiotic stresses. Interestingly, we noted that several genes identified in the literature as S-genes, such as *HyPRPI* and *WATI* (Hanika et al., 2021; Yang et al., 2018), were down-regulated in both resistant and susceptible genotypes during various plant stress. Therefore, we hypothesized that S-genes were predominantly down-regulated during stress and that the down-regulation levels make the difference in plant susceptibility or resistance.

A Germin like protein (Solyc07g041720), involved in oxalate-oxidase production and cell wall modification during plant stress, resulted repressed by biotic and abiotic stresses, in accordance with a study of Moon et al. (2018), in which Solyc07g041720 was repressed by drought stress. A gene coding for a glucan endo-1,3beta-glucosidase-12 (Solyc07g049370) resulted down-regulated in susceptible genotypes to Bacteria, abiotic stress, and resistant genotypes to *C. fulvum*, *P. infestans,* and drought. This gene is involved in sucrose synthase and accumulation. Gong et al.

(2020) found that Solyc07g049370 was up-regulated in plants treated with a halotolerant *Streptomyces sp.* that enhanced salt tolerance.

The role of peroxidases in plant stress interaction was widely investigated. These genes are involved in antioxidant activity and ROS control (Pandey et al., 2017). Interestingly, we identified two peroxidases (Solyc03g006700 and Solyc02g083630) respectively up and down-regulated in different datasets. Furthermore, Solyc02g083630 was found down-regulated in resistant and susceptible genotypes under biotic stress and drought. Landi et al. (2017) reported that this gene reduced oxidative stress during drought stress. A chitinase defensive protein (Solyc02g082920) was up-regulated during tomato challenge with bacteria, virus, pest, low temperature, and oxidative stress. This gene was also found up-regulated in susceptible genotypes infected with *F. oxisporum* (Mazzeo et al., 2014). An RLK (Solyc11g006040) and a metallothionein (Solyc04g058150) were down-regulated in bacteria, drought, and salt stress in resistant and susceptible genotypes. Meng et al. (2020) reported that the lipoxygenase and the RLK proteins could be involved in the plant's salt stress response, while the metallothionein can have a role in interaction with *R. solanacearum* (Myer et al., 2020).

Interestingly, the *WATI* gene was down-regulated in our dataset. In particular, it was down-regulated during bacterial infections and in resistant-stressed genotypes to *R. solanacearum*, *P. infestans,* and drought. Notably, *WATI*, Solyc04g058150 (metallothionein), and Solyc10g049580 were down-regulated in susceptible and resistant genotypes to biotic and abiotic stress. A common repressed gene in resistant genotypes infected with fungi and bacteria was Solyc07g007600 (down-regulated also in bacteria-susceptible genotypes). This gene is a K^+ pump that plays a fundamental role in homeostasis and Na^+ circulation in plant stress response (Asins et al., 2012; Hauser and Horie, 2010). Furthermore, a gene coding for a GDP mannose (*SlGME1*) involved in cell wall biosynthesis (Mounet-Gilbert et al., 2016) resulted down-regulated in fungi and bacteria susceptible genotypes and in *C. fulvum*, *R. solanacearum,* and Drought resistant genotypes. Our study also identified 77 DEGs involved in different biotic and abiotic stress. Thus, our list of DEGs could be used as a starting point to further genetic characterizations.

3.4 Conclusions

Our comparative analysis allowed us to identify several genes differentially regulated in response to various tomato stress. We identified the basal response activated by susceptible and resistant genotypes during biotic and abiotic stress. Furthermore, we found key genes functioning in the regulation of defensive crosstalk between pathogens and adverse environmental factors. This work provided a list of one hundred and twenty-six DEGs identified in tomatoes exposed to several biotic and abiotic stress. Forty-nine DEGs were found DE in other plant stress-related studies, confirming that they could play a role in processes related to resistance or susceptibility to various stresses. In addition, seventy-seven tomato DEGs involved in biotic and abiotic stress responses, have never been studied before. The most interesting genes identified could be further characterized through genetic engineering approaches to assess their role in plant multiple stress response.

3.5 Materials and methods

3.5.1 Bibliographic research, studies selection, and transcriptomic datasets downloading

Large-scale literature research was performed to find publicly available tomato (Solanum lycopersicum) RNA-seq experiments. To find transcriptomic studies, we used a number of single keywords such as "plant stress," "resistance genes tomato," "tomato stress susceptibility," "Biotic stress tomato," "multiple stress tomato," as well combinations of the above words. Bibliographical research was conducted using both Google Scholar and Scopus search engines. Hundreds of papers evaluating tomato stress response were collected. In particular, thirty tomato transcriptomic studies were evaluated in detail. However, a further screening was made using the following criteria: i) tomato plants were subjected to biotic or abiotic stress; ii) Sequencing was performed with Illumina technology; iii) at least three biological replicates for treatment were used. Hence, twelve studies of tomatoes exposed to different stresses were collected, and raw transcriptomic data were downloaded. For some experiments, samples were sequenced at different time points. A total of 134 samples were analyzed through a common pipeline. Studies included both pair-and and single-end reads.

Transcriptomic studies reported on SRA (Sequence Read Archive) repository of NCBI (https://www.ncbi.nlm.nih.gov/sra) were considered further. SRA accession number was used to access the corresponding raw sequencing data (fastq file). SRR files corresponding to the sample's runs were downloaded using SRA-toolkit (http://ncbi.github.io/sra-tools/). The last available Linux version of SRA Toolkit (2.10.8) was installed (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software). Sample's SRR IDs were saved in a .txt file in the bin folder of SRA Toolkit. Data were downloaded using SRA-toolkit through the Linux command line (opened from the bin folder) using the following script: $ cat SRR (name of txt file).txt | while read id; do (write the path to open fasterq-dump program in the SRA Toolkit bin folder) $id; done. Alternatively, a single SRR file was downloaded directly through the following steps: open the Linux command line directly from the SRA Toolkit bin folder, then insert the path for fasterq-dump toll and write the SRR accession name. After raw data collection, samples were grouped for experiments and comparative conditions.

3.5.2 Data re-processing and differential expression analysis

Raw data collected were analyzed with a common pipeline through the online bioinformatics software A.I.R. (Sequentia Biotech, Barcelona, Spain) using the RNA-seq package (https://transcriptomics.sequentiabiotech.com/). The analysis and comparison of RNA samples were conducted through the following steps: read quality check, mapping against the tomato genome version SL3.0, raw count calculation, and DEG analysis. For DEGs statistical analysis, we used the DESeq2 algorithm for comparing samples. This workflow allowed us to compare and analyze different tomato studies starting from transcriptomic raw data.

3.5.3 DEGs Filtering criteria

DEGs from Biotic and abiotic experiments, obtained after A.I.R. analysis, were downloaded and grouped. Comparisons of different RNA-seq studies were made searching for common DEGs among datasets using the Microsoft Excel software.

3.5.4 Pathway analysis

The differentially expressed genes were mapped to pathways using MapMan software version 3.6.0 (http://mapman.gabipd.org, Thimm et al., 2004). In order to assign MapMan ontology to DEGs, we first downloaded the tomato protein annotation file in .fasta format (ITAG3.2_proteins.fasta), (https://solgenomics.net/ftp/tomato_genome/annotation/ITAG3.2_release/) From Solgenomics, and then, we used Mercator tool (version 3.6) to create an ad hoc protein annotation file (Lohse et al., 2014). The Mercator file was then uploaded in MapMan version 3.6 and used as a mapping file together with the list of DEG considered for each comparison analyzed in this study.

CHAPTER IV

IMPROVING TOMATO RESPONSE TO MULTIPLE STRESS: TARGET SELECTIONS AND IMPAIRING OF WATI AND HYPRPI GENES

4.1 Introduction

Tomato is one of the most important horticultural crops worldwide, and it is typically exposed to various stress during growth. At least 24 fungi, 10 viruses, 7 bacteria, and several nematodes have been described among the most harmful tomato diseases (Jones et al., 1991), and newly emerging diseases have been reported in the last decades (Oladokun et al., 2019), while drought, high temperatures, and salinity are reported among the most frequent abiotic stress (Krishna et al., 2019). FAO estimated that globally, adverse effects caused by plant pathogens and biotic stress lead to economic losses of up to two hundred billion euros (FAO, 2021). Despite the intensive use of fertilizers and pesticides, in most cases, the synergistic occurrence of biotic and abiotic stress can lead to production losses ranging from 70 to 100% (Krishna et al., 2019; Rana et al., 2016). Good Agricultural Practices (GAP) are essential to managing the detrimental effects of biotic and abiotic stress combinations. However, the simple use of agronomic practices may be inadequate to prevent several crop-damaging.

Furthermore, extensive use of pesticides and fertilizers can be detrimental for the environment and human health (Rani et al., 2021). Hence, introducing resistant and highly productive varieties represents the way forward to boost crop production in the following years. Plant breeding for developing elite crops requires many selections and backcrossing steps, taking about ten years to develop a new improved variety (Wolter et al., 2019). Despite, new selection techniques such as MAS and GS reduced the selection time (Cappetta et al., 2020; Kim et al., 2020; Lenaerts et al., 2019; Lema 2018; Yamamoto et al., 2017) the processes for obtaining a new variety remain complex.

Plant genetic improvement programs rely on plant genomic modifications to introduce or remove particular traits. Genetic engineering techniques can selectively edit plants DNA, silencing or introducing specific genes. Recently, clustered regularly interspaced short palindromic repeats (CRISPR) associated-Cas protein (CRISPR-Cas) system stood out for its effectiveness in generating DNA site-specific mutations, leading to gene knockout and thus enabling geneticists to obtain improved plants rapidly and with reduced costs (Ahmad et al., 2020; Das et al., 2019; Durr et al., 2018). The use of CRISPR/Cas technology is mainly based on the identification and destruction of genes involved in disease susceptibility (S-genes) (Zaidi et al., 2018). Pathogens can employ S-genes to promote penetration and spreading through the cells. Alternatively, under specific conditions, S-genes can negatively affect the regulation of the immune signaling cascade, inhibiting a proper plant stress response (van Schie and Takken, 2014). A key result of S-gene inactivation is related to the broad-spectrum resistance of mutated plants (Wang et al., 2018; Denance et al., 2013). However, the impairment of S-genes is not without risks. Indeed, despite their role in plant susceptibility, these genes are often highly conserved among plants (Sun et al., 2016; Appiano et al., 2015), indicating their relevance in other essential plant functions. In order to minimize the adverse effects of S-genes silencing, a multiplexing approach for simultaneously targeting multiple traits could be used (Li et al., 2018; Wang et al., 2018; Li et al., 2017).

This study was aimed to increase tomato resistance to biotic and abiotic stress using CRISPR/Cas9 technology. Therefore, we performed large-scale bibliographical research identifying several genes involved in plant resistance or susceptibility to different stress to identify target genes. Hence, we identified two possible target genes for genome editing by the cross-comparison of bibliographical data and our transcriptomic study reported in Chapter III.

1) *WATI* (*Walls are thin 1*), first reported by Ranocha et al. (2010) in *Arabidopsis.* Mutated plants showed a broad-spectrum resistance against various vascular pathogens such as *Verticillium dahliae*, *Verticillium albo-atrum, Ralstonia solanacearum*, *Xanthomonas campestris*, and *Plectoshaerella cucumerina*.

2) *HyPRPI,* which silencing improved plant resistance to different abiotic stress such as salinity and oxidative stress (Tran et al., 2021; Liu et al., 2016).

Finally, different vectors to inactivate *SlWATI* and *SlHyPRPI* (singularly or in combinations) were produced to edit two different tomato genotypes (SanMarzano2 and MoneyMaker) and improve their response against biotic and abiotic stress.

4.2 Results

Recently introduced genome editing technologies allowed generating precise genetic modifications, leading to selective insertion/deletions (InDels) in crucial gene sites. Therefore, literature research was carried out to find candidate genes implicated in different stress responses for their potential editing. Twenty-seven promising genes characterized in different plant species and involved in response to biotic and abiotic stress were identified (Table 16) (keywords used are described in the Materials and methods section).

Most of them were key regulators of hormone metabolism (four), kinase and membrane proteins (three), transcription factors (six), and cell wall components (three).

In particular, *WATI* (*Walls are thin 1*) identified by Ranocha et al. (2013), resulted localized on tonoplast and is involved in IAA accumulation. Mutant plants for *AtWATI* showed a broad-spectrum resistance against various vascular pathogens such as *Verticillium dahliae*, *Verticillium albo-atrum, Ralstonia solanacearum*, *Xanthomonas campestris*, and *Plectoshaerella cucumerina*.

Recently, Hanika et al. (2021) exploited the role of *SlWATI* in tomato, highlighting its participation in vascular immunity. However, mutated plants showed a dwarf and stunned phenotype despite an increasing resistance to *Fol*, *Verticillium dahliae,* and *Verticillium albo-atrum*. Potential secondary effects related to *WATI* silencing were already predicted by Ranocha et al. (2013), which reported deficiencies in secondary cell wall formation and thickening. Interestingly, also *HyPRPI* increased *V. dahliae* susceptibility through ROS accumulation and disorders related to cell wall thickening. In addition, *HyPRPI* silenced plants showed improved resistance to different abiotic stress such as salinity and oxidative stress (Tran et al., 2021; Liu et al., 2016). Our comparative transcriptomic study (described in Chapter 3) revealed that *HyPRPI* resulted down-regulated during drought, salt, low temperatures, and oxidative stress, suggesting a negative role in several plant abiotic stress responses. In addition, both genes were involved in plant hormonal regulation. Indeed, silencing of *WATI* increased SA levels while decreasing IAA contents, while silencing of *HyPRPI*

increased ABA and ROS-scavenging levels (Tang et al., 2019; Liu et al., 2016; Ranocha et al., 2013).

Table 16. List of genes characterized in published studies, involved in response to multiple stress.

Gene name	Locus ID	Function in	Resistance/susceptibility	Plant	Reference
MEDIATOR 18	AT2G22370	Mediator complex	Susceptibility to: *Fusarium oxysporum*	A. thaliana	(Fallath et al., 2017)
MEDIATOR 20	AT2G28230	Mediator complex	Susceptibility to: *Fusarium oxysporum*	A.thaliana	(Fallath et al., 2017)
CYP83B1	Solyc09g092640	Glucosinolate, tryptophan metabolism, and callose deposition	Resistance to: *Fusarium oxysporum*	Solanum lycopersicum	(Manzo et al., 2016)
COI1	AT2G39940	JA-ile receptor	Susceptibility to: *Fusarium oxysporum*	A.thaliana	(Cole et al., 2014)
ARF2	AT5G62000	Auxin responsive factors	Susceptibility to: *Fusarium oxysporum*	A.thaliana	(Lyons et al., 2015)
PRX33	AT3G49110	Required for ROS formation	Susceptibility to: *Fusarium oxysporum*	A.thaliana	(Lyons et al., 2015)
ETR1	AT1G66340	Ethylene receptor	Susceptibility to: *Fusarium oxysporum*	A.thaliana	(Pantelides et al., 2013)
ESR1	AT5G53060	An upstream regulator in jasmonate signaling	Susceptibility to: *Fusarium oxysporum*	A.thaliana	(Thatcher et al., 2015)
AFB1 ***AFB3*** ***AXR4***	AT4G03190 AT1G12820 AT1G54990	Auxin transporters	Susceptibility to: *V. dahliae*	A.thaliana	(Fousia et al., 2018)
PR-5x	Solyc08g080620	Disrupt the lipid bi-layer of fungi	Resistance to: Fusarium Wilt Disease	Solanum lycopersicum	(de Lamo et al., 2018)
Unknown protein	*Solyc08g075770*	Small transmembrane protein	Resistance to: Fusarium Wilt Disease	Solanum lycopersicum	(Prihatna et al., 2018)
PUB12 and PUB13	AT2G28830 AT3G46510	regulation of the chitin receptor complex	Silencing, enhance callose deposition and chitin-induced ROS production	A.thaliana	(Yamaguchi et al., 2017)

GbRLK	AT5G63930 LOC105800342	receptor-like kinase (RLK) gene	Resistance to: drought and high salinity and Verticillium wilt	A.thaliana; Gossypium barbadense	(Jun et al., 2015; Zhao et al., 2013)
BOS1	AT3G06490	encoding an R2R3MYB transcription factor	Resistance to: *B. cinerea* and abiotic stress tolerance	A.thaliana	(Luo et al., 2010)
OsNAC5	Os11g08210	NAC family TFs	Tolerance to abiotic stresses	Oryza sativa	(Takasaki et al., 2010)
ATAF1	AT1G01720	NAC family TFs	Susceptibility to: *Botrytis cinerea*, *Pseudomonas syringae,* and *Alternaria brassicicola* Resistance to salt in Rice	A.thaliana; Oryza sativa	(Wang et al., 2009; Liu et al., 2016)
CRPK1	AT1G16670	Protein kinase	Susceptibility to: cold	A.thaliana	(Liu et al., 2017)
PgSAM	Unknown	*S*-adenosyl-L-methionine synthetase (SAMS)	Resistance to: Salt, salicylic acid, abscisic acid, and chilling	Panax ginseng	(Pulla et al., 2009)
miRNA 5300	slmiR5300	Target nucleotide-binding (NB) domains	Susceptibility to: *Fusarium oxysporum*	Solanum lycopersicum	(Ouyang et al., 2014)
WRKY33	GhWRKY33	Transcriptional repressor to ABA sensitivity3	Susceptibility to: drought	Gossypium hirsutum	(Wang et al., 2013)
WRKY 45	OsWRKY45	WRKY family TFs	Resistance to: *Pseudomonas syringae*, salt, and drought stresses	A.thaliana	(Qiu and Yu, 2009)
HyPRPI	Solyc12g009650	cell wall protein	Susceptibility to: salt stress	Solanum lycopersicum	(Tran et al., 2021)
WAT1	AT1G75500	secondary cell-wall deposition	Susceptibility to: vascular pathogens	A.thaliana	(Denancé et al., 2013)
ERF72	AT3G16770	ROS response	Susceptibility to: *Fusarium oxysporum* Resistance to: *B.cynerea*	A.thaliana	(Li et al., 2021; Chen et al., 2014)

4.2.1 Identification of target genes to increase tomato resistance to multiple stress

Literature search results were compared with the results of the transcriptomic analysis. In our data, *WATI* resulted down-regulated in resistant genotypes to *R. solanacearum*, *P. infestans*, drought, and susceptible genotypes to *P. syringae* and *R. solanacearum*. *HyPRPI* was down-regulated in susceptible genotypes to abiotic stress, to fungi, and in resistant genotypes to *C. fulvum*, *P. infestans,* and drought. This cross-comparison led to the selection of the two genes as targets in the CRISPR/Cas9 knockout experiments. *WATI* was first identified in *Arabidopsis* as AT1G75500 (Ranocha et al., 2010). Therefore, we carried out an orthology analysis to reveal the *AtWATI* orthologs in tomatoes (Solanum Lycopersicum). The Amino acid sequence of AT1G75500 was acquired from the TAIR database and used as a query in a blast protein research against Tomato Genome protein (ITAG release 3.20) in the SolGenomics database finding 48 putative homologs genes. In particular, Solyc04g080940 showed 72.89% of similarity with *AtWATI*. Thus, we collected and grouped the protein sequences of all putative AT1G75500 orthologs to perform a phylogenetic analysis (Figure 26).

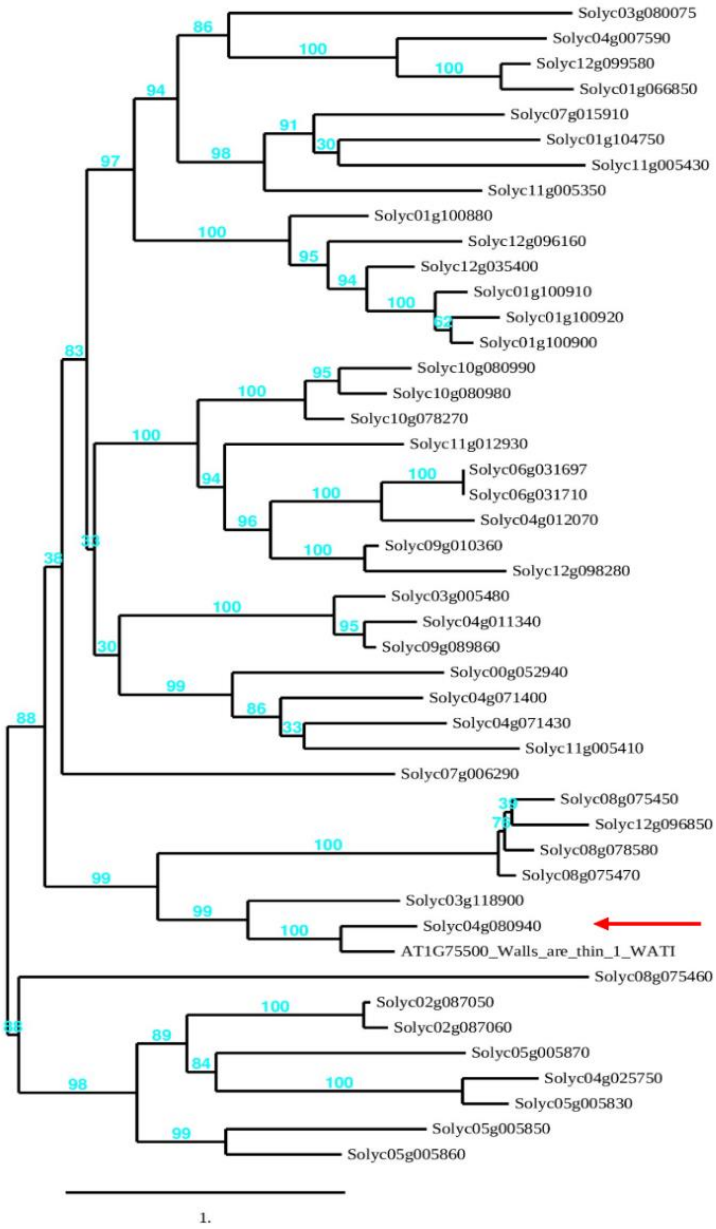


Figure 26. Phylogenetic tree of Arabidopsis *WATI* (at1g75500) and Solanum lycopersicum genes. Total lengths of amino acid sequences were aligned using Phylogeny.fr. The red arrow shows the *AtWATI* ortholog in tomato.

Results showed that Solyc04g080940 (from now on, *SlWATI*) was the closest orthologs to at1g75500 in tomato. On the other hand, *SlHyPRPI* (Solyc12g009650) was already used in previous tomato experiments (Li et al., 2016) and did not require phylogenetic comparison. Thus, both cDNA sequences of *SlHyPRPI* and *SlWATI* were retrieved from SolGenomic Network and investigated. In particular, both genes displayed a reverse orientation in the genome, as shown in Figure 27. Sequence analysis showed that *SlWATI* was composed of 6 exons for a total CDS length of 1188bp while *SlHyPRPI* had a large exon of 778bp and a small second exon of 20bp.

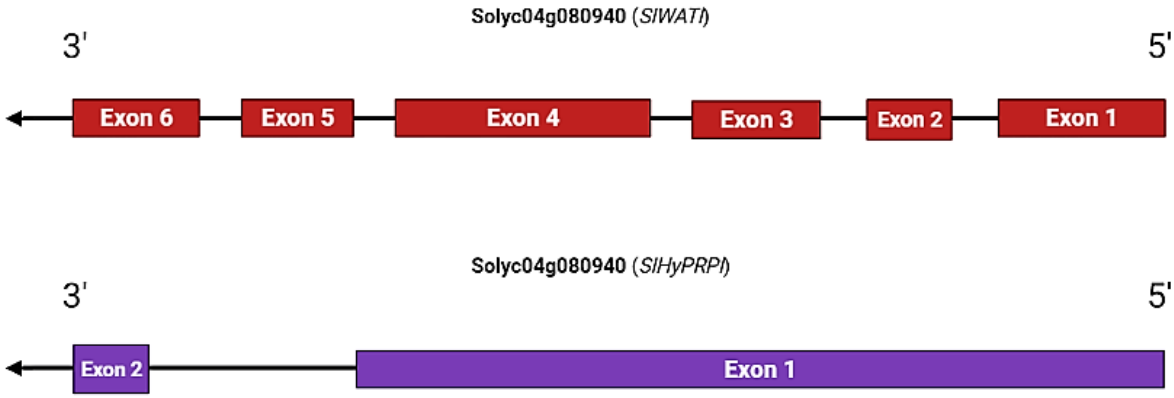


Figure 27. Schematic representation of *SlWATI* and *HyPRPI* genes. Colored boxes represent exons, while introns are represented as black lines.

4.2.2 Genetic sequence investigations of *SlWATI* and *SlHyPRPI* for CRISPR/Cas9 experiment

The design of suitable gRNAs is a key factor to effectively knockout gene expression. Thus, four single guide RNA (sgRNA) were selected evaluating the following parameters: 1) proximity to start codon (gRNAs close to the start codon were preferred) 2) 'G' as first nucleotide, 3) high efficiency predicted in generating deleterious mutations, 4) minimum number of matches with other genomic sequences (off-targets). Hence, to identify reliable sgRNAs, we used two different tools: Benchling and Cas-Offinder. Benchling was used to investigate the first four exons of *SlWATI* and exon one of *SlHyPRPI*. The analysis resulted in seventy-two suitable gRNAs for *SlWATI* and more than one hundred usable gRNAs for *SlHyPRPI*. However, gRNAs starting with a 'G' were preferred because 'G' presence at the start site increased transcription efficiency when a U6 promoter is used to promote gRNAs expression in CRISPR/Cas9 cassette. This filtering resulted in a total of twenty-five gRNAs for *SlWATI* and twenty-two available sequences for *SlHyPRPI*. Then, sequences were sorted for higher values of predicted knock-out efficiency (on-target score) and specificity (off-target score). Hence, the top ten gRNAs were selected and used for Cas-Offinder tool crosschecking to analyze possible off-target sites. In particular, a maximum number of mismatches of three was set up. Tables 17 and 18 reported selected gRNAs with their respective predicted values of on-target and off-target scores and the number of off-target identified after Cas-Offinder analysis.

Table 17. Selected gRNAs for *SlWATI* knockout. Higher values (numbers range from 0 to 100) correspond to better efficiency and fewer identified off-target sites. The last column reports the number of the most likely off-targets predicted by Cas-Offinder. Bold = selected sequences.

Distance from ATG (bp)	Strand	Sequence	PAM	On-target score	Off-target score	Off-targets (Cas-Offinder)
1220	+	GGCTTGCAACACGAGCCACC	CGG	60,6	50	3
1345	+	GAGTGAACTAGCCAAGCCTG	AGG	63,9	49,9	0
1251	-	GAAGTACCCGGCTAGGCTCT	CGG	58	49,9	0
1331	-	GTGAAAGGGACCCTCAGGCT	TGG	54,8	49,8	0
1261	+	GACGTGACCGAGAGCCTAGC	CGG	49,3	49,6	0
805	+	GCCATGAGAAATGTTATGGC	AGG	57,9	48,9	1
1198	-	GGACATTGTTTATCATGGGC	CGG	48,4	48,7	2
1114	+	**GGTGAACTCCTTTGTAGTGG**	TGG	55,5	48,6	1
771	+	**GTATGGCAGAAGCAAAAGTA**	GGG	51,2	47,8	2
1194	-	GATCGGACATTGTTTATCAT	GGG	51,3	47,6	1

Table 18. Selected gRNAs for *SlHyPRPI* knockout. Higher values (numbers range from 0 to 100) correspond to better efficiency and fewer identified off-target sites. The last column reports the number of the most likely off-targets predicted by Cas-Offinder. Bold = selected sequences.

Distance from ATG (bp)	Strand	Sequence	PAM	On-target score	Off-target score	Off-targets (Cas-Offinder)
209	-	**GATCGGTGGAATTCCGATAG**	GGG	58,6	99,0	1
391	-	GTGACCGGTGGAATCCCAAC	AGG	47,7	98,9	1
164	-	GATTGGTGGAATTCCGATAG	GGG	58,2	95,9	2
149	-	**GATAGGGGGCAAATCGACAG**	GGG	70,0	95,3	0
130	-	GGGGGTTTGACAATGGGAGT	GGG	63,7	93,0	1
478	-	GGTGATGGTTTAATTACTGG	TGG	61,3	92,5	2
398	+	GCCACCTGTTGGGATTCCAC	CGG	58,4	91,7	3
451	-	GTGACTGGTGGAATTCCAAT	AGG	57,1	90,6	3
100	-	GGCTTTTTGGGCTTGTGAGA	AGG	40,9	48,2	5

Following the above considerations, four sgRNAs were selected. Two gRNAs were chosen for *SlWATI* knockout: GTATGGCAGAAGCAAAAGTA (WgRNA1) and GGTGAACTCCTTTGTAGTGG (WgRNA2), while two gRNAs were selected for knockout of *SlHyPRPI*: GATAGGGGGCAAATCGACAG (HgRNA1) and GATCGGTGGAATTCCGATAG (HgRNA2). Guides for *SlWATI* silencing (WgRNA1 and WgRNA2) were designed on exon three and exon four, respectively, and were spaced from 343bp to each other. On the other hand, HgRNA1 and HgRNA2 were only distant 60bp and were located close to the start codon (around 150nt away). Moreover, these sequences exhibited good efficiency levels for editing and low off-target effects. WgRNA1 showed an "on-target" score of 51.2, suggesting an excellent probability of inducing a frameshift in the target sequence, while two off-target sites were predicted starting from three nucleotides of mismatches in the seed sequence (first 8-12nt proximal to the PAM sequence). Furthermore, this guide was the closest sequence to the start codon (ATG). WgRNA2 had a higher value of on-target score (55.5) and only a possible off-target site. HgRNA1 showed the highest level of on-target score in Benchling (70.0) and only a predicted off-target sequence, starting from three nucleotides of mismatches. Finally, HgRNA2 was the best gRNA predicted in Benchling with an off-target score of 99 out of 100 and a good level of the on-target score (58,6). Indeed, Cas-Offinder analysis showed that the only predicted off-target site is a similar sequence on the same target gene, partially overlapping the HgRNA1. Predicted off-target sites were reported in the supplementary material (Supplementary Table 3).

4.2.3 Vector construction for SlWATI and HyPRPI knockout

The efficiency of gRNAs can be highly variable and often cannot be well-predicted (Xiang et al., 2021; Soyars et al., 2018). Therefore, to enhance the odds of success in the silencing of *SlWATI* and *SlHyPRPI* genes, chosen gRNAs were paired and assembled in three different vectors. In particular, other studies showed that the simultaneous effect of two sgRNAs could lead to complete fragment deletions (Durr et al., 2018; Wang et al., 2017). Hence, our strategy was based on three different vectors carrying all required transcriptional units (TUs) for silencing of target genes (Figure 28).

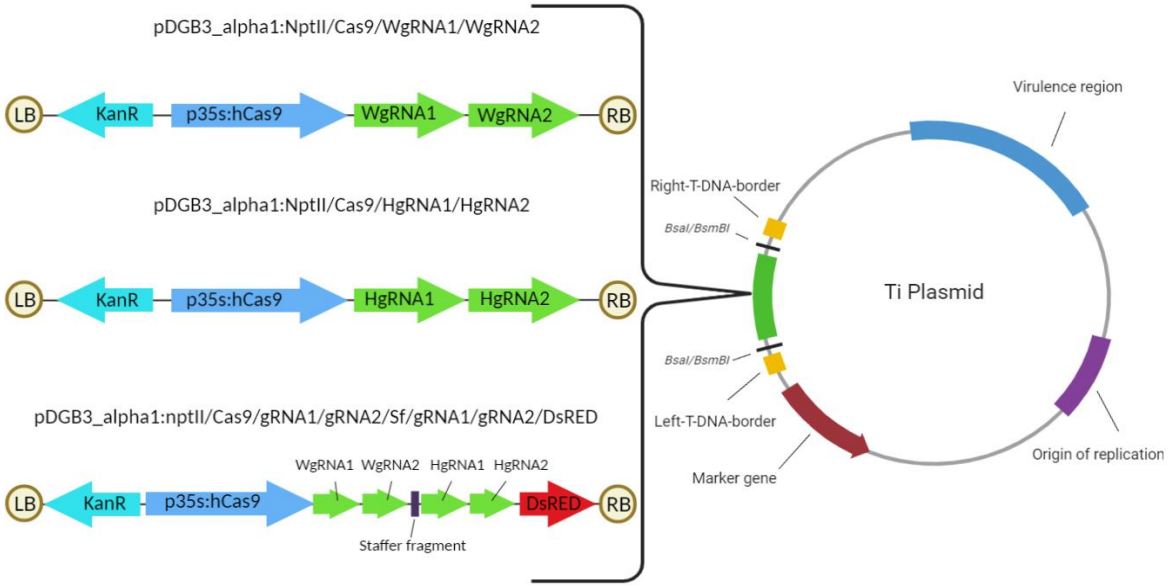


Figure 28. Schematic representation of vector used for *SlWATI* and *SlHyPRPI* knockout.

Binary vectors assembled were composed by the combination of TUs for the expression of *hCas9*, marker genes, and guide RNAs. In particular, we designed two vectors for the individually knockout of *SlWATI* and *SlHyPRPI* and a third vector carrying the TUs to silence both target genes simultaneously (named CF vectors). Expression of *hCas9* was placed under the control of the *Cauliflower Mosaic Virus* (*CaMV*) 35S promoter, which conferred an optimal gene constitutive expression in plants. The expression of sgRNA sequences was ensured by the AtU6-26 promoter, which showed a strong capability to induce the sgRNA transcription. *Neomycin phosphotransferase II* (*NPTII*) gene was used as plant antibiotic resistance, and its expression was ensured by a *nopaline synthase* (*nos*) promoter. CF vector was assembled

in the institute for plant molecular and cell biology (IBMCB) of Valencia at the Antonio Granell Laboratory. In this vector, a second marker gene encoding for a red fluorescent protein (*DsRed*) was introduced, in the proximity of the right border, to facilitate the selection of transformed plants. Finally, the three assembled vectors were validated through both methods of sequencing and enzymatic digestions. Figure 29 shows two sections of the vectors carrying the information for silencing of *SlWATI* and *SlHyPRPI* genes. In particular, the insertion of the four gRNA sequences is shown in Figure 29A, while Figure 29B shows the bands derived from enzymatic digestion of the CF vector. Results showed two and three bands respectively (left), according to in silico prediction (right). Once validated, plasmids were used for *A. tumefaciens* transformations (described in Supplementary materials).

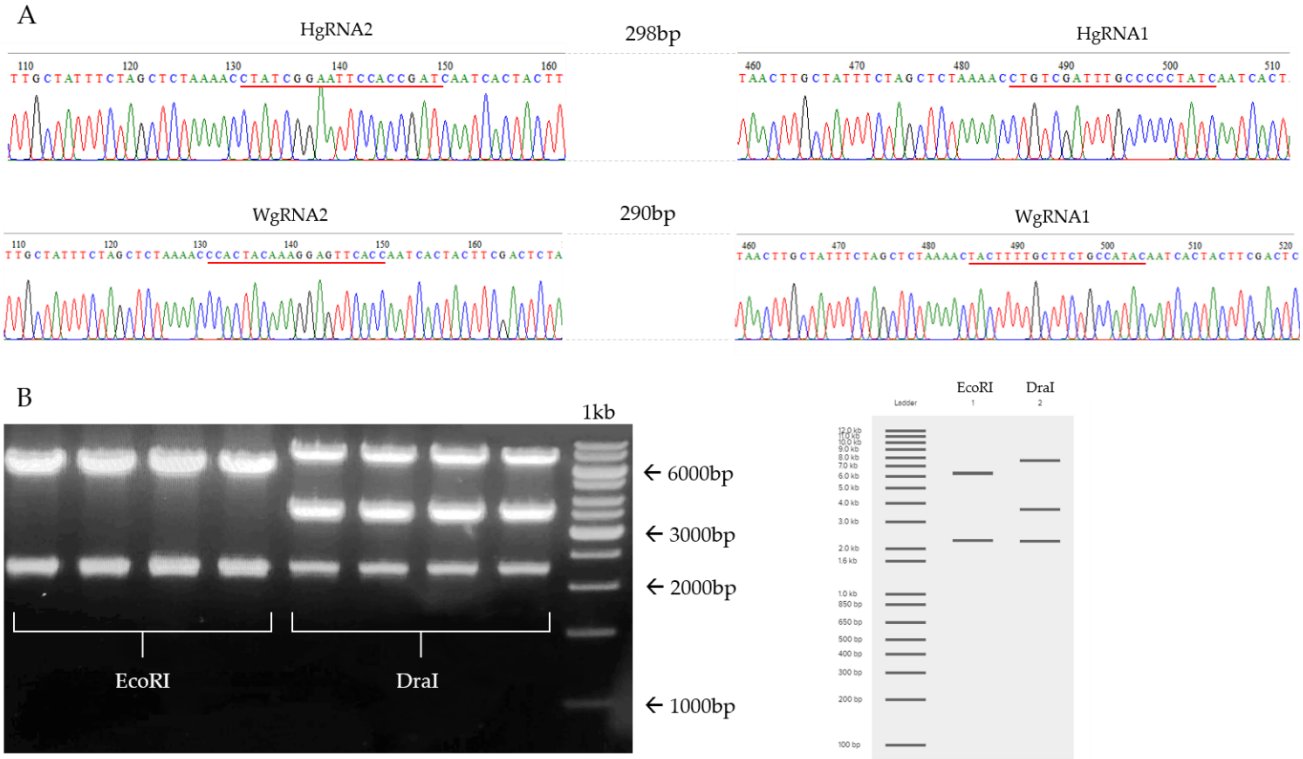


Figure 29. Plasmid construction and validation. A) Sanger sequencing chromatograms of vector for *SlWATI* and *SlHyPRPI* knockout. Sequences were obtained using a reverse primer (RB-TDNA Rv-see supplementary table 4). The red lines highlight the four gRNAs (HgRNA1, HgRNA2, WgRNA1 and WgRNA2). B) DNA digestion of four "CF" colonies, using EcoRI and DraI enzymes of vector carrying all the four gRNAs for simultaneously. On the right predicted bands from a virtual vector digestion in Benchling

4.2.4 Plant transformation efficiency

In our experiment, we used SanMarzano2 variety and MoneyMaker as control. Out of four hundred fifty cotyledons used, one hundred two kanamycin-resistant plants were obtained, with a percentage of transformed cotyledons of around 22.8%. A schematic representation of the transformation process was reported in Figure 30.

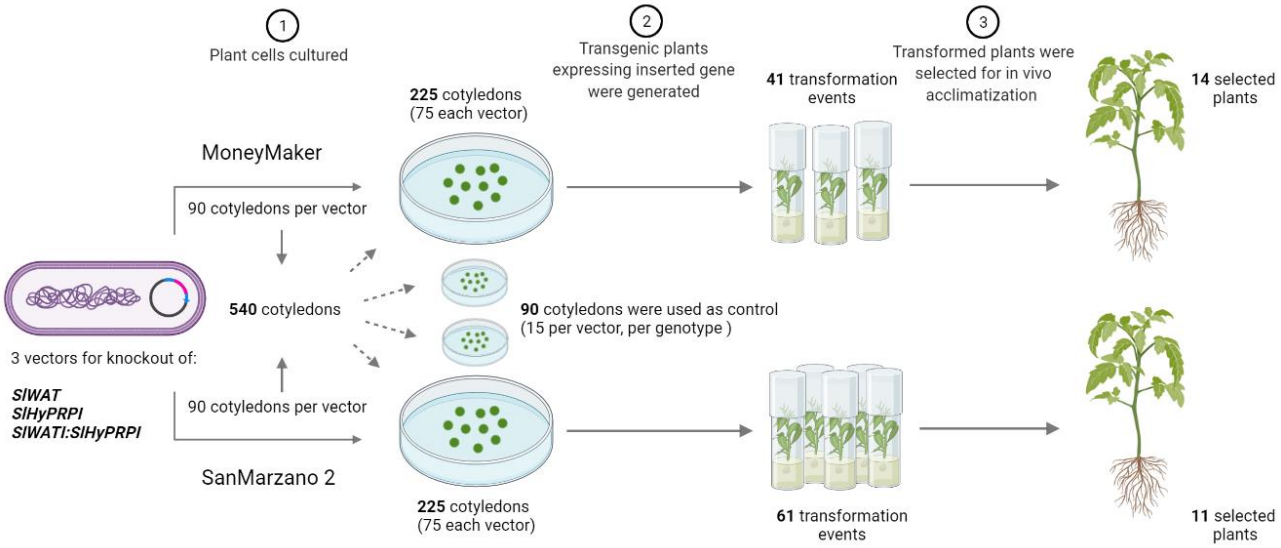


Figure 30. Transformation process and the number of transformed events for both MoneyMaker and SanMarzano2 genotypes. In particular, three different vectors were used. Seventy-five cotyledons were used for each vector transformation, and fifteen cotyledons were used as a control for a total of five-hundred forty cotyledons used. A total of 102 transformed plants were obtained, 25 plants were chosen for "in vivo" acclimatization (14 MoneyMaker and 11 SanMarzano 2).

In particular, we observed that SanMarzano2 showed a greater regeneration rate when compared with Moneymaker. Indeed, we found that regardless of the vector used, Moneymaker developed a lower number of regenerated plants compared with SanMarzano2. Results showed that forty-one new transformed seedlings were produced by Moneymaker, while SanMarzano2 originated sixty-one transgenic plants (Figure 30). Leaf samples were collected by rooted plants to assess the mutation entities and extracted genomic DNA from kanamycin-resistant plants was used to amplify and sequence *SlWATI* and *SlHyPRPI* genes (primer used for amplification and sequencing are listed in Supplementary materials). Furthermore, leaves of kanamycin-resistant plants, transformed with CF vector (from now on called CF plants), were observed under a fluorescence microscope. Transformed plants that exhibited a red fluorescent light (due to the expression of the *DsRed* gene) were selected for the molecular evaluation of the *SlWATI* and *SlHyPRPI* loci (Figure 31).

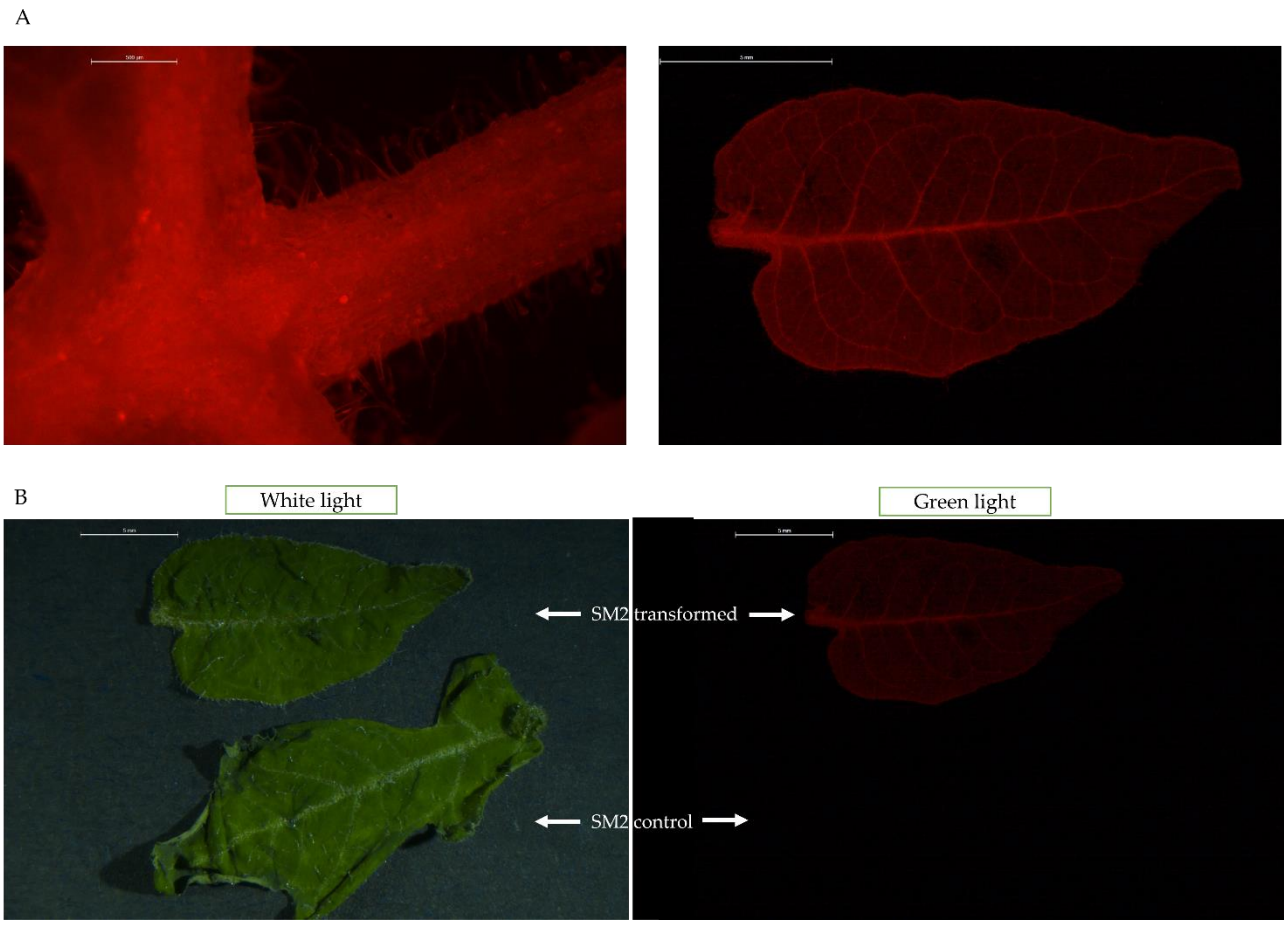


Figure 31. Plants transformed with vector to simultaneously silencing of *SlWATI* and *SlHyPRPI*. Transformed plants illuminated with green light expressed the red fluorescence due to the presence of *DsRed* protein. Panel A) a detail of veining and leaf expressing *DsRed*. Panel B) a comparison between two leaves of San Marzano 2 transformed (up) and not transformed (down). On the left, both leaves were illuminated with white light; on the right, leaves were observed through a DsRed filter under green light illumination.

Extracted DNA was used for the PCR amplification of target genes. In particular, *SlWATI* and *SlHyPRPI* showed amplicons of 1497bp and 975bp, respectively (Figure 32).

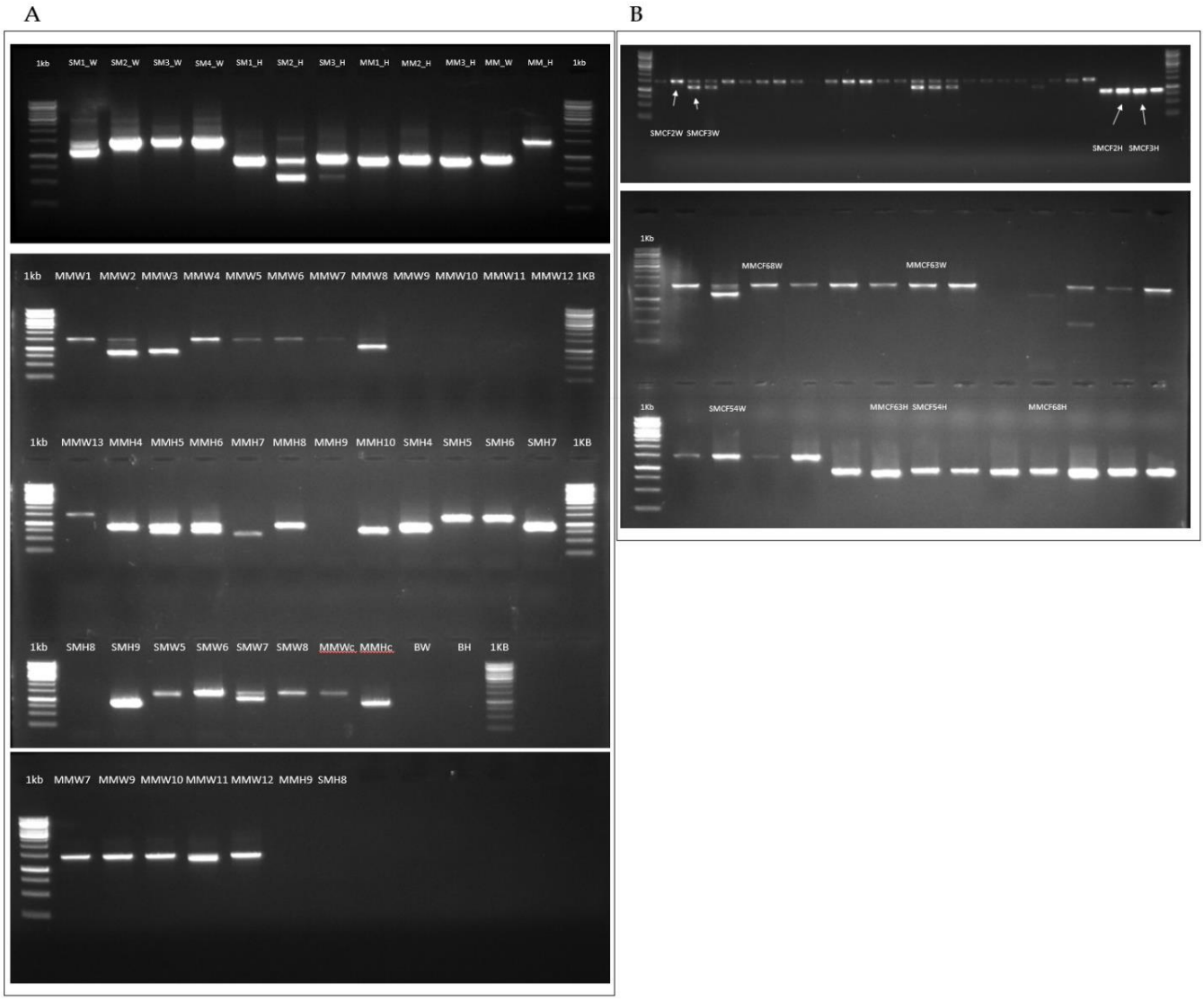


Figure 32. PCR amplification of SlWATI and SlHyPRPI loci. Panel A: single transformed plants analysed for different SlHyPRPI and SlWATI mutations . Panel B: transformed CF plants were analysed for both loci. The most interesting CF plants are pointed out by arrows.

Out of one hundred two analyzed plants, eighty-one showed the amplicon bands and were subjected to sequencing. Table 19 reports the size and frequency of mutations retrieved by Sanger sequencing. Instead, Table 20 showed the first two most likely predicted mutations with their predicted percentages of contribution. Our results showed that mutations occurred in 78.7% of the sequenced loci (eighty-nine out of a total of one hundred thirteen). Synthego results showed that T0 plants carried different mutations on both alleles of the same locus. Moreover, not all plants mutated *SlWATI* and *SlHyPRPI* genes.

Table 19. The frequency of most probable mutation occurred in *SlHyPRPI* and *SlWATI* loci. CF plants were analyzed for both loci. In bold, first three classes of frequency.

Locus	Number of samples	Mutation (bp)	Frequency (%)
SlHyPRPI	10	-60	**18.2**
	1	-15	1.8
	5	-59	**9.1**
	1	-7	1.8
	4	-11	7.3
	3	4	5.5
	1	-9	1.8
	1	-64	1.8
	14	0	**25.5**
	3	-3	5.5
	2	-8	3.6
	1	-16	1.8
	1	10	1.8
	2	-4	3.6
	1	-12	1.8
	1	-6	1.8
	3	-67	5.5
	1	-62	1.8
SlWATI	10	0	**17.2**
	1	1	1.7
	2	-1	3.4
	1	-10	1.7
	4	-11	6.9
	1	-17	1.7
	2	2	3.4
	1	-2	1.7
	1	-28	1.7
	1	3	1.7
	6	-3	10.3
	2	-342	3.3
	7	-343	**12.1**
	7	-4	**12.1**
	3	-5	5.2
	3	-6	5.2
	3	-7	5.2
	1	-8	1.7
	2	-9	3.4

Table 20. Selected plants with indication of most likely InDel that occurred in guide sequence 1 or 2. Low percentages are probably due to noises in the Sanger sequencing.

SlHyPRPI mutated plants		
Genotype	Sequence contribution	Mutation size
MMH1	33%	-60:md-0[g1],-0[g2]
	28%	-15[g1]
MMH4	63%	-59:md+1[g1],+0[g2]
	20%	-60:md-0[g1],-0[g2]
MMH5	46%	-60:md-0[g1],-0[g2]
	25%	-8[g1]
MMH6	19%	-7:m-4[g1],-3[g2]
	15%	-21[g1]
MMH8	17%	-11[g1]
	13%	-15[g1]
	12%	-17[g2]
SMH1	31%	4[g1]
	21%	-3[g1]
SMH5	24%	4[g1]
	10%	-26[g1]
SMH9	54%	-64:md-1[g1],-3[g2]
	37%	-60:md-0[g1],-0[g2]
MMH51	50%	-59:md+1[g1],+0[g2]
	45%	-60:md-0[g1],-0[g2]
SMH51	35%	-16[g1]
	27%	-4[g1]

SlWATI mutated plants		
Genotype	Sequence contribution	Mutation size
MMW2	39%	-2:m-1[g2],-1[g1]
	4%	1[g2]
MMW7	100%	-3:m-2[g2],-1[g1]
MMW9	99%	-3:m-2[g2],-1[g1]
MMW10	33%	-11[g2]
	17%	-13:m-5[g2],-8[g1]
MMW12	33%	-11[g2]
	19%	-13:m-5[g2],-8[g1]
MMW50	52%	-4:m-1[g2],-3[g1]
	21%	-4:m-1[g2],-3[g1]
SMW1	47%	-342:md+1[g2],+0[g1]
	5%	2[g2]
SMW2	36%	-11[g2]
	23%	-12:m-7[g2],-5[g1]
SMW6	30%	-4:m-3[g2],-1[g1]
	22%	-4:m-2[g2],-2[g1]
SMW7	12%	-342:md+1[g2],+0[g1]
	8%	-347:md-1[g2],-3[g1]

SlWATI:SlHyPRPI mutated plants			
Genotype	Gene	Sequence contribution	Mutation size
SMCF 2	HyPRPI	29%	-4[g1]
		8%	4:m+2[g1],+2[g2]
		7%	-3:m-2[g1],-1[g2]
	WatI	12%	-8:m-3[g2],-5[g1]
		9%	-9:m-3[g2],-6[g1]
SMCF 3	HyPRPI	42%	-60:md-0[g1],-0[g2]
		28%	-7[g1]
	WatI	29%	-343:md-0[g2],-0[g1]
		2%	-11[g2]
SMCF 54	HyPRPI	46%	-60:md-0[g1],-0[g2]
		24%	-18[g2]
	WatI	37%	-6:m-5[g2],-1[g1]
		14%	-6:m-4[g2],-2[g1]
MMCF 63	HyPRPI	51%	-62:md-1[g1],-1[g2]
		19%	-28[g1]
	WatI	29%	-343:md-0[g2],-0[g1]
		3%	-342:md+1[g2],+0[g1]
MMCF 68	HyPRPI	36%	4[g1]
		7%	-26[g1]
	WatI	23%	-4:m-1[g2],-3[g1]
		2%	-4:m-2[g2],-2[g1]

It is important to highlight that plants carrying small nucleotides deletion in numbers multiples of three were discarded since they led to few aminoacid eliminations but not the formation of premature stop codons (we only relied on those sequencing producing either a frameshift or 21+ bp InDel). Moreover, some CF plants (transformed with the double-silencing vector) showed mutations in only one of the two target genes; therefore, these plants have been eliminated. Thus, we selected the best-transformed plants for the next step of *in vivo* acclimatization.

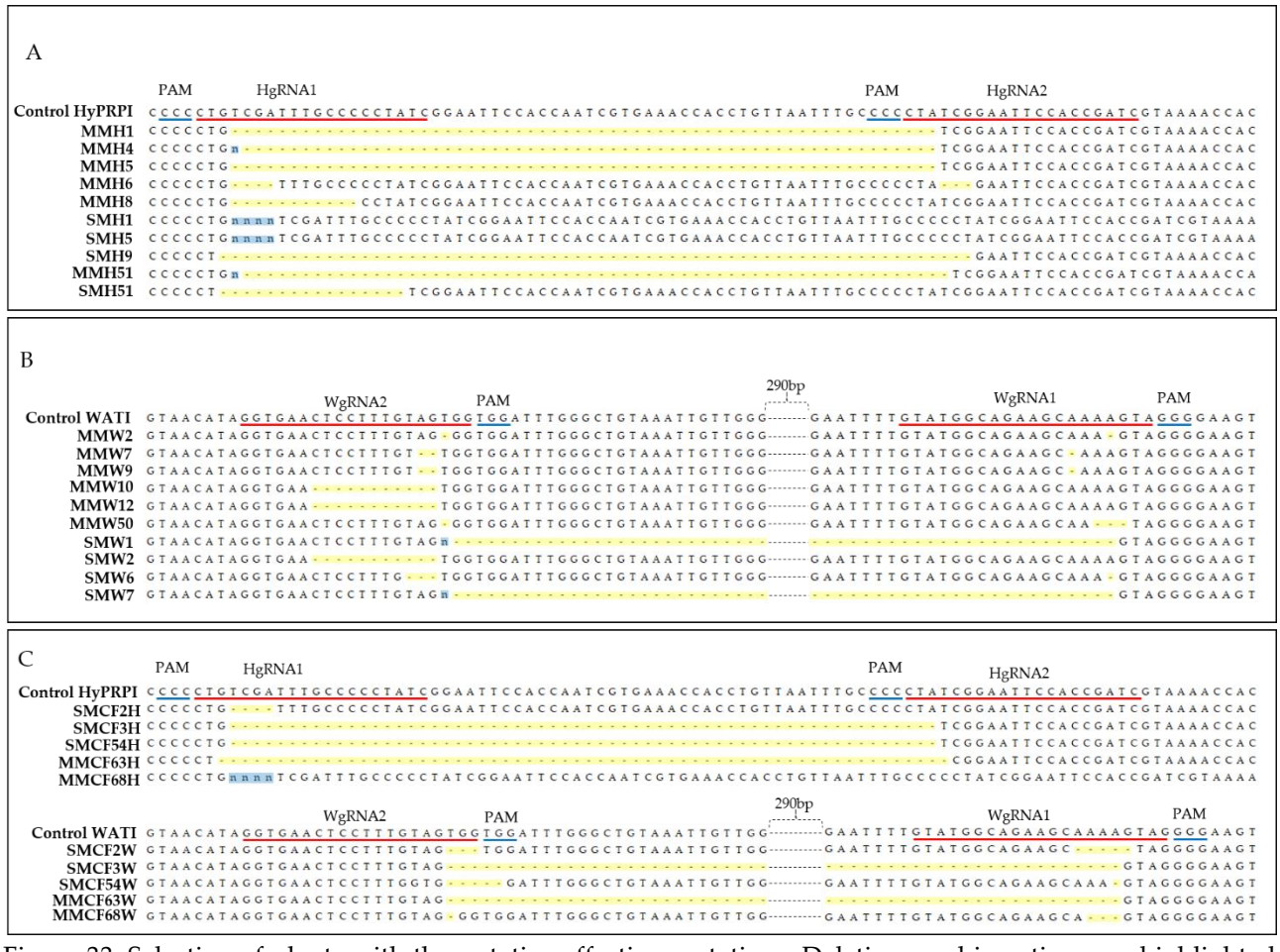


Figure 33. Selection of plants with the putative effective mutations. Deletions and insertions are highlighted in yellow and blue, respectively. The guide sequence is underlined in red, the PAM is underlined with a blue line. A) Mutations occurred in ten selected plants mutated for *SlHyPRPI* gene; B) Mutation occurred in ten selected plants mutated for *SlWATI* gene; C) five selected plants carrying mutation *SlHyPRPI* and *SlWATI* loci simultaneously.

Figure 33 shows the plants selected for the in vivo transfer and their most likely mutation on the target gene. All the selected plants showed potential InDels, which could cause frame-shifting and early transcription terminations. Thus, sequencing analysis of

mutated *SlHyPRPI* genes revealed that two SanMarzano plants (SMH1 and SMH5) carried the insertion of 4 nucleotides at the level of gRNA1, leading to a premature stop codon formation few bases upstream the guide RNA. Other plants such as SMH9 were subjected to deletions of more than sixty nucleotides. In particular, SMH9 showed the deletion of sixty-four nucleotides, indicating an efficient expression of *hCas9* and gRNAs that led to the deletion of the entire gene portion. Similarly, in SMW1 and SMW7 transformed plants, large gene deletions occurred. These plants showed the losses of 342nt and the simultaneous insertion of a nucleotide at the level of gRNA2.

Furthermore, other transformed plants such as MMW7 showed deletions of a few nucleotides near both gRNAs. In particular, MMW7 and MMW9 showed the deletion of three nucleotides (gRNA2 -2 + gRNA1 -1). Therefore, we compared wild type sequence of *SlWATI* and its mutated form in MMW7, finding that this mutation led to the formation of a stop codon 199 amino acids upstream of the start codon (Figure 34). In addition, mutations in MMW7 and MMW9 plants have been predicted as the only suitable, suggesting that the same mutation occurred in both alleles. Thus, T1 plants derived from MMW7 and MMW9 could be directly used for further analysis.

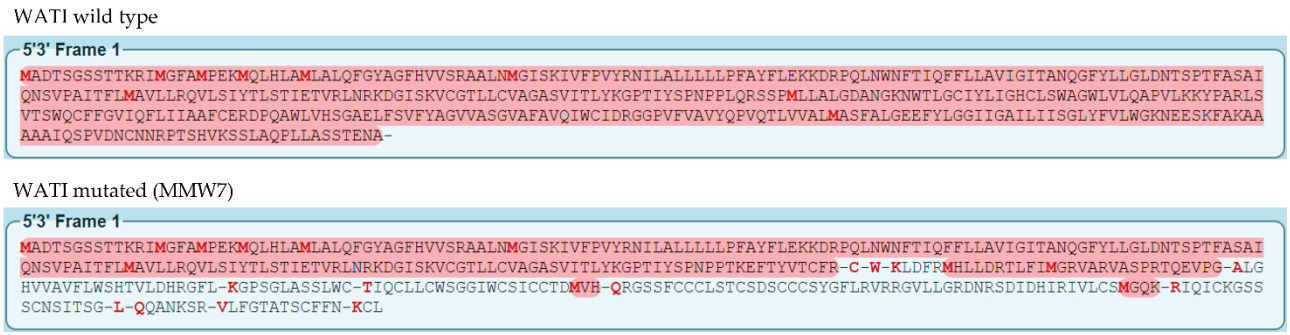


Figure 34. Comparison between aminoacidic sequence of WATI (not mutated) and WATI mutated in MMW7.

Finally, five CF plants mutated in *SlWATI* and *SlHyPRPI* loci were selected. In particular, MMCF63 showed a deletion of 62nt in the *SlHyPRPI* gene and 343nt in the *SlWATI* gene, resulting in two simultaneously large deletions on the same plant. Similarly, in the SanMarzano transformed plant SMCF3, deletions of 60bp in the *SlHyPRPI* gene and 343nt on the *SlWATI* gene occurred. Hence, plants reported in Figure 33 were transferred in larger pots and grown in the greenhouse to be further investigated.

4.2.5 Early observations on in vivo transformed plants

Several T0 plants grown in the greenhouse carried different mutations on both alleles. For obtaining stable (homozygotic), biallelic mutations next generations (T1 or T2) are needed. However, results showed that MMW7 and MMW9 (T0 plants) had a homozygote mutation at target loci, facilitating selection. Thus, early phenotyping of T0 transformed plants was performed. After greenhouse transfer (22 of October), plants were observed for their growth habitus and morphological traits.

Interestingly, mutated plants for *SlWATI* showed a dwarf phenotype compared with plants mutated for *SlHyPRPI.* Unfortunately, the absence of control plants in the greenhouse did not allow the comparison of mutated plants with wild-type genotypes. However, *SlWATI* plants resulted much shorter than *SlHyPRPI* mutated plants, while the SMCF54 plant (mutated for both genes) showed an intermediate height compared with plants mutated for two genes separately (Figure 35). Furthermore, a few days after transplant (07/09/2021), the daily temperature raised over 25C° (Figure 36) while, due to a failure in the ventilation system, greenhouse temperatures raised 30°C for a few hours. Interestingly, plants mutated for *SlWATI* showed a seriously stressed phenotype compared with plants mutated for *SlHyPRPI,* speculating a role for *SlWATI* in heat stress response (Figure 37).

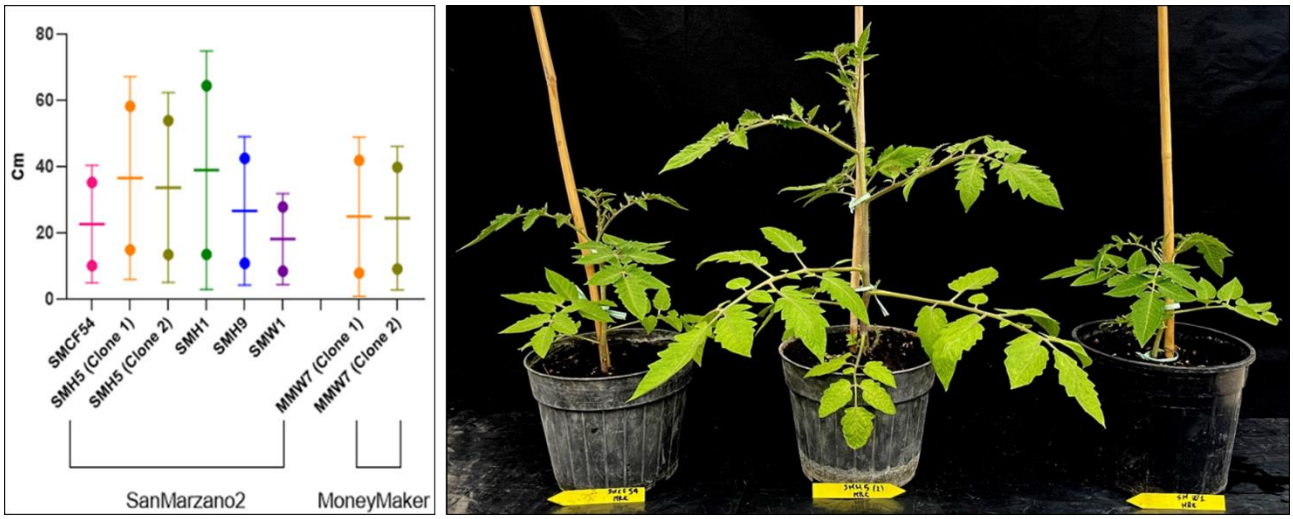


Figure 35. Comparison among height of mutated plants. The graph on the left showed plant height at 2 weeks and 6 weeks after transplant (measurement times are shown as colored points). On the right, comparison among three SanMarzano2 mutated plants, double mutated (CF) (left), *slhyprpI* (in the center), and *slwatI* (right). Significant differences in growth emerged from SMW1, SMH5, and SMCF54 plants.

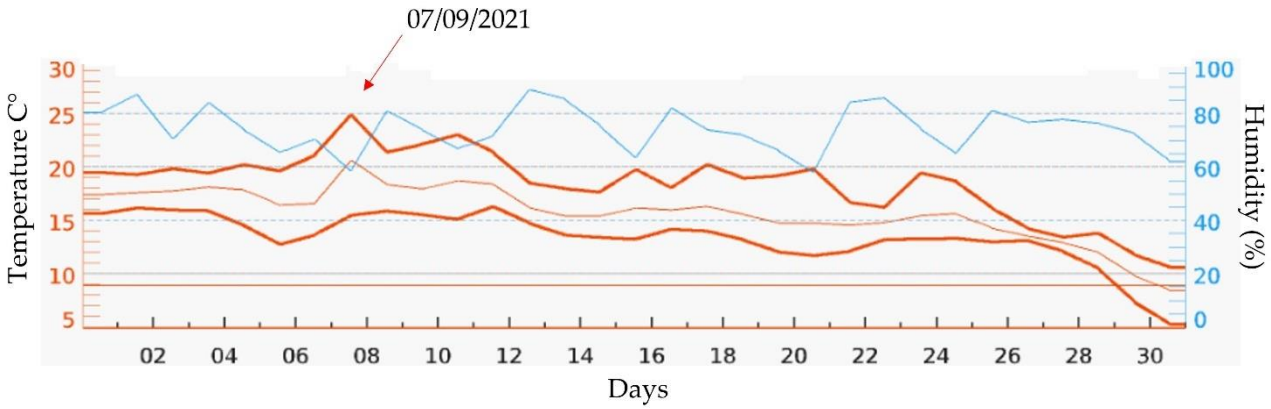


Figure 36. Temperature and air humidity during November 2021 in Naples (Italy).

Figure 37. Transformed plants stressed with high temperatures. A) During heat stress; B) 5 hours after heat stress.

4.2.6 Off-target analysis

Currently, analysis of three potential off-target sites (reported in supplementary materials) is in progress. However, none of the predicted off-targets show nucleotide mismatches outside the seed sequence (first 8-12 nucleotides distal to the PAM sequence). Seed sequence is known to be critical for Cas9-sgRNA binding. Therefore, mismatches in this portion could impair cleavages (Jamal et al., 2018; Naito et al., 2015).

4.3 Discussion

4.3.1 Literature search for multiple stress-responsive genes

Plants usually face multiple biotic stress such as fungi, bacteria, viruses, or insects, which can simultaneously interact with adverse environmental factors. As the first step of our study, a literature search for genes involved in abiotic and biotic stress response was carried out. We found twenty-seven genes described as potentially involved in vascular pathogens or multiple stress responses. In particular, five genes were involved in multiple stress susceptibility (*PUB12*, *PUB13*, *ATAF1*, *HyPRPI*, and *WAT1*).

PUB12 and *PUB13* (At2g28830 and At3g46510) resulted in functional E3 ubiquitin ligases (Lu et al., 2011). These enzymes participated in the ubiquitination and degradation of different membrane receptors involved in plant-pathogen perception. In *Arabidopsis, PUB13* interacted with the *Lysin Motif Receptor Kinase 5* (*LYK5*), a chitin receptor that was required to activate *Chitin Elicitor Receptor Kinase 1* (*CERK1*) to induce a MAPK cascade (Yamaguchi et al., 2017; Liao et al., 2017). Their silencing induced strong ROS production and callose deposition (Liao et al., 2017). Other studies suggest that *PUB12* and *PUB13* also interact with *BAK1* in *FLS2* (a flagellin-receptor gene) modulation (Lu et al., 2011). *PUB13* also resulted involved in the ubiquitination of the *BRASSINOSTEROID RECEPTOR INSENSITIVE1* (*BRI1*) (Zhou et al., 2018). Interestingly, *PUB13* mutants showed early flowering time and increasing salicylic acid (SA) contents.

Several studies demonstrated that the transcription factor *ATAF1* contributed to both abiotic and biotic stress responses. *ATAF1* containing a specific NAC domain (Lu et al., 2007) negatively regulated plant response to necrotrophic fungi and bacterial (Wang et al., 2009). Resistance increased with chimeric repression of *ATAF1* domains, while overexpression of *ATAF1* showed increased susceptibility to *P. syringae*, *B. cinerea*, and *Alternaria brassicicola* (Wu et al., 2009). Several stress-responsive proteins containing ABA (abscisic acid) binding factors were induced by *ATAF1* (Wu et al., 2009; Lu et al., 2007). By contrast, its expression was down-regulated by treatments with SA, Jasmonic acid (JA), and by one ethylene biosynthesis precursor (*1-amino cyclopropane-1-carboxylic acid*). *ATAF1* increased the expression of critical stress-responsive genes during salt stress in rice (Liu et al., 2016) but could promote drought susceptibility (Lu et al., 2018).

Several genes involved in signalings, such as RLKs, Ca^{2+} channels, ion transporters, and hormone metabolism, modulating the response network against different stresses, were characterized. In particular, *Walls are thin1* (*WATI*, at1g75500), a vacuolar auxin transporter required to auxin homeostasis was studied in detail. *WAT1* mutants were defective in transporting Indole-3-acetic acid (IAA) from the vacuole to the cytosol, which could explain defections on secondary cell wall formation (Ranocha et al., 2013). Mutants for *WATI* altered SA and auxins levels favoring SA and thus mediating resistance to vascular pathogens (Denancé et al., 2013). Further studies proved the participation of the *WATI* gene in *Xanthomonas campestris*, *Ralstonia solanacearum*, and Verticillium wilt susceptibility. Tang et al. (2019) showed that silencing three orthologs of *WATI* in cotton (*GhWAT1*, *GhWAT2*, and *GhWAT3*) plants exhibited a significant SA up-regulation and a *V. dahliae* resistance. They also confirmed that mutated plants displayed a shorter phenotype and increased xylem lignin content. In tomato, Hanika et al. (2021) demonstrated that silencing of *WATI* using CRISPR/Cas9, plants displayed various vascular pathogen resistances despite defects in plants growth. We also found a *Hybrid Proline-rich Protein1* (*HyPRPI*) during our literature research first identified in *Capsicum annuum* and *Nicotiana benthamiana* (Yeom et al., 2012). This gene showed a down-regulation after SA exposure and up-regulation after Methyl jasmonate (MeJA) treatments (Yeom et al., 2012). *HyPRP1* can induce plant cell deaths through the terminal 8 CM domain, and its silencing enhanced basal defense against pathogens and abiotic stress (Yang et al., 2018). Li et al. (2016) showed that silencing of *HyPRPI* increases the expression of antioxidant genes improving resistance against salinity and oxidative stress. Recently, (Tran et al., 2021) developed a salt stress resistance tomato, removing functional domains of *HyPRPI* using CRISPR/Cas9 technology.

4.3.2 Impairing WATI and HyPRPI genes in tomato

Traditional plant breeding mainly relies on introducing resistant genes conferring crop protections to biotic or abiotic stresses. However, resistance genes introgression in elite tomato varieties is often compromised by the simultaneous introduction of undesirable traits, which requires multiple selections and backcrossing steps for their removal, making the process expansive, tedious, and time-consuming (Wolter et al., 2019). Recently, CRISPR/Cas technology emerged as a promising tool in genome editing programs,

providing the opportunity of selectively suppress genes involved in plant stress susceptibility (S-genes).

Hence, the identification of novel S-genes is highly desirable in the coming future. These genes are often conserved across different species and could be involved in relevant plant processes. Therefore, their silencing could affect the overall plant fitness, including yields and other desired phenotypic characteristics (Zaidi et al., 2018). In the previous chapter, we performed an RNA-seq comparative analysis among thirteen tomato stresses, which allowed the identification of potential susceptibility genes involved in response to multiple stressors. Comparative analysis results were compared with the bibliographical research described above, leading to select two genes down-regulated during different biotic and abiotic stress. *SlWATI* was first identified in *Zinnia elegans* plants (Pesquet et al., 2005) and then characterized in Arabidopsis by Ranocha et al. (2010). In our study, phylogenetic analysis revealed several *SlWATI* homolog genes. In particular, Solyc04g080940 (*SlWATI*) showed to be orthologs to *AtWATI*, confirming data obtained by Hanika et al. (2021), which recently published a study using *slwatI* plants stressed with *Fol* and Verticillium *spp*. Another negative regulator of both biotic and abiotic stress responses is *SlHyPRPI* (Liu et al., 2016; Tran et al., 2021). In cotton, *GbHyPRP1*, an ortholog of *SlHyPRPI,* enhanced *V. dhalie* susceptibility favoring ROS accumulation and affecting cell wall thickening (Yang et al., 2018b). Hence, *SlWATI* and *SlHyPRPI* were used (individually and in combination) to perform a CRISPR/Cas9 experiment to increase tomato resistance to multiple stresses. In particular, two gRNAs for each gene were used, resulting in a high number of mutated plants. The design of multiple gRNAs sequences on the same gene could increase the odds of producing deleterious mutations (Alok et al., 2021; Lowder et al., 2015). Moreover, gRNAs gene location and distance could induce a type of change than others. Indeed, mutation obtained on *SlHyPRPI* genes showed a high frequency of large fragment deletions (around 60bp) in agreement with other studies (Ordon et al., 2017; Nekrasov et al., 2017; Kosicki et al., 2018). On the other hand, in *slwatI* gene, gRNAs were placed more distant (343bp), and both point mutagenesis (due to the effect of a single gRNA) and larger deletions occurred with similar frequency. These results suggest that the increasing distance between gRNAs, affects the mutation efficiency, increasing the number of point mutagenesis and reducing the number of large gene deletions.

Several T0 *slwatI* plants carried allelic mutations inducing premature stop codons formations. In particular, MMW7 and MMW9 showed homozygous mutated loci, while all other mutated plants showed different mutations on both alleles, highlighting that Cas9 expression efficiency can vary depending on the genomic structure, the insertion site, and environmental parameters (LeBlanc et al., 2018). The discovery and the introduction of new Cas proteins could increase mutations efficiency (Lin et al., 2021; Bernabé-Orts et al., 2019).

Therefore, T0 plants carrying the deleterious mutation on target loci were used for in *vivo* acclimatization (in supplementary materials are reported possible mutation occurred in sequenced loci, with their respective likelihood ratio). Hence, early phenotyping was performed on mutated plants. In particular, we noted that *slwatI* plants showed dwarfisms and stunted structures, especially in the early stages of development. In Arabidopsis, aberrations in *watI* arise in adult plants (Ranocha et al., 2010). However, in our experiment, impairment in the growth of *watI* plants was quite evident in the early phases of development and slightly decreased during the time, according to other studies performed in cotton and tomato (Hanika et al., 2021; Tang et al., 2019). These growth defects could be related to weakness in hormone balancing. Indeed, auxins are involved in cell growth and differentiation (Gomes and Scortecci, 2021), while high levels of SA negatively regulate plant growth and development (Koo et al., 2020). Thus, low auxins concentrations and high SA levels could affect plant growth.

Furthermore, our plants were subjected to accidental heat stress, showing a heavily stressed phenotype. We supposed that compromised cell wall thickening in *slwatI* plants could increase water losses, negatively influencing the heat stress response. However, a plant mutated for both *SLWATI* and *SLHyPRPI* genes (SMCF54), stressed with heat, showed a phenotype similar to that of *watI*-mutated plants.

In cotton, transient silencing of *GPHyPRPI* led to a drastic increase in cell wall thickening during *V. dhalie* infections Yang et al., (2018). Hence, it might be the case that double mutated plants increase broad-spectrum resistance during multiple stress by restoring cell wall thickness, overcoming defects due to the impairment of *SlWATI* and *SlHyPRPI* genes.

In the near future, *watI*, *hyprpI,* and *watI:hyprpI* plants with homozygous mutated alleles will be further characterized to assess the resistance to both biotic and abiotic stress. In particular, T1 or T2 plants will be studied for their resistance to vascular pathogens and

combinations of various abiotic stress. Furthermore, the evaluation of differences in the cell wall structure and thickening among *watI*, *hyprpI,* and *watI:hyprpI* mutated plants will be carried out. In addition, an evaluation of edited-plants production will be carried out to assess the potential use of these modified cultivars for company purposes.

In conclusion, different strategies may be adopted to obtain plants resistant to both and abiotic stress with reduced pleiotropic effects. For example, multiple targeting of genes involved in opposite functions could help restore deficits induced by the single S-gene silencing. Shen et al. (2017) simultaneously knock out eight genes in rice, enhancing plant yields and quality. The simultaneous editing of four MPK genes (Moustafa et al., 2014) or five GABA genes, enhanced plant abiotic stress tolerance (Li et al., 2018). In tomato, different S-genes have been silenced, deleterious mutations in *SlMlo1* and *DMR6* genes provided resistance against *powdery mildew*, *P. syringae*, *P. capsici*, and *Xanthomonas spp.* (Nekrasov et al., 2017; Thomazella et al., 2016). Therefore, S-gene knockout through CRISPR/Cas technology could be a powerful tool for rapidly generating improved elite varieties resistant to multiple stressors,

4.4 Materials and methods

4.4.1 Bibliographical research

In our research, bibliographical research to find out genes involved in plant multiple stress response was conducted using different keywords such as “plant stress,” “resistance genes tomato,” “tomato stress susceptibility,” “Biotic stress tomato,” “multiple stress tomato” or combinations of above words. Bibliographical research was conducted using both Google Scholar and Scopus search engines. Hundreds of papers evaluating plant stress response in different plant species such as *Arabidopsis*, tomato, cotton, and rice have been collected. These studies were. Plants were exposed to various pathogens such as fungi, bacteria, viruses, or pests in these experiments. Alternatively, we selected studies evaluating plants' responses to both biotic and abiotic stresses.

4.4.2 Phylogenetic analysis to identify AtWATI ortholog in tomato

The amino acid sequence of Arabidopsis *AtWAT1* (At1g75500) was retrieved from the TAIR archive and used as a query to perform a blast protein search against the Sol Genomics Network database (ITAG release 3.20). The full-length protein sequences of putative homologs of At1g75500, originating from Blastp search, were obtained from Sol Genomics FTP site using ITAG3.2_proteins.fasta (https://solgenomics.net/ftp/tomato_genome/annotation/ITAG3.2_release/) file. For comparative propose, a phylogenetic tree was constructed using the Phylogeny.fr tool using the "One-click mode" (Dereeper et al., 2008). By itself, Phylogeny.fr in "One-click mode" follow a default pipeline using MUSCLE for sequence alignment, PhyML for phylogeny, and TreeDyn software for tree rendering (Chevenet et al., 2006; Edgar 2004; Guindon and Gascuel 2003).

4.4.3 Targets selection and vector construction

4.4.3.1 Target gRNAs identification and designing

Guide RNAs design was performed using Benchling and Cas-Offinder tools. Genomic regions of *SlWATI* and *SlHyPRPI* were loaded In Benchling (https://benchling.com/). Exons of both genes were identified and used for searching suitable gRNAs as described by (Pellegrini 2016). For gRNAs analysis, we set up the following parameters: Design type: single guide, Guide length: 20nt, Genome: SL3.0, PAM: NGG. Other parameters were left to their default values.

On-target and Off-target scores were calculated using algorithms proposed by Doench et al. (2014) and Hsu et al. (2013) attributed to each sgRNA. These values ranged from 0 to 100, where higher scores were considered as better. These parameters led to the selection of ten top-ranked gRNAs. Hence, Cas-Offidner (Sangsu et al., 2014) was used to validate the selected gRNAs (http://www.rgenome.net/cas-offinder/). This tool allows searching potentially off-target sites in the entire genome of interest. In particular, a maximum number of mismatches of three for potentially off-target identification was set up. The identified off-target sequences for selected gRNAs, were reported in supplementary materials.

4.4.3.2 Cell strains, growth conditions, and transformation

For vector cloning and assembly, competent Escherichia coli (DH5α) cells were used. *E. coli* strains were grown in LB (Luria-Bertani) medium at 37°C under agitation (220rpm). Selection of transformed colonies was performed using Kanamycin (50 µg/mL), ampicillin (100 µg/mL), and spectinomycin (50 µg/mL) antibiotics. White and blue colonies were selected using 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (40 µg/mL) and isopropylthio-β-galactoside (0.5 mM), placed on LB plates. Finally, *Agrobacterium tumefaciens* electro-competent cells (strain LBA4404) were used for plant transformations. The final plasmids were inserted in LBA4404 cells using a Bio-Rad MicroPulser Electroporator (1652100). Transformed cells were incubated in LB medium and selected using Rifampicin, streptomycin, and kanamycin at the concentration of 50 µg7mL.

4.4.3.3 Restriction-Ligation Assembly Reactions

Reactions for vectors assembly were performed as described by Sarrion-Perdigones et al. (2011). In particular, BsaI and BsmBI restriction enzymes (Thermo Fisher) were used for vector restrictions, while ligation was obtained using the T4 Ligase enzyme (Thermo Fisher). Reactions were set up using the following parameters: 2 min at 37 °C and 5 min at 16°C (for a total of 25 cycles). Then, one microliter of reaction was used to transform DH5α electro-competent cells. Hence, plasmid DNA was extracted from positive colonies using the Exprep™ Plasmid SV Midi kit (GeneAll). The correct vector assembly was evaluated through restriction analysis and sequencing. In both cases, results were compared with those predicted in Benchling (https://benchling.com).

4.4.3.4 Golden Braid (GB) vector construction

For plasmids assembly, the GoldenBraid strategy was used. In particular, all vectors used were assembled using Golden Braid 3.0 platform as described in other works (Vazquez-Vilar et al., 2016; Sarrion-Perdigones et al., 2014; 2013). GB method is based on different levels of assembly. In our experiment, vectors were assembled, alternating α and Ω levels. Plasmids required to perform GoldenBraid reactions were retrieved from the GoldenBraid 2.0 kit (https://www.addgene.org/kits/orzaez-goldenbraid2/). Vector sequences were downloaded in the "search DB" elements section (https://gbcloning.upv.es/search/features/) using the vectors ID.

4.4.3.5 Vectors construction: Guide RNAs assembled in α-Level Plasmids

Alpha vectors (pDGB3α) were used in the assembly of following vectors: pDGB3α1_WgRNA1, pDGB3α2_WgRNA2, pDGB3α1_HgRNA1, pDGB3α2_HgRNA2. At first, 20nt of four different single-guide RNA: WgRNA1 (GTATGGCAGAAGCAAAAGTA), WgRNA2 (GGTGAACTCCTTTGTAGTGG), HgRNA1 (GATAGGGGGCAAATCGACAG), and HgRNA2 (GATCGGTGGAATTCCGATAG), were suspended in sterile Milli-Q Water at a final concentration of 1µM and left in incubation at RT for 30 minutes. Alpha vectors were restricted using the BsaI enzyme. Thus, GB reactions included the following components: 75ng of GB1001 (U626 promoter), 75ng of GB0645 (scaffold RNA), 75ng of pDGB3α1 destination vector, 0.6µl of primers, 3u of BsaI enzyme, 3u of T4 ligase enzyme,

and 1µl of ligase buffer in a total volume of 10 µl. The gRNAs "1" were introduced in α1 vectors while gRNAs "2" were introduced in α2 vectors. Tubes were placed in the thermocycler using the parameters described above: 25 cycles × (37 °C 2 min, 16 °C 5 min). Then, one microliter of reaction was inserted in DH5α electro-competent cells. Transformed cells were grown overnight at 37°C on LB plates containing selective antibiotics. The day after, white colonies were selected and grown in liquid LB supplied with antibiotics at 37°C overnight. After plasmid DNA extraction, colonies were evaluated through restriction reactions and sequenced. Positive colonies were used for subsequent reactions.

4.4.3.6 Vectors construction: assembly of multiple guide and nptII:Cas9 in Ω Level Plasmids

Alpha plasmids were combined in Ω-level vectors. In particular, vectors assembled in omega (Ω) level were: pDGB3Ω2_ WgRNA1:WgRNA2, pDGB3Ω2_ HgRNA1:HgRNA2 and pDGB3 Ω1_nptII:Cas9. Reactions were performed using 75ng of the destination vectors (pDGB3_ Ω1 or pDGB3_ Ω2), 75ng of the two previously assembled alpha vectors, 3u BsmBI and 3u T4 DNA ligase; Milli-Q Water was added up to a final volume of 10µl. As for the alpha vectors, reactions were placed in a thermocycler for 25 cycles at 37 °C for 2 minutes and 16 °C for 5 minutes. Then, one microliter of reaction was used to transform *E.coli* (DH5α) electro-competent cells. The day after, White colonies were placed in liquid LB containing selective antibiotics overnight. Hence, plasmid DNA was extracted and analyzed through restriction digestions and sequencing.

4.4.3.7 Vectors construction: the combination of gRNAs and nptII:Cas9 vectors in α-Level Plasmids

Finally, vectors assembled in Ω-level were joined in an α-level plasmid. Final vectors assembled in α-level were: pDGB3α1_nptII:Cas9:WgRNA1:WgRNA2 and pDGB3α1_nptII:Cas9:WgRNA1:WgRNA2. Reactions were carried out by combining: 75ng of the α1-vector as the final destination plasmid, 75ng of the previously assembled Ω vectors, 3u BsaI, and 3u T4 Ligase in a final volume of 10µL. Tubes were placed in a thermocycler at 37 °C for 2 minutes and 16 °C for 5 minutes (25 cycles). Then, one microliter

of reaction was used for *E.coli* (DH5α) electro-competent cells transformation. Cells were placed on LB plates containing antibiotics (overnight), the day after white colonies were collected, and grown in liquid LB (containing antibiotics) overnight. Hence, plasmid DNA was extracted and digested for validation. Positive colonies were subjected to sequencing.

4.4.3.8 Vectors construction: the combination of four gRNAs, nptII:Cas9 and DsRed TUs

Four sgRNAs were combined to target SlHyPRPI and SlWATI simultaneously. To this purpose, we first transferred the TUs for the HgRNA1 and HgRNA2 expression in an α2 vector using a staffer fragment carried by a pDGB3Ω1 vector. Thus, we combined pDGB3Ω_Sf with pDGB3Ω2_ HgRNA1:HgRNA2 to obtain a pDGB3α2_Sf:HgRNA1:HgRNA2. Hence this vector was combined with the previously assembled pDGB3α1_nptII:Cas9:WgRNA1:WgRNA2 in a destination vector pDGB3Ω1 to obtain: pDGB3Ω1_nptII:Cas9:WgRNA1:WgRNA2:HgRNA1:HgRNA2. Finally, using an α1 level vector as a backbone, we combined pDGB3Ω1_nptII:Cas9:WgRNA1:WgRNA2:HgRNA1:HgRNA2 with TU for DsRed gene (GB0406) to develop the final vector pDGB3α1_nptII:Cas9:WgRNA1:WgRNA2:HgRNA1:HgRNA2:DsRed. All the reactions have been assembled using the same condition described above.

4.4.4 *Agrobacterium tumefaciens* (LBA4404) mediated plant transformation

Seeds of *Solanum lycopersicum* San Marzano 2 and MoneyMaker were sterilized using ethanol 70% (v/v) for 2 min and then placed in sodium hypochlorite solution 2% (v/v) for 10 min. After that, seeds were washed with Milli-Q sterile Water five times. Sterile seeds have been placed for 48h at 27°C in the dark on MS medium (Murashige & Skoog solid medium) containing 15g of sucrose and 9g/l of agar (pH of 5.8). Germinated seeds were then grown under long-day photoperiod (16 h light, 8 h dark) at 24°C. The growth chamber was supplied with cool white fluorescent tubes (110 μmol m−2 s−1). The final vectors (pDGB3_alpha1:NptII/Cas9/HgRNA1/HgRNA2, pDGB3_alpha1:NptII/Cas9/WgRNA1/WgRNA2 and pDGB3_alpha1:nptII/Cas9/WgRNA1/WgRNA2/Sf/HgRNA1/HgRNA2/DsRED) for genes

silencing were transferred into Agrobacterium tumefaciens (LBA4404). 15ng of plasmid DNA was used for the electroporation of 50 μL of *A.tumefaciens*. Then, 500 μL of SOC medium were added, and the mixture was placed in 15ml tubes and incubated in agitation (200rpm) at 28°C for 2h. Hence, 50μL of the mixture was spread on LB plates containing selective antibiotics and incubated for two days at 28°C. Colonies were then evaluated through PCR or restriction reactions.

Transformed *A.tumefaciens* colonies were transferred in a pre-culture adapted MGL liquid medium (yeast extract 2.5 g/l, tryptone 5 g/l, mannitol 5g/l, NaCl 0.1 g/l, KH2PO4 0.25 g/l, glutamic acid 1.15 g/l, biotin 1 mg/l,MgSO4.7H2O 100 g/l with pH 7). Pre-culture media was supplied with selection antibiotics: Rifampicin and kanamycin (50mg/l) and left for overnight incubation at 28°C. TY medium was prepared (yeast extract 3 g/l, tryptone 5 g/l, MgSO4.7H2O 0.5 g/l, and acetosyringone 200μM with a pH of 5.8) and incubated overnight in agitation at 28°C.

For plants transformations, bacterial optical density was measured at 600 nm (OD600) and diluted to obtain a density of 0.1. Each transformation experiment started with fifty seeds per genotype. Around ninety cotyledons per genotype were collected and used for subsequent *Agrobacterium* infections. Of these, fifteen were infected with not transformed *Agrobacterium* cells and used as a control. Seventy-five cotyledons per genotype were used in the co-culture process with each constructed vector (for a total of two hundred and twenty-five cotyledons per genotype).In particular, cotyledons from germinated in vitro plants (MoneyMaker and San Marzano 2) were placed in co-culture with transformed *A.tumefaciens* for 20min, dried using filter paper, and transferred on Petri dishes containing co-culture media (MS basal salt mixture 4.5 g/l, phytoagar 10 g/l, Gamborg vitamin mixture 1 ml/l, sucrose 30 g/l, trans-zeatin 2 mg/l, acetosyringone 200μM and IAA 0.1 mg/l with pH 5.8). Plates were then left in the dark for two days at 24°C. After two days, explants were placed on induction media for organogenesis (MS basal salt mixture 4.5 g/l, phytoagar 10 g/l, sucrose 30 g/l, IAA 0.1 mg/l, trans-zeatin 2 mg/l, Gamborg vitamin mixture 1 ml/l, carbenicillin 500 mg/l and kanamycin 100 mg/l, pH 5.8). Explants were transferred to a fresh medium every 2–3 weeks. Each shoot produced was considered a unique transformation event. A total of 142 putatively transformed plants were transferred in 200ml vessels containing rooting and elongation media, prepared as described in (Muktadir et al., 2016), and adding carbenicillin 500 mg/l and kanamycin 100mg/l as antibiotics and 0.2 mg/l of

indolebutyric acid. And they were grown under long-day photoperiod (16 h light, 8 h dark) at 24°C.

4.4.5 Plant sequencing and analysis

Rooted plants on kanamycin-containing media were assessed for mutations in *SlHyPRPI* and *SlWATI* loci. Samples from MoneyMaker and San Marzano 2 genotypes were collected once the plants were big enough to harvest 150mg of plant material, immediately frozen in liquid nitrogen, and stored at −80 °C to perform genomic DNA extraction. Plant DNA was extracted as described by Murray and Thompson (1980). Extracted DNA was used for *SlHyPRPI* and *SlWATI* PCR amplification using MyTaq™ DNA polymerase (Bioline) and primers sequences reported in supplementary materials. PCR was performed using the follow conditions: initial denaturation 95°Cx 4 min (1x), denaturation 95 °C x 30 sec, annealing 62 °C x 30 sec, extension 72 °C x 45 sec (30x), final extension 72 °C x 8 min (1x). Gel electrophoresis (agarose 1%) was used for PCR evaluation, and positive samples were purified adopting the ExoSAP-IT™ purification kit (ThermoFisher), following the steps described in the manufacturer's protocol. In order to confirm that our T0 plants were edited, purified PCR amplification of *SlHyPRPI* and *SlWATI* loci were subjected to Sanger sequencing. The Sequence evaluations and possible modifications in target loci were analyzed using the ICE algorithm (https://www.synthego.com/products/bioinformatics/crispr-analysis). The analysis of Sanger sequencing data was performed using the Synthego tool "ICE" (www.synthego.com) which provided the total editing efficiency rate and the different percentages of contribution related to each possible sequence (sequences with low percentage values were probably due to noisiness in the Sanger sequencing and were discarded). Primers used for amplification of target genes and sequencing are listed in supplementary materials. Developed seedlings were then moved to the soil for the gradually in vivo acclimation.

4.4.6 Phenotypic analysis of T0 plants

T0 plants growth was measured considering the distance between plants collar and apical meristems. Weekly observations were made for main plant morphological traits.

Mean temperature and humidity recorded in October 2021 to January 2022 were retrieved from the historical range of temperatures at: https://www.3bmeteo.com/meteo/portici/storico.

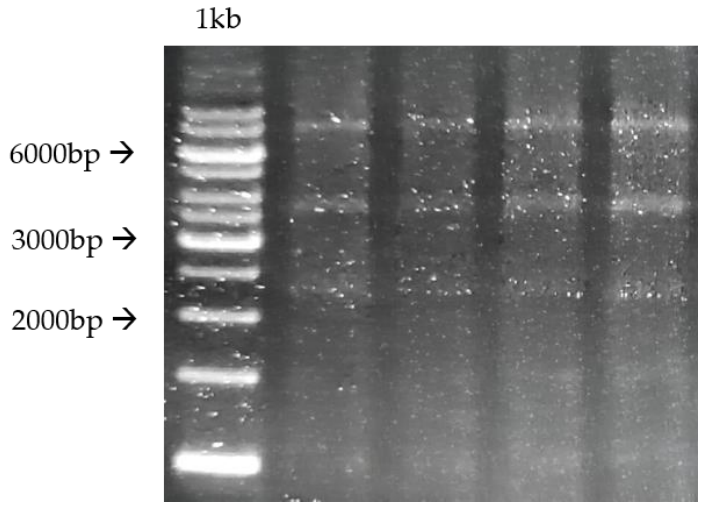
4.5 Supplementary materials

Supplementary Table 3. List of possible off-target genes. In red: mismatch nucleotides. * This predicted off-target is on the same gene of the gRNA. In particular, this sequence overlaps in 14 nucleotides downstream the chosen gRNA.

sgRNA	Off-targets	Mismatches	Position
GGTGAACTCCTTTGTAGTGG	GGTGAACTCCTTTGTtaTGaGGG	3	chr8-16586965
GTATGGCAGAAGCAAAAGTA	GTATGGCAtAtGCAAAAaTATGG	3	chr6-3917123
	GTAcGGCAGAAtCtAAAGTAAGG	3	chr10-21244318
GATCGGTGGAATTCCGATAGGGG	GATtGGTGGAATTCCGATAGGGG*	1	chr12-2923372
GATAGGGGGCAAATCGACAGGGG	None		

Supplementary Table 4. Primers used in this study. * Used for sequencing analysis.

Primer name	Sequence
SlHyPRPI Fw	GGAACTGTGACCGGTGGAAT
SlHyPRPI Rv*	GGCCATATAGCCCCTCATGT
SlWATI Fw*	GATGCCACCACTCCCTGTAT
SlWATI Rv	GGAAAGACAGGCCACAACTT
LB-TDNA Fw	TGGCAGGATATATTGTGGTG
RB-TDNA Rv*	TTACCCGCCAATATATCCTG



Supplementary Figure 2. DNA Enzymatic digestion of four colonies of A. tumefaciens transformed with the CF vector(for simultaneously knockout of SlWATI *and* SlHyPRPI*) . Digestions were performed with the DraI restriction enzyme. As expected, three bands of 7700, 3600, and 2200bp were visible. The intensity of DNA bands was low due to the poor amount of DNA extracted by* Agrobacterium *cells with the standard plasmid DNA extraction kits.*

CHAPTER V

GENERAL CONCLUSIONS

The global population is constantly growing, with nine billion people expected by 2050, while malnutrition and food insecurity remain the leading cause of death in many countries. A rising in food demand between 60% and 100% is expected in the coming years (Fukase and Martin 2020; Pawlak and Kołodziejczak 2020). Tomato (*Solanum lycopersicum*) is grown worldwide, and it is one of the most important crops with global production levels of around 180 million tons. Tomato stresses can induce tremendous production losses, especially in untreated conditions. New plant breeding strategies are needed for developing varieties able to have good performance in limiting conditions. To this purpose, the use of novel technologies to facilitate environmental adaptation against multiple stress could represent a proper solution to ensure high yield levels.

This Ph.D. program was conducted at UNINA in collaboration with the seed company "La Semiorto Sementi" and the CSIC, Valencia-Spain, to develop tomatoes with increased tolerance to biotic and abiotic stresses. To this purpose, three main studies were carried out: 1) the phenotypic, molecular, and *in vitro* evaluation of ten genotypes of company interest for selecting a suitable line to be edited for genetic improvements; 2) the identification of genes conferring resistance or susceptibility to different biotic and abiotic stresses; 3) the selection and knockout of the target genes using CRISPR/Cas9 tech. Results obtained are summarized in the following points:

- A protocol to evaluate the appropriateness of elite lines to be employed in genome editing experiments was set up.

- SanMarzano2, a variety with high commercial value in Italy, resulted as one of the most suitable for our purpose both for its susceptibility to main tomato disease and for its good ability to produce new shoots *in vitro*.

- The regeneration survey also allowed the identification of four genotypes (1006, 1081, 1009, and 1027) with high regeneration rates.

- Several differentially expressed genes involved in multiple stress responses were identified through the cross investigation of twelve transcriptomic experiments of tomato plants exposed to the most common biotic and abiotic stress

- The basal response activated by plants to different stress were elucidated by analyzing both resistant and susceptible genotypes. The data obtained were arranged and explored to set up a strategy for prioritizing genes involved in multiple stress responses.

- Comparison of common DEGs datasets allowed the identification of 176 target genes. Clues on their role were provided for 49 of them. However, the function of the remaining should be experimentally validated.

- The extensive literature search and the transcriptomic investigation allowed the selection of two genes: *WATI* and *HyPRPI*, with complementary functions to reduce the deleterious effects caused by S-gene CRISPR/Cas9 impairments.

- Three vectors for single and simultaneous knockout of target genes were obtained and used for genetic transformations.

- Early phenotyping of *slwatI* and *watI:hyprpI* mutated plants (T0) showed dwarf phenotypes compared to *slhyprpI* plants. In the near future, T2 plants will be stressed with different biotic and abiotic factors to evaluate plant tolerances to multiple tomato stresses.

In conclusion, our work provided a pipeline to quickly and accurately modify specific genes in elite genotypes to improve resistance against various plant stresses. Furthermore, new knowledge on the basal mechanisms activated by plants in response

to different stress has emerged, while new target genes that could be used as a starting point for further investigations related to plant stress-response, using biotechnological approaches, were pointed out.

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