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CYCLE

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Development and Application of Genome Editing Tools in *Solanaceae*

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CONTENTS

Experimental works described in this thesis are part of scientific papers published or submitted to international journals:

Chapter I:

CRISPR/Cas9 mediated knock-out of SlDMR6-1 in tomato promotes a drought-avoidance strategy and increased tolerance to Late Blight

Maioli, A., De Marchi, F, Valentino, D., Gianoglio, S., Patono, D., Miloro, F., Bay, Y., Comino, C., Lanteri, S., Lovisolo, C., Acquadro, A., Moglia, A. (2023). *Submitted*.

Chapter II:

Simultaneous CRISPR/Cas9 Editing of Three PPO Genes Reduces Fruit Flesh Browning in *Solanum melongena* L.

Maioli, A., Gianoglio, S., Moglia, A., Acquadro, A., Valentino, D., Milani, A. M., Prohens, J., Orzaez, D., Granell, A., Lanteri, S., & Comino, C. (2020). *Frontiers in Plant Science*, *11*.

Chaper III:

Setting up of Virus-Induced Genome Editing (VIGE) protocol in eggplant.

Maioli, A., Nagalakshmi, U., Dinesh-Kumar, S. P., Moglia, A. The results of this chapter are currently in a draft paper that is *under preparation*.

INTRODUCTION

Plant breeding

For millennia, humans have been engaged in a rudimentary form of plant breeding, inadvertently selecting and propagating plants with desirable traits through traditional farming practices. However, the scientific progresses of genetics in the late 19th and early 20th centuries propelled plant breeding into a more systematic and effective endeavor. Traditional plant breeding methods have served as the backbone of crop improvement for centuries, relying on the natural genetic variation available in plants to develop new varieties with desirable traits. These methods have played a crucial role in meeting the needs of a growing population. Introduction of the semidwarf gene, semi-dwarf1 (sd1), which encodes a gibberellin biosynthesis enzyme, made significant contributions to the "green revolution" in rice.

However, the traditional methods present some important limitations. First they rely on natural genetic variation within a species or its closely related relatives (Rommens et al, 2007). While this variation can be extensive, it is not limitless. The available genetic diversity might not encompass all the desired traits or combinations of traits required to address specific challenges. For example, traits like resistance to certain biotic diseases or tolerance to extreme environmental conditions may be absent or limited within the existing gene pool, making it difficult to breed for these traits through traditional crossing.

Another limitation lies in the time and resources required to develop new varieties through traditional breeding approaches (Rommens et al, 2007). It typically takes several years, if not decades, to breed and select plants with the desired agronomic traits. This long process is due to the need for repeated cyclesa of crossing, selection, and backcrosses to stabilize the

desired traits and eliminate unwanted genetic backgrounds (due to the linkage drag phenomena). Additionally, traditional breeding often involves the screening of a huge populations of plants to increase the chances of finding desirable individuals, requiring substantial land, labor, and financial resources.

Furthermore, traditional plant breeding methods are subjected to the laws of Mendelian genetics, based on random assortment and recombination of genes during sexual reproduction. While these phenomena contribute to genetic variability, it might introduce unpredictability and limitations in controlling the inheritance of specific genetic traits (Rommens et al, 2007). Breeders may face challenges in transferring traits from one generation to the next one or maintaining the desired combination of traits, especially when dealing with complex genetic traits influenced by multiple genes such as quantitative ones. Another crucial factor to consider is the limitations imposed by plant reproductive biology (Lopez-Caamal et al - 2014). Some plants have unique reproductive mechanisms, such as self-incompatibility or complex pollination requirements, that hamper the successful hybridization or cross-breeding of desired traits.

In recent years, technological advancements in molecular biology and biotechnology have mitigated some of the key limitations imposed by traditional breeding methods. Techniques like marker-assisted selection (MAS) and genetic engineering allow to identify and transfer specific genes or genomic regions associated with desired traits. These tools enable faster and more precise breeding outcomes by bypassing the need for extensive phenotypic screening or relying solely on natural genetic variation (Rommens et al, 2007). However, it's important to note that these genetic engineering techniques have their own set of limitations, including regulatory concerns and public acceptance (Becker et al, 2023). Another

important limitation in plants' genetic engineering is the requirement of an established protocol for Agrobacterium tumefaciens-mediated stable transformation. These protocols are species and genotype specific and require an important amount of time and resources to be fully developed.

Genome editing

In recent years, plant breeding research has witnessed a groundbreaking advancement in the field of genome editing. This revolutionary technology allows scientists to make precise and targeted modifications within the plant genome, offering unprecedented control over their traits and characteristics. Compared to traditional breeding methods, genome editing holds immense promise for accelerating crop improvement and addressing agricultural challenges in an efficient and targeted manner (Figure 1).

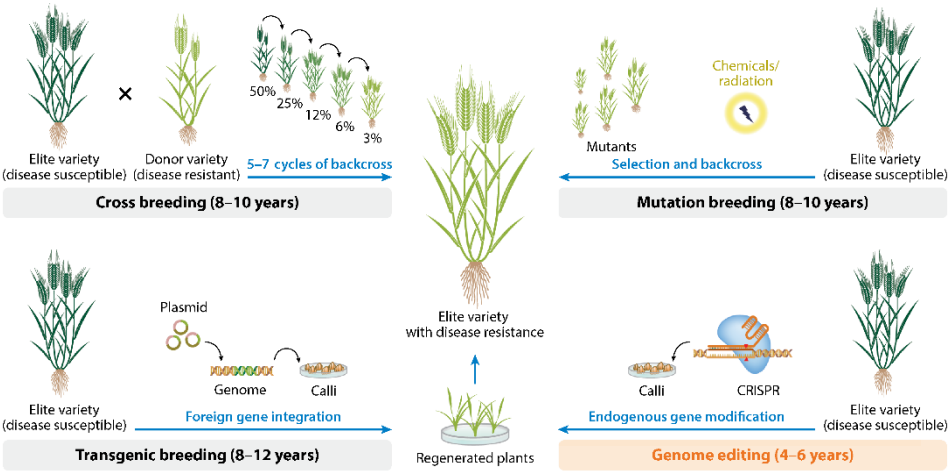

Figure 1: breeding methods and time required (Chen et al, 2019)

Genome editing offers plant breeders an array of possibilities for crop improvement. It has been already applied to crops for the improvement of agronomic traits, including enhanced resistance to diseases, altered

nutritional composition, increased potential for higher yields, and strengthened ability to withstand environmental challenges (Das et al, 2023). By directly manipulating specific genes or regulatory elements, breeders can accelerate the development of new plant varieties with desired traits, potentially bypassing the long and unpredictable process of traditional breeding.

At the heart of genome editing lies the powerful tool CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9; Figure 2).

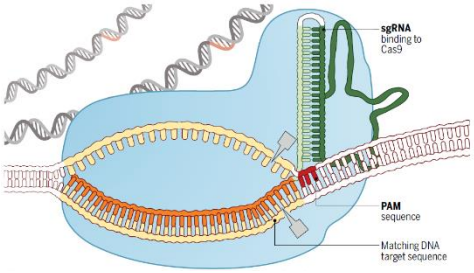

Figure 2: Cas9 sgRNA–mediated DNA recognition and cleavage (Doudna et al, 2014)

This system, adapted from the bacterial defense mechanism against viral infections (Doudna & Charpentier, 2014), has been repurposed as a precise and versatile molecular tool for the genome editing of various organisms, including plants (Jinek et al, 2012; Belhaj et al, 2015). The process of genome editing begins with the design and synthesis of a small RNA molecule called single guide RNA (sgRNA). This sgRNA serves as a molecular guide, directing the Cas9 enzyme to the target gene sequence. Once the Cas9 enzyme is bound to the target gene, it introduces a double-strand break (DSB) at the desired genome location. The plant's natural DNA repair mechanisms then intervene. Plant’s cell can rejoin the break by two processes (Jiang & Doudna, 2017): non-homologous end joining

(NHEJ) or homologous recombination (HR). Through NHEJ the damaged DNA is just reconnected by small insertions or deletions leading to a functional *knock-out*, while by HR a DNA molecule ("donor DNA") homologous to the damaged region is used as template to reconstruct the target region (Figure 3). If the donor DNA is correctly designed, it is possible to substitute a portion of the sequence or insert a new one (*knock-in*).

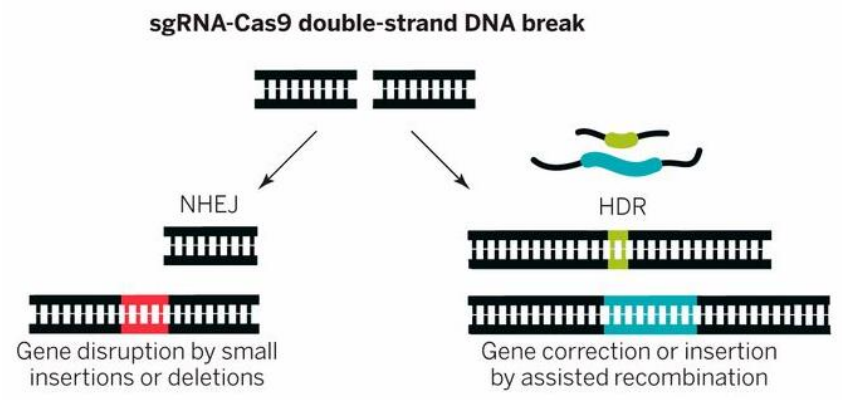

Figure 3: cell's reparation system upon Double strand break (Doudna & Charpentier, 2014)

New Cas variants are arising to widen the possible inducible modification (summarize in Tao et al, 2023). It is possible to induce precise single-nucleotide modification through base editing. It relies on a modified Cas protein, able to nick the non-edited DNA, bound to a single-strand-specific cytidine or adenine deaminase (Gaudelli et al, 2017). This directs cellular DNA repair machinery to replace the modified nucleic acid inducing a modification: from C-G → T-A (cytosine base editing) or A-T → G-C (adenine base editing). It is also possible to edit RNA. This process is based on an adenosine deaminase acting on RNA (ADAR) fused to dCas13. This can achieve precise adenosine-to-inosine editing of target

RNA. Due to its affinity for RNA, Cas13 can also be used for gene knock-down. The mechanism relies on a sgRNA targeting a RNA that will be degraded by Cas13, therefore reducing its level in the cells (Tao et al, 2023). Unlike genome editing, which is permanent, the effects of RNA editing are transient and reversible (Tao et al, 2023). Another interesting option is represented by prime editing. This revolutionary approach allows the reverse transcription of a sequence (provided by a modified sgRNA called pegRNA) directly into the target locus, introducing single or multiple base substitutions, indels or long deletions (Tao et al, 2023).

One of the major advantages of genome editing is its ability to create targeted modifications without introducing foreign sequences from unrelated/sexually incompatible organisms. Unlike traditional genetic engineering, which often relies on the insertion of foreign transgenic DNA, genome editing focuses on making changes within the plant's own genetic material. The versatility of the CRISPR-Cas9 system also allows breeders to target multiple genes simultaneously or sequentially, opening up new possibilities for complex trait modifications and trait stacking within a single plant variety.

CRISPR/Cas9 technique has been already applied to a wide range of crop species, including major staples like rice (Sun et al, 2016), wheat (Gil-Humanes et al, 2016), corn (Svitashev et al, 2015), soybeans (Li et al, 2015), tomato (Cermak et al, 2015) and potato (Butler et al, 2016).

The most solid application of CRISPR/Cas9 in plant is based on *Agrobacterium tumefaciens*-mediated stable transformation. In this process plant explants are transformed by the bacterium and regenerated on selective media. While protocols are available for different plant species, its feasibility for research is at the moment suffering for different flaws. Major issues are represented by the recalcitrance of most of the

species to *Agrobacterium tumefaciens* transformation, the integration of transgenes, low editing efficiency and the availability of an *in vitro* regeneration protocol (Antony Caesar & Ignacimuthu, 2023). Although concerns regarding off-target effects were common about genome editing by CRISPR/Cas9, several studies have consistently shown minimal to no impact on non-targeted loci within the DNA (Sturme et al, 2022).

New frontiers of genome editing

Novel approaches to genome editing application in plant are focused on alternative methods of CRISPR/Cas9 elements delivery into plant tissues. The main goal is to obtain transgene free plant. For sexually propagated plant it is possible to get rid of any foreign DNA through genetic segregation, while in clonally propagated plant or in plant with long life cycle this approach could be unfeasible. To overcome this problem different technological solutions have been proposed.

Ribonucleoprotein (RNPs) transfection of protoplasts represents a validated method to obtain transgene-free edited plants and has been already applied in different plant species (summarized by Zhang et al, 2021). It relies on protoplast transfection with pre-assembled Cas9 protein and gRNA complex, which acts in the cells before being degraded swiftly by endogenous proteases.

A different strategy is based on the utilization of nanoparticles (NPs) in plant genome editing. NPs usage has shown significant potential in improving the efficiency of delivering biomolecules into plant cells. NPs act as carriers or delivery vehicles for key components involved in genome editing, such as CRISPR-Cas9 complexes, guide RNAs, or DNA templates (Vats et al, 2022).

One of the primary benefits of using NPs in plant genome editing is their ability to protect and deliver these biomolecules to the desired genome target sites within plant cells. NPs can encapsulate and shield the biomolecules, safeguarding them from degradation or inactivation during the delivery process. This protection ensures that the editing components reach the intended location and increase their chances of successfully modifying plant's genome.

Various types of NPs have been explored for plant genome editing, including liposomes, polymeric NPs, metallic NPs, and carbon-based NPs (Vats et al, 2022). Each type of NP offers unique properties and advantages, such as size, surface charge, stability, and biocompatibility. The choice of NP depends on the specific requirements of the experiment or application.

Despite NPs have been more commonly used on protoplasts, this method appears to be feasible also for other tissues (Vats et al, 2022).

However, the primary challenge in achieving edited plants lies not in the delivery step but in the tissue response to *in vitro* regeneration. Both RNPs and NPs indeed require successful tissue regeneration for the recovery of edited plants. This process can be extremely tricky and requires a protocol adapted to the species and the genotype under research.

The establishment of a highly efficient and genotype-independent plant *in vitro* regeneration system is crucial for an easier application of genome editing in crop improvement (Maren et al, 2022).

To enhance or enable regeneration efficiency, the ectopic overexpression of developmental regulators (DRs) emerged as a potential solution (Maren et al, 2022). The regulators operate in concert with the hormones within the cultivation media to induce the totipotency of the tissue leading to organogenesis or embryogenesis (Gordon Kamm et al, 2019). Different

DR have already been used for this purpose, as Baby boom, Wuschel (Lowe et al, 2016) or GRF and its cofactor GIF1 (Debernardi et al, 2020). DRs-enhanced regeneration approach has been applied to a wide range of species, like *Arabidopsis thaliana* (Daimon et al, 2003), *Capsicum annuum* (Heidmann et al, 2011), *Nicotiana tabacum* (Kyo et al, 2018) and *Solanum lycopersicum* (Maher et al, 2020).

An interesting alternative method for genome editing is the usage of viral constructs in the process named Virus Induced Genome Editing (VIGE). Viral vectors, such as plant viruses or modified viral systems, can be engineered to carry the necessary editing machinery, including CRISPR-Cas9 components or other genome editing systems (Figure 4).

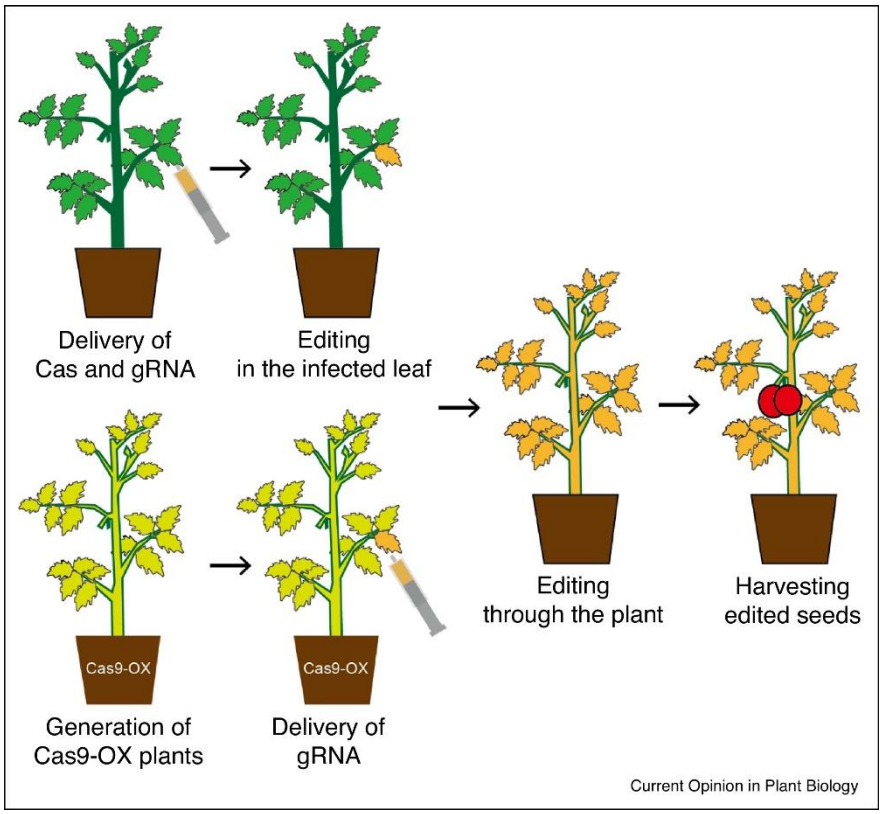

Figure 4: VIGE workflow (Oh et al, 2021)

One advantage of viral constructs in plant genome editing is their ability to infect a wide range of plant species and cell types, including those that are traditionally difficult to be transformed or regenerated. Moreover, due to their self-replication ability and efficient systemic movement through plant tissues, viruses are emerging as a highly effective alternative for the transient delivery of CRISPR-Cas reaction components into entire plants (Uranga & Daros, 2023). The first VIGE publication dates back to 2015 (Ali et al, 2015). In this research, **Δ***PDS3* edited *Nicotiana benthamiana* lines have been obtained using a Tobacco Rattle Virus (TRV) – based construct. While the gRNA was carried by the virus, Cas9 was instead expressed by the plant itself thanks to a previous stable transformation.

The need of plants expressing Cas from their genome is based on the cargo capacity of the viral construct. Space inside viral particles is an important limiting factor and only few viruses are able to carry Cas9 gene within their genomes.

Even if constructs based on different viruses have already been used to obtain editing (summarized in Uranga & Daros, 2022), TRV remains one of the most interesting option due to its extensive use.

An interesting possibility is to use viral construct to edit germinal line's cells in fully grown plant and therefore obtain completely edited plants from seeds (Dinesh-Kumar & Voytas, 2020; Figure 4).

Since viruses are rarely able to move into meristems, it is necessary to move and express editing machinery in that tissue. It is possible to move gRNA or Cas9's mRNA into meristems using so called "Mobile Element" (ME), genes whose RNA can be found inside floematic stream. The fusion of ME with gRNA or Cas9 can lead to a targeting into meristems and editing in germline tissue. This approach resulted in successful in editing *Arabidopsis thaliana* (Nagalakshmi et al, 2022) and *Nicotiana*

benthamiana (Ellison et al, 2020) by TRV-based construct. In both the cases, plants were already expressing Cas9.

Research is now focusing on the widening of Cas proteins available for editing. Cas12e and Cas12j, formerly known as CasX and CasΦ, could be small enough to be adapted for VIGE using TRV, although they have been used only in animal model (Liu et al, 2019; Pausch et al, 2020).

Genome editing in *Solanaceae* crop: state of the art

For centuries, numerous plant species from *Solanaceae* family have been used as important crops, such as tomatoes, potatoes, peppers, and eggplants. These are staple food for many populations worldwide and contribute to food security by providing a reliable source of calories, essential nutrients, and nutraceutical compounds. Even though is not consumed as food, also tobacco represents an important economic crop. Tomato and tobacco are also a fundamental model species in genomics and biological research (Liu et al, 2022; Zan et al, 2023).

A reference genome is available for all these plants (Bombarely et al, 2012; Hosmani et al, 2019; Barchi et al, 2021). Although genomic information allows easier research for breeding, it is still necessary to further expand the gene functional characterization. Research progress and genomic tools available strongly differ in Solanaceae species.

Tomato (*Solanum lycopersicum* L.) is the most economic important *Solanaceae* crop. It is considered a model species for different biological research (Liu et al, 2022). Its genome is available since 2012 (The Tomato Genome Consortium, 2012) and it is constantly updated (Hosmani et al, 2019). Genome editing in tomato is well established. It has been applied in tomato since 2014 (Brooks et al. 2014; Lor et al. 2014) and has greatly facilitated the characterization of gene function and precision breeding.

Genome editing has been carried out in tomato for improving fruit yield and quality, increasing stress resistance, accelerating the domestication of wild tomato, and recently customizing cultivars for urban agriculture (Xia et al. 2021).

The advancement of research is reflected in the array of tools already available in tomato. Indeed, protocols for CRISPR/Cas9 mediated by *Agrobacterium*-transformation are well consolidated, and new alternative technical approaches such as protoplast transient RNP transfection (Liu et al, 2020) and VIGE (Dahan Meir et al, 2018) have been already published. *N. benthamiana*'s genome was published as a draft version in 2012 (Bombarely et al, 2012). Because of the fast growth, small dimensions and ease of growth in climate chamber, this plant has been used as a model species for developing genome editing tools. Protocols for ribonucleoprotein (RNP) transformation of protoplasts (Hsu et al, 2021) and VIGE (Ali et al 2018; Ellison et al, 2020) have been already published. It is noteworthy that Ellison et al, 2020 obtained an edited transgene free line by using TRV-based construct. Also, in *N. tabacum* these genome editing tools are already feasible (Gao et al, 2015; Woo et al, 2015; Baltes et al, 2014).

Potato (*Solanum tuberosum* L.) is the second most important *Solanaceae* crop after tomato. Genome editing has been achieved by stable transformation (Wang et al, 2015), protoplast RNP transfection (Andersson et al, 2017) and VIGE (Butler et al, 2016).

The progress in the development of editing tools in pepper (*Capsicum annuum* L.) and eggplant (*Solanum melongena* L.) lags behind compared to other *Solanaceae*.

Even if pepper's first genome was published in 2014 (Kim et al, 2014), genome edited lines in this species have not been obtained yet. Due to the

recalcitration to *in vitro* regeneration process, only edited calli through stable transformation (Park et al, 2021) or edited protoplasts by RNPs transfection (Kim et al, 2020) were obtained.

Despite its growing economic relevance, genetic tools in eggplant's have been developed only recently (Gramazio et al, 2018). Eggplant's draft genome was released in 2014 (Hirakawa et al, 2014) and improved in 2021 (Barchi et al, 2021).

At present, the progress of CRISPR/Cas9-mediated genome editing in this species is falling behind when compared to the advancements made in tomato and potato. Two are the examples of gene editing published so far (Maioli et al, 2020, Kodackattumannil et al, 2023). Maioli et al, 2020 (as described in Chapter 2) achieved to edit eggplant by CRISPR/Cas9 system for the first time. It has been carried out through *Agrobacterium*-mediated stable transformation of cotyledons explants followed by *in vitro* regeneration. Editing strategy was focused on a single sgRNA targeting three genes of PPO family (*SmelPPO4*, *SmelPPO5*, *SmelPPO6*). In eggplant regeneration process is challenging because of polyphenol oxidation and vitrification (Kodackattumannil et al, 2023). Despite that, edited regenerated plantlets (T_0) were recovered.

The development of novel genetic tools in eggplant has the potential to significantly enhance comprehension of gene functions and revolutionize plant breeding strategies. This advancement holds the promise of shedding light on gene roles and facilitating a more effective approach for the improvement of eggplant varieties.

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AIMS AND THESIS STRUCTURE

The aim of my PhD Thesis was to develop and apply new approaches for genome editing in *Solanaceae*. While several genome editing techniques were already available in tomato, no examples of gene editing were described in literature at the beginning of my PhD activity. Progresses in genome editing could offer the opportunity to clarify gene functionalities and enable a more efficient strategy for enhancing crop varieties.

Chapter I: **CRISPR/Cas9 mediated knock-out of *SlDMR6-1* in tomato promotes a drought-avoidance strategy and increased tolerance to Late Blight**

The DOWNY MILDEW RESISTANCE 6 (DMR6) protein is a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase, involved in salicylic acid (SA) metabolism. In tomato its inactivation was found to increase SA levels and to confer disease-resistance against several pathogens. Due to the potential role of SA as an abiotic stress-tolerance enhancer, we conducted experiment to test the tolerance to drought stress in SlDMR6-1 tomato mutants generated through the CRISPR/Cas9 technique. The results of this chapter are drafted and are currently **under peer review process**.

Chapter II: **Simultaneous CRISPR/Cas9 editing of three PPO genes reduces fruit flesh browning in *Solanum melongena* L.**

This chapter describes the first example of use of the CRISPR/Cas9 system for mutagenesis studies in eggplant. The polyphenol oxidase enzymes (PPOs) are implicated in undesirable enzymatic browning of eggplant fruit due to the oxidization of polyphenols after cutting. Three genes belonging

to PPO gene family were edited (*SmelPPO4*, *SmelPPO5* and *SmelPPO6*) and their role on fruit's flesh browning elucidated.

The results of this chapter have been published:

Maioli, A., Gianoglio, S., Moglia, A., Acquadro, A., Valentino, D., Milani, A. M., Prohens, J., Orzaez, D., Granell, A., Lanteri, S., & Comino, C. (2020). Simultaneous CRISPR/Cas9 Editing of Three PPO Genes Reduces Fruit Flesh Browning in Solanum melongena L. *Frontiers in Plant Science, 11*. **https://doi.org/10.3389/fpls.2020.607161**

Chapter III: **Setting up of Virus-Induced Genome Editing (VIGE) protocol in eggplant**

To overcome both *in vitro* regeneration bottleneck and transgene integration, an intriguing solution might be represented by the usage of viral constructs to deliver CRISPR/Cas machinery, at the basis of Virus-Induced Genome Editing (VIGE). This chapter describes the first example of VIGE application in eggplant based on the use of Tobacco Rattle Virus (TRV). To maximize viral expression and persistence inside the plant tissue, we first optimized VIGS approach previously developed in eggplant by targeting a reporter gene (*SmelChl*). The results of this chapter are currently in a draft paper that is **under preparation**.

CHAPTER I

CRISPR/Cas9 mediated knock-out of *SlDMR6-1* in tomato promotes a drought-avoidance strategy and increased tolerance to Late Blight

ABSTRACT

The DOWNY MILDEW RESISTANCE 6 (DMR6) protein is a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase, involved in salicylic acid (SA) metabolism, and its inactivation in tomato was found to increase SA levels and to confer disease-resistance against several pathogens. SA is also recognized as an abiotic stress-tolerance enhancer, and we tested the resistance to drought stress in *SlDMR6-1* tomato mutants generated by the CRISPR/Cas9 technique.

Wild-type (WT) tomato cultivar 'San Marzano' and its *Sldmr6-1* mutants were subjected to water deprivation for 7 days. While WT plants exhibited severe wilting, the T_2 *Sldmr6-1* mutant plants showed fairly turgid leaves and maintained higher soil Relative Water Content (RWC). Ecophysiological measurements highlighted that *Sldmr6-1* mutants adopted a water saving behaviour reducing both assimilation rate (A) and transpiration rate (E), by stomatal conductance (Gs) reduction, while still increasing Water Use Efficiency (WUE). This made it possible to support a proper photosynthetic metabolism under drought stress, resulting in no alteration of the CO_2 concentration in the sub-stomatal chamber (Ci). Compared to WT, the *Sldmr6-1* mutants under drought stress showed up-regulation of the genes *SlPOD* and *SlGST* (anti-oxidant related) as well as down-regulation of *SlCYP707A2* gene, which is involved in ABA catabolism. Our results suggest that the disabling of *SlDMR6-1* in tomato plants leads to a drought-avoidance strategy through tight control of stomatal closure and thus reduction of water loss. Furthermore, it was

highlighted, for the first time in tomato, that *Sldmr6-1* mutants showed reduced susceptibility to *Phytophthora infestans*, the causal agent of Late Blight.

INTRODUCTION

Rising temperatures and changes in precipitation patterns are a serious problem in many parts of the world, causing dramatic reduction of water availability during the crop growing season. Drought stress represents one of the key limiting factors affecting plant growth and concurs with plant pathogen or pest attacks to cause severe plant yield reductions (Borràs et al., 2021; Cappetta et al., 2020). World food security in the coming years will hence largely depend on the availability of biotic and abiotic stress-tolerant plants. In the past, research has been focused on elucidating the mechanisms involved in the response to individual stress, and more recently on identifying new forms of resistance to multiple stress, and several genes participating in recognizing both biotic and abiotic stresses have been characterized (Saijo & Loo, 2020; Sunarti et al., 2022). Tomatoes suffer severe yield losses due to both abiotic and biotic stresses (Kissoudis et al., 2016), thus the development of elite genotypes endowed of tolerance towards them is a major objective for tomato breeders (Egea et al., 2022).

To this end, a significant contribution can be provided by the emergent CRISPR/Cas9 technology for genome editing, which may greatly contribute to precision breeding and reducing product development costs compared to complex, imprecise and lengthy conventional breeding strategies (Lassoued et al., 2019; Zhu et al., 2020). Genome editing has been applied to tomato since 2014 (Brooks et al., 2014; Lor et al., 2014), and has greatly facilitated the functional characterization of genes involved

in fruit yield and quality, development and ripening, stress response and domestication processes (Vu et al., 2020; Lobato-Gómez et al., 2021; Salava et al., 2021).

Negative regulators of abiotic stress response pathways have been targeted in tomato through CRISPR/Cas9, and key genes involved in drought (*SlLBD40*, *SlMAPK3*, *SlNPR1*, *SlARF4*, *SlGID1a*), salinity (*SlARF4*, *SlHyPRP1*) and chilling stress (*SlCBF1*, *SlBZR1*) response have been identified (Bouzroud et al., 2020; Salava et al., 2021; Tran et al., 2021; Yin et al., 2018).

One mechanism of pathogen plant resistance is due to the loss-of-function of genes required for the onset of pathogenesis, referred to as plant susceptibility (S) genes (Pavan et al., 2010), which have been proposed as key targets for genome editing approaches (Engelhardt et al., 2018; Zaidi et al., 2018; Chaudhary et al., 2022). A classic example of a S-gene is Mildew resistance locus O (*Mlo1*) (Bü & Hollricher, 1997), of which loss-of-function natural mutants have been used for over 70 years in barley breeding programs (Piffanelli et al., 2002).

In tomato, successful examples of CRISPR/Cas9 genome editing aimed at disabling S-genes have been reported to confer resistance against different classes of pathogens such as viruses (Atarashi et al., 2020; Yoon et al., 2020; Kuroiwa et al., 2022), bacteria (Ortigosa et al., 2019; Paula De Toledo Thomazella et al., 2021), fungi (Nekrasov et al., 2017; Santillán Martínez et al., 2020; Salava et al., 2021) and oomycetes (Paula De Toledo Thomazella et al., 2021; Li et al., 2022).

One of the most intriguing S-genes is DOWNY MILDEW RESISTANCE 6 (*DMR6*), encoding a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase, which has a salicylic acid (SA) 5-hydroxylase activity and thus reduces the active SA pool (Van Damme et al., 2008; Zhang et al., 2017).

In tomato, *Sldmr6-1* mutants display enhanced resistance against evolutionarily distinct classes of pathogens: bacteria, oomycetes, and fungi (Paula De Toledo Thomazella et al., 2021). CRISPR/Cas9 knock-out mutants of *DMR6* have been generated in different species such as *Arabidopsis thaliana* (Zeilmaker et al., 2015), *Vitis vinifera* (Giacomelli et al., 2022), *Ocimum basilicum* (Hasley et al., 2021), *Musa spp.* (Tripathi et al., 2021), *Solanum tuberosum* (Kieu et al., 2021), and *Citrus spp.* (Parajuli et al., 2022).

In tomato, impairment of *SlDMR6-1* has resulted in resistance to *Pseudomonas syringae*, *Xanthomonas gardneri*, *Xanthomonas perforans*, *Phytophthora capsici* and *Pseudoidium neolycopersici* (Paula De Toledo Thomazella et al., 2021; Kieu et al., 2021) have also reported that inactivation of the potato *DMR6-1* gene resulted in plants with increased resistance to Late Blight (LB) caused by *Phytophthora infestans.* Notably, disease resistance correlates with increased SA levels and transcriptional activation of immune responses (Paula De Toledo Thomazella et al., 2021).

SA is a natural phenolic compound and a signalling regulator, which plays various regulatory roles in mediating plant development, growth, and defences to environmental stresses. SA has been shown to improve plant tolerance to major abiotic stresses such as salinity, metal, osmotic, drought and heat stresses (Khan et al., 2015; J. Liu et al., 2022). Furthermore, it has been observed that exogenous application of SA in low concentration in tomato could mitigate the oxidative stress generated by the water stress (Chakma et al., 2021; Aires et al., 2022). Interestingly, tomato plants pre-treated with SA and submitted to water deficit had an improved water-use efficiency and net photosynthetic rate, leading to an increased fruit weight, fruit numbers, and biomass (Lobato et al., 2021).

In this study, we applied CRISPR/Cas9 editing for disabling the *SlDMR6-1* gene in the tomato 'San Marzano' variety. We assessed both the overall mutational status and potential unintended off-target effects through Illumina whole genome sequencing (WGS) of one selected T_1 mutant, characterized by *Cas9* absence. Due to the link between SA and drought stress response, we tested the potential drought resistance of *Sldmr6*-1 tomato mutants. In addition, we characterized their tolerance to Late blight, a devastating disease that can destroy an entire unprotected tomato crop within 7–10 days after infection.

MATERIALS AND METHODS

Target identification and vector construction

Three gRNAs (Data S1) targeting the first three exons (Figure S1) of *SlDMR6-1* (ID Solyc03g080190) were designed using the online tool CRISPR-P 2.0 (hzau.edu.cn). The transformation vector was assembled through a Golden Braid (GB) cloning system (Sarrion-Perdigones et al., 2011, Sarrion-Perdigones et al., 2013; Vazquez-Vilar et al., 2017; Maioli et al., 2020) following GB software-directed procedures (https://gbcloning.upv.es/). Within the vector, the expression of h*Cas9* and *NptII* was driven by the CaMV 35S and *nos* promoters, respectively, while the gRNAs were placed in a polycistronic gRNA array under the control of the *At*U6-26 RNA PolIII promoter.

Plant material and genetic transformation

Seeds of the cultivar 'San Marzano' were provided by Agrion (www.agrion.it) and were maintained in the Germplasm Bank of DISAFA (University of Torino, Italy). Fifty tomato seeds were sterilized in 2.5% sodium hypochlorite soaking for 20' and then rinsed in sterile water three

times. Sterile seeds were placed on sterile germination medium (1/2 MS + 15 g/l sucrose + 8 g/l plant agar) in plastic boxes, that were kept at 25°C in the dark for 72h before being transferred to a day/night cycle of 16/8 hours. After 10 days, plantlets presented fully grown cotyledons that were used for plant genetic transformation.

The final vector *pDGB3_alpha1_Tnos:NptII:Pnos_U6-26:tRNA:gRNA1-2-3_P35S:hCas9:Tnos* was transformed by heat shock into the *Agrobacterium tumefaciens* LBA4404 strain. Bacteria inoculum was prepared as follows. On the first day, *A. tumefaciens* was cultured in MGL (Data S1) added with streptomycin 50mg/l and kanamycin 50mg/l and incubated at 28°C ON. On the second day, an aliquot of the culture was inoculated (1:50) in TY (Data S1) supplemented with 200µM acetosyringone and incubated at 28°C ON. The optical density of the culture at 600nm (OD_{600}) was checked and the bacterial culture was diluted to a final OD_{600} of 0.10-0.15 in TY medium supplemented with 200µM acetosyringone. Cotyledons of the seedling were cut in pieces of about 0.5 cm, which were dipped in bacterial culture for 10', blotted dry on sterile paper and placed for 48h on a co-culture medium in the dark. Callogenesis, shoot induction, elongation and rooting were obtained as previously described (Qiu et al., 2007). After regeneration, fully developed plantlets (T_0 plants, Data S1) were moved to soil and gradually acclimated to *ex vitro* conditions. By selfing T_0_2 plant (selected on the basis of editing outcome, Data S1), 14 plantlets were obtained (T_1 generation) (Data S1). T_2 plants were obtained from selfing of T_1_6 (homozygous, Cas positive) and T_1_7 plant (homozygous, Cas free).

Molecular Screening

Genomic DNA from T_0/T_1 plants' leaves was extracted using E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek, Norcross, USA) following instruction. The screening for h*Cas9* presence was performed using primers reported in Data S1 by PCR using KAPA HIFI Taq (Kapa Biosystems, Boston, USA) with the following program: 95°C/3', 30 cycles of 98°C/15'', 60°C/20'', 72°C/1' and 72°C/5'.

Mutation frequencies at the target sites were evaluated through PCR amplification using primers designed on gRNAs flanking regions (Data S1). DNA was amplified using KAPA HIFI Taq with the following PCR program: 95°C/3', followed by 30 cycles of 98°C/20'', 60°C/20'', 72°C/45'' and 72°C/5'. After purification with DNA/RNA Clean Up E.Z.N.A.® kit (Omega Bio-Tek, Norcross, USA), PCR products were sequenced by Sanger method. Chromatograms were analyzed using the TIDE online tool (Brinkman et al., 2018).

Whole Genome Sequencing and analysis

A T_1 plant (T_1_ 7), showing a homozygous mutation at two target gRNA regions and h*Cas9* segregation, and a wild type (WT) plant underwent a WGS analysis with an Illumina sequencer (Illumina Inc., San Diego, USA). One µg of DNA was used for the preparation of short insert (length 350 bp) genomic libraries (Novogene, Hong Kong), which were sequenced with paired-end chemistry (2×150 bp). Raw reads were cleaned with Scythe (v0.991, https://github.com/vsbuffalo/scythe) in order to remove contaminant residual adapters and Sickle (v1.33, https://github.com/najoshi/sickle), which allows poor quality ends reads to be removed with (Q < 30). Raw data were submitted to NCBI as SRA files (Project: PRJNA846963).

A *de novo* genome assembly was performed through MegaHit assembler (v1.2.9, https://github.com/voutcn/megahit), using specific parameters (k-min = 27, k-max= 141, k-step = 10, disconnect-ratio = 0 and cleaning-rounds = 1). A Blast analysis was performed on the assembled scaffolds (T_1 and WT) to identify any possible insertion, with the T-DNA sequence as a query.

Target, off-target analyses and SNP statistics

The identified target genomic variants and allele frequencies were examined with CRISPResso2 (http://crispresso2.pinellolab.org, Clement et al., 2019) using fastq reads extracted in the 100 bp window surrounding each gRNA. The CasOT script (Xiao et al 2014, https://github.com/audy/mirror-casot.pl) was run to identify any putative off-target region in the tomato genome (SL4.0). All the designed gRNAs were considered as bait in a single-gRNA mode, with default PAM type (NGG=A) and specific numbers of permitted mismatches in the non-seed (2) seed (2) regions allowed. All the candidate target/off-target genomic region coordinates were intersected with the vcf file through the bedtools (https://bedtools.readthedocs.io) intersect command to filter-out monomorphic regions among edited and WT plants. Results were inspected through custom bash scripts.

Clean reads obtained from the edited plants were mapped to the tomato reference genome (SL4.0, https://solgenomics.net) using the Burrows-Wheeler Aligner (BWA, v0.7.17,https://sourceforge.net/projects/bio-bwa/files) program and the 'mem' command with the default parameters. BAM files were processed and used for the SNP calling by means of Samtools (v1.9-166-g74718c2) mpileup using default parameters except

for the minimum mapping quality (Q = 20) and filtering out multimapping events (-q > 1). A vcf (variant call format) file was produced.

Evaluation of agronomic traits

Nine WT and nine T_2_7 plants were grown in a greenhouse (mean temperature 25°C), in 10 l pots containing a substrate mixture of horticultural substrate and perlite (3:1 v/v) from March to September 2022. Standard horticultural practices were applied. Experimental plots were arranged in a randomized complete block design with nine replications. Two different classes of variables were analyzed: growth variables (plant height) and yield component variables (fruit weight; number of fruits per plant). Fruits were collected at the commercial ripening stage. Each value represented the mean of nine biological replicates compared through a one-way analysis of variance (ANOVA) test ($p \leq 0.05$).

Drought stress analysis

Six WT, six T_2_6 and six T_2_7 plants were grown in a climate chamber (temperature 25°C, RH 60%, 16 h light: 8 h dark photoperiod cycle, light intensity of 300 µmol m^{-2} s^{-1} PPFD) in pots containing perlite and soil-substrate (van Egmond universele potgrond) 1:4 v/v (Figure S2). A quote of this soil was used to determine the maximum water holding capacity of the pots (Patono et al., 2022). Plants were grown in a well-watered status by watering to field capacity (above 75% of soil relative water content SRWC, daily at 8 am) for 6 weeks prior to the experimental imposed drought. Starting the drought, plants were allowed to slowly develop water stress by withholding irrigation. Steady state measurements of plant-to-atmosphere gas exchange were conducted on replicate plants from 10:0 am to 02:00 pm with a portable Infra Red Gas Analyzer - IRGA (GFS-3000,

Walz, Germany) on single leaves, under 300 ± 5 µmol m^{-2} s^{-1} light, adjusted by the additional IRGA light source (Patono et al., 2023).

Assimilation rate (A), transpiration rate (E), stomatal conductance (Gs) and CO_2 concentration in the sub-stomatal chamber (Ci) were calculated following von Caemmerer and Farquhar's equations (von Caemmerer & Farquhar, 1981); water use efficiency (WUE) was calculated as A/E.

Measurements were taken in watered condition (Day 0) and daily following drought stress application (Day 1-7). One leaf per plant was sampled at each measurement, frozen in liquid nitrogen and stored at -80°C. Pots' weight was also measured daily, and soil relative water content (soil RWC) calculated as percentage of moisture in the soil compared to the maximum water holding capacity.

RNA was extracted from leaf samples (three biological replicates for each genotype) using Spectrum Plant Total RNA Kit (Sigma Aldrich, Saint Luis, USA) following instruction. cDNA was synthesized from 1 µg of RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, USA). PCR reactions were carried out by using the StepOnePlus Real-Time PCR System (Thermo Fisher, Waltham, USA). The following PCR program was used: 95°C/10', followed by 40 cycles of 95°C/15'' and 60°C/1'. Chosen targets belong to two groups: anti-oxidant related genes (*SlGST*, *SlPOD*, *SlSOD*, *SlAPX*, *SlCAT*) and ABA-related genes (*SlNCED1*, *SlNCED2*, *SlNCED3*, *SlCYP707.A1*, *SlCYP707.A2*, *SlCYP707.A3*). Tomato *Actin* and *β-Tubulin* were used as housekeeping genes. Information about primer sequences and target can be found in Data S1. Transcript levels were quantified through the $2^{-\Delta\Delta Ct}$ method. Each value represented the mean of three biological replicates compared using Student's t-test ($p \leq 0.05$).

Pathogen assay with *Phytophtora infestans*

The isolate of *Phytophtora infestans* (Westerdijk Fungal Biodiversity Institute strain CBS 120920) was maintained in Cornmeal medium (Data S1) at 18°C in dark. *P. infestans* was inoculated on Rye Agar (Data S1) one week before pathogenic assay and kept at 18°C in the dark. The plate was then flooded with chilled tap sterile water and kept for 2-3h at 4°C to induce zoospore release. The liquid from the plate was then filtered through two layers of cheesecloth and the concentration of zoospores was assessed using a hemocytometer. The concentration was adjusted to 2.5 x 10^4 spores/ml (Karki et al., 2021).

A detached leaf assay was set up using 5 leaves from the six selected T_1 plants showed in Figure 5 and WT plants according to the procedure described by Foolad (Foolad et al., 2015b). A panel of T_1 plants were used to compare the effect of different mutated alleles on pathogen tolerance. Leaves were rinsed in sterile water, blotted dry on sterile paper and placed in plastic trays containing water agar (20 g/l). About 250 zoospores (10µl) were placed on each leaf. Plastic trays were then covered with lids and incubated at 20°C in a growth chamber in the dark. The trays were examined on a daily basis. Picture and samples were collected three days post inoculation.

To quantify the pathogen infection rate, the ratio between fungal and plant DNA was evaluated according to Pavese et al. (Pavese et al., 2021). Disk samples around infection site were taken and DNA extraction was performed using an E.Z.N.A.® Stool DNA Kit (Omega Bio-Tek, Norcross, USA) following the manufacturer's protocol. Standard curves were prepared for the quantification of DNAs by qPCR using primers designed as follows: *SlActin* for tomato DNA, *PiO8* (Llorente et al., 2010) for *P. infestans* DNA. Extracted DNAs were analyzed through real-time

qPCR both with pathogen gene (*PiO8*) and tomato's one (*SlActin)*. qPCR reaction was carried out as described in the previous paragraph and information about primer sequences can be found in Data S1. Fungal and plant DNA was quantified using standard curves and the ratio fungus DNA/plant DNA calculated. IBM SPSS statistical software was applied to perform a one-way analysis of variance test (ANOVA). Each value represented the mean of 5 biological replicates compared using Tukey's HSD Test ($p \leq 0.05$).

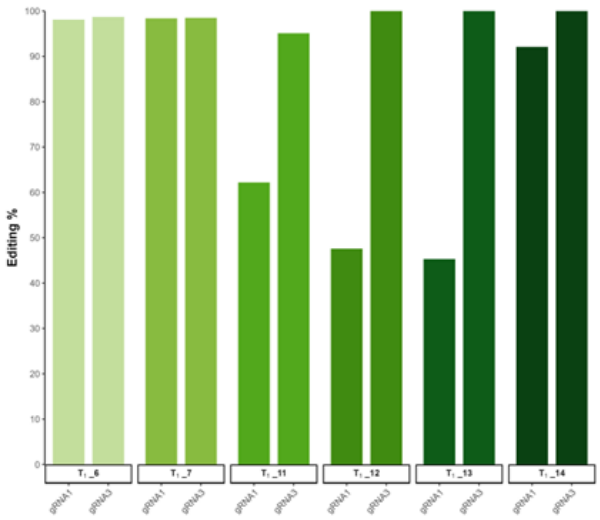
RESULTS

Molecular screening of *Sldmr6-1* mutants

A CRISPR-Cas9 vector containing the h*Cas9* gene, the selective marker (*NptII*) and the polycistronic tRNA–gRNA structure with the 3 gRNAs targeting the *SlDMR6-1* gene (Figure S1) was introduced via *A. tumefaciens*-mediated transformation into the tomato cultivar 'San Marzano'. These gRNAs target the first three exons of *SlDMR6-1* in order to disrupt the protein's catalytic site. Editing efficiency spanned greatly between the targets in T_0 plants: it was higher for gRNA1 and gRNA3, while no editing was detectable for gRNA2 (Data S1).

T_1 plants were sequenced by Sanger approach at the target loci to evaluate editing efficiency and transmission pattern of CRISPR/Cas9-induced mutations using the web tool TIDE (Brinkman et al, 2018). Out of the 14 analysed individuals, 6 showed homozygous, heterozygous and biallelic mutations at the target sites of gRNA1 and gRNA3 (Figure 5; Data S1).

a



b

Plant	*Cas9* +/-	gRNA	Editing %	Mutation	Allelic state
T_1_6	+	gRNA1	98,1	-3	Homozygous
		gRNA3	98,7	+1	Homozygous
T_1_7	-	gRNA1	98,4	+1	Homozygous
		gRNA3	98,5	+1	Homozygous
T_1_11	+	gRNA1	62,2	+1, WT	Heterozygous
		gRNA3	95,1	+1	Homozygous
T_1_12	+	gRNA1	47,6	WT, -6	Heterozygous
		gRNA3	100	-266	Homozygous
T_1_13	+	gRNA1	45,3	WT, -3	Heterozygous
		gRNA3	100	+1, -266	Bi allelic
T_1_14	+	gRNA1	92,1	+1	Homozygous
		gRNA3	100	+1, -266	Bi allelic

Figure 5: Genotyping of targeted gene mutations induced by CRISPR/Cas9 in selected T_1 plants. (a) Mutagenesis frequencies (%) at gRNA1 and gRNA3 targets in six plants of the T_1 progenies. (b) h*Cas9* presence, observed mutations and allelic forms. Data were retrieved through TIDE analysis of Sanger sequences.

The T_1_6 plant showed homozygous mutations for gRNA1 (-3/-3) and gRNA3 (+1/+1), and the presence of h*Cas9*. The T_1_7 plant was then selected for further molecular analyses due to both its homozygous mutations for gRNA1 (+1/+1) and gRNA3 (+1/+1), and the absence of any transgene.

Whole genome resequencing of a *Sldmr6-1* mutant

T_1_7 and WT plants were sequenced through Illumina WGS. Genome sequencing of T_1_7 yielded 196,4 million raw paired-end reads (29.5 Gb), with an average length of 150 bp. These were reduced to 196,1 million after filtering and trimming high-quality reads. The sequence depth of coverage ranged from 37.7X (T_1_7) to 42.8X (WT) (Data S1).

A *de novo* genome assembly of T_1_7 was produced and the presence of T-DNA integration was inspected through the scanning of the scaffolds with Blast analysis, which did not identify any T-DNA insertions. These results clearly demonstrated h*Cas9* segregation.

Scanning of *SlDMR6-1* in the gRNA1 region revealed a 100% editing effect with homozygous mutations (a 1 bp insertion at position SL4.0ch03:46628534) and no reference alleles, confirming the TIDE analysis (Figure 6).

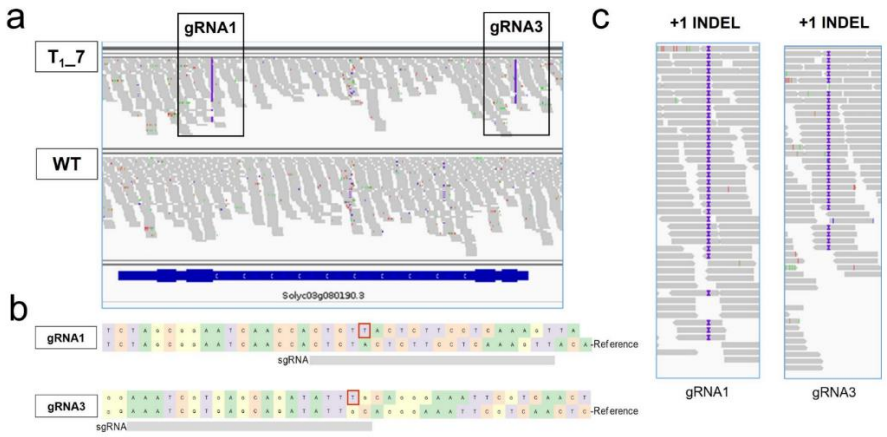


Figure 6: Whole Genome Sequencing of T_1_7 *Sldmr6-1* mutant (a) Sequence alignment view of the edited *SlDMR6-1* gene at the level of the two sgRNAs (gRNA1 and gRNA3) in T_1_7 plant and WT plant. (b) Genotyping of targeted gene mutations induced by CRISPR/Cas9 in the T_1_7 plant. (c) Focus on sgRNA1 and sgRNA3 is shown on the right-hand side.

Scanning of *SlDMR6* in the gRNA3 region highlighted an editing efficiency of 100% with homozygous mutations (a 1 bp insertion at position SL4.0ch03:46624776) and no reference alleles, in agreement with the TIDE analysis (Figure 5). The mutations impacting *SlDMR6-1* result in a premature stop codon in exon 1 leading to a truncated protein.

Off-target and SNP analyses in *Sldmr6-1* mutant

To confirm that T_1_7 displayed mutations only in *SlDMR6-1* locus and to get a deep insight into possible nonspecific editing activity, we analysed the candidate off-target loci by using the resequencing data. At first, we generated a list of 53 potential off-targets for the gRNA1, gRNA2 and gRNA3 used to target the *SlDMR6-1* locus (Data S1). All the 53 candidate off-target regions showed a number of mismatches higher than 2 bp with respect to the gRNAs. They fell in both coding (7) and non-coding (46) regions (Table 1).

gRNA	Number of off target in the genome	In coding	Non coding	SNP/Indel
gRNA1	21	2	19	0
gRNA2	22	4	18	0
gRNA3	10	1	9	0
Total	53	7	46	0

Table 1: Analysis of *SlDMR6-1* off targets in plant T_1_7.

The off-target analysis was conducted by mapping the Illumina reads from WT and T_1_7 genomes to the tomato Heinz 1706 reference genome. All the 53 candidate off-target regions were fully covered by Illumina reads in both the wild type and *Sldmr6-1* mutant, discarding the possibility that large deletions occurred. Side-by-side comparison of DNA alignments in

the WT and mutant revealed that in the candidate off-target regions no SNPs/indels nor big deletions occurred. Even if some indel/SNPs were present in the surrounding regions, they did not indicate off-target effects being always: i) conserved SNP/indels between mutants and WT, while polymorphic in comparison with the Heinz 1706 genome; ii) outside of the 20 bp window related to the gRNA-like sequence. On the basis of our analyses, we can confirm the specificity of Cas9-mediated *SlDMR6-1* gene editing, as highlighted by the lack of off-target effects.

Based on resequencing data, polymorphisms in T_1_7 and WT were searched and identified, using the Heinz tomato genome as a reference genome. 42,196 SNPs were identified in T_1_7 (88.5% of which in heterozygous state) and 40,998 SNPs in WT were observed (91.3% of which in heterozygous state). The average SNP number across edited (53.9 SNPs per Mb) and not edited plants (52.4 SNPs per Mb) was comparable, as was the average mutation rate (0.0054% for edited plants, 0.0052% for unedited plants) (Table 2).

Genotype	Plant type	SNPs	Homozygous	Heterozygous	SNP (%)	SNP per Mb
T_1_7	edited	42,2	4,86	37,336	0.0054	53.93
WT	*in vitro*	41	3,57	37,432	0.0052	52.40

Table 2: SNPs statistics of WGS. WT and T_1_7 plants were compared at genomic level with reference genome (Heinz).

Agronomic performance of *Sldmr6-1* mutants

With the aim to highlight potential differences in agronomic traits between WT and T_2_7 plants, different parameters of these plants growing under greenhouse conditions were measured. Two groups of variables were analysed: growth variables (plant height) and yield component variables

(fruit weight, number of fruits per plant). No statistically significant differences emerged by analysing these traits (Figure 7).

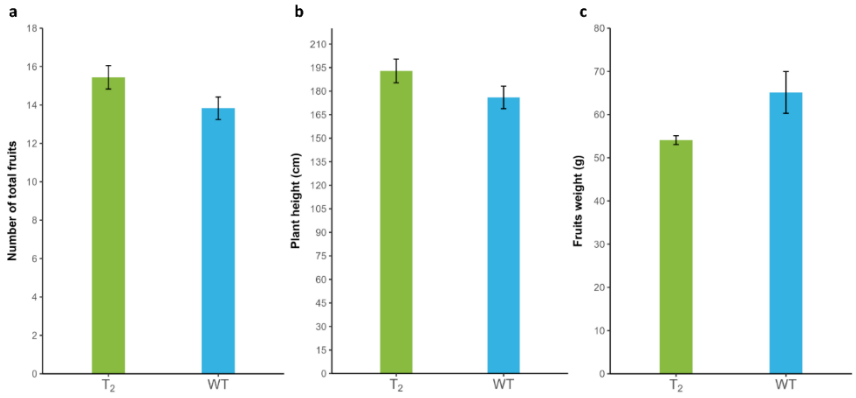


Figure 7: Comparison of agronomic performances of T_2_7 *Sldmr6-1* mutants and wild type lines. Two different classes of variables were analysed: growth variables (plant height) and yield component variables (fruit weight; number of total fruits per plant). Each value represents the mean of nine biological replicates ± SE. No significant differences were observed in the three analysed parameters (ANOVA test; $p \leq 0.05$).

Effects of *SlDMR6-1* knock-out on drought resistance

To investigate the role of *SlDMR6-1* in drought stress resistance, six-week-old WT and T_2_6 and T_2_7 (Figure S2) were subjected to drought stress conditions by withholding water during a further week. After 7 days of drought stress, T_2 plants remained turgid whereas WT plants exhibited severe wilting at days 6 and 7. This behaviour persisted at least until 10 days of stress (Figure 8; Figure S3).

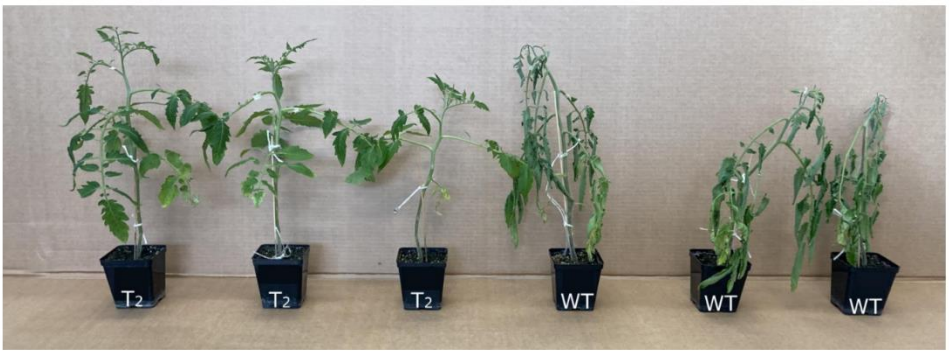


Figure 8: Drought stress analysis. T_2_7 *Sldmr6-1* plants and WT plants growing in a greenhouse after 10 days of withholding water.

As shown in Figure 9, the Relative Water Content (RWC) of the soil of both the WT and the T_2_7 plants decreased after drought treatments, as expected. However, at each dehydration time point the rate of water loss in the T_2_7 plants was lower than that of WT, indicating that edited plants were superior to WT in maintaining soil RWC. Although T_2_7 was slightly superior to T_2_6, no statistically significant differences between the 2 T_2 lines were observed (Figure S4).

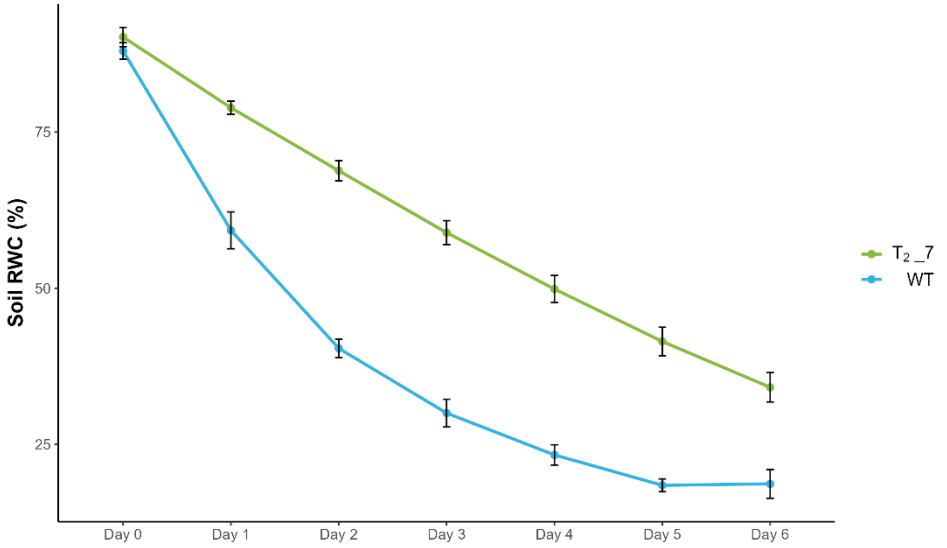


Figure 9: Soil relative water content (RWC) of WT and *Sldmr6-1* lines (T_2_7) during the drought period. Each value represents the mean of six biological replicates ± SE.

To gain insights into the physiological response of *Sldmr6-1* mutants assimilation rate (A), transpiration rate (E), stomatal conductance (Gs) and CO_2 concentration in the sub-stomatal chamber (Ci) were measured for 7 days after stress application (Figure 10; Figure S4); Water Use Efficiency (WUE) was calculated as A/E. Ecophysiological data were compared on the basis of RWC values. 80-95% RWC was considered a well watered condition, 50-65% RWC represents a moderate stress while at 25-40% RWC plants suffered a severe drought stress (Secchi et al, 2013).

E and Gs were significantly reduced in T_2_7 plants with respect to the WT at any soil RWC range.

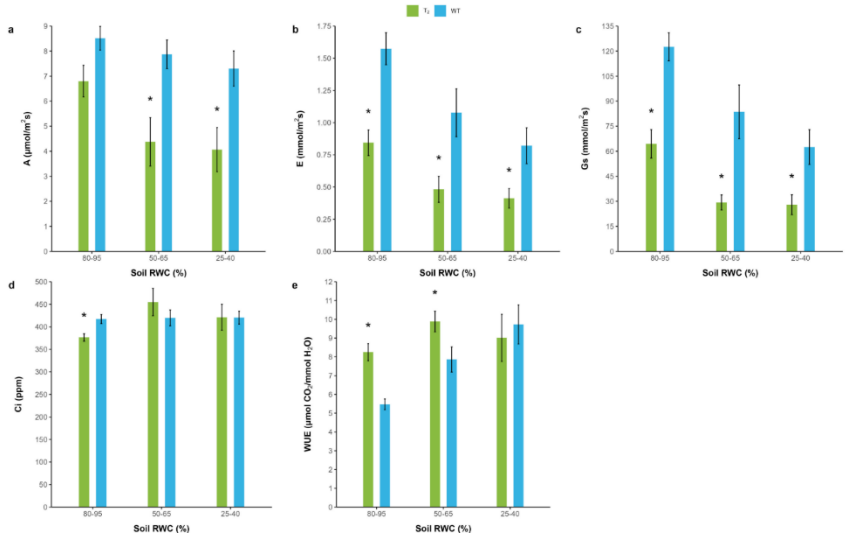


Figure 10: Leaf gas exchange of WT and *Sldmr6-1* lines (T_2_7) during the drought period, according to the decreasing trend of soil relative water content (soil RWC). Plants under water stress were analysed to determine different eco-physiological traits: (a) assimilation rate (A), (b) transpiration rate (E), (c) stomatal conductance (Gs), (d) CO_2 concentration in the sub-stomatal chamber (Ci) and (e) Water Use Efficiency (WUE). Data are means of six biological replicates ± SE. Asterisk indicates a significant difference based on ANOVA test ($p \leq 0.05$).

A was significantly reduced at soil RWC range 50-65% and 25-40%, whereas Ci did not show significant differences at soil RWC range 50-65% and 25-40%. WUE significantly increased in T_2_7 at soil RWC range 80-95% and 50-65%. Ecophysiological traits of WT plants had an abrupt collapse concurrently with wilting at day 6; at this time point soil RWC for WT plant was around 20% while was higher in T_2_7 plants (around 40%). No significant differences in ecophysiological traits were underlined by comparing T_2_6 and T_2_7 (Figure S4). In *Sldmr6-1* plants, despite under conditions of reduced stomatal conductance, no metabolic damage was conceivable and this resulted in a Ci trend similar to what measured in WT controls, and a gain in WUE till to a moderate stress condition.

The increased drought resistance of *Sldmr6-1* lines prompted us to examine whether the expression of genes involved in ABA biosynthesis (*SlNCED1*, *SlNCED2*, *SlNCED3*) and catabolism (*SlCYP707.A1*, *SlCYP707.A2*, *SlCYP707.A3*) was altered in the edited lines under drought conditions. Moreover, we examined the transcript levels of key anti-oxidant related genes (*SlGST*, *SlPOD*, *SlSOD*, *SlAPX1*, *SlCAT1*) (Figure 11).

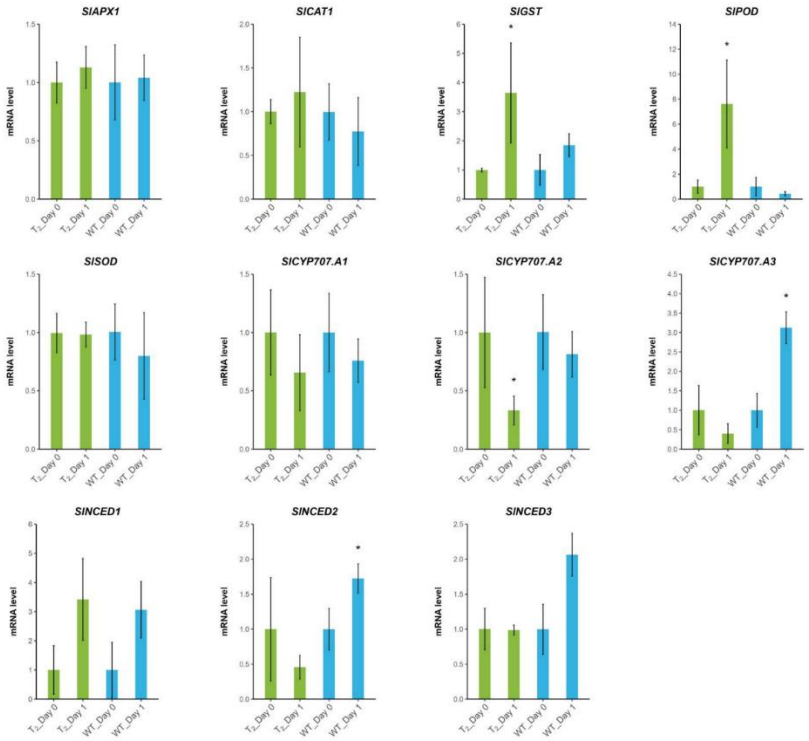


Figure 11: Transcriptional levels of anti-oxidant related genes (*SlAPX1*, *SlCAT1*, *SlGST*, *SlPOD*, *SlSOD*) and ABA-related genes (*SlCYP707.A1*, *SlCYP707.A2*, *SlCYP707.A3*, *SlNCED1*, *SlNCED2*, *SlNCED3*) during the drought assay. The values are expressed as relative mRNA abundance 1 day after drought stress application and compared to time 0 (just before stress application). Tomato actin and tubulin genes were used as reference genes. Data are means of three biological replicates ± SE. Data refer to T_2_7 line. Asterisk indicates a significant difference based on Student's t-test ($p \leq 0.05$).

Among anti-oxidant related genes, a significant up-regulation in T_2 plants was detected for *SlGST* and *SlPOD*. Among genes related to ABA biosynthesis, a significant up-regulation was highlighted only for *SlNCED2* (in WT). Among genes related to ABA catabolism, *SlCYP707.A2* was down-regulated in the T_2 line, while *SlCYP707.A3* up-regulated in WT.

Knock-out of *SlDMR6-1* improves tolerance against *P. infestans*

The impairment of S-genes leads to resistance or tolerance against several biotic stresses. *SlDMR6-1* knock-out in tomato is related to tolerance against a wide array of pathogens (Paula De Toledo Thomazella et al., 2021). In this work we assessed tolerance against *P. infestans* in six selected T_1 lines (T_1_6, T_1_7, T_1_11, T_1_12, T_1_13, T_1_14). A pathogenic assay was performed by using a detached leaf assay (Foolad et al., 2015). 72 hours after inoculation the edited T_1 lines showed reduced susceptibility to *P. infestans* as highlighted by smaller chlorotic and necrotic foliar lesions than the control plants (Figure 12a). Genomic DNA was extracted from foliar disks cut around infection site and qPCR was used to quantify the fungal biomass (Figure 12b). Edited T_1 lines showed a clear reduced fungal biomass, from 64% (T_1_6) to 95% (T_1_7, T_1_13) reduction compared to WT.

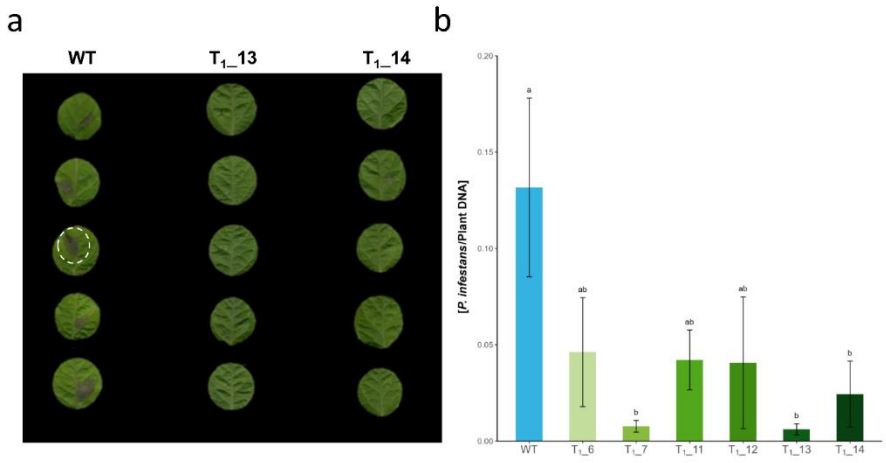


Figure 12: Pathogen assay on WT and *Sldmr6-1* lines (a) Detached leaves assay with *Phytophthora infestans* performed on two *dmr6-1* mutants and a WT plant as a control group at three days post-inoculation. The circle indicates the pathogen lesion (b) q-PCR pathogen DNA quantification after *Phytophthora infestans* infection. Data were quantified using the $2^{-\Delta\Delta Ct}$ method based on the Ct values of fungal genes (*PiO8*) with *SlActin*-7 as a housekeeping gene. Data are the means of five biological replicates ± SE. Letters indicate significant differences based on Tuckey's HSD Test.

DISCUSSION

Climate change and the resulting limited water availability represent the most limiting factors for in field tomato production. It is thus pivotal to implement breeding strategies aimed at developing more resilient elite genotypes, able to deal with water shortage while maintaining yield. Although many studies have provided information on the genetic mechanisms involved in tolerance and adaptation to drought stress, the limited genetic variation within *S. lycopersicum* has not, to date, allowed to enhance the drought tolerance of the species through traditional breeding strategies.

Recent advances in sequencing technologies and the availability of breakthrough tools, such as the genome editing through CRISPR/Cas9, has

made the functional validation of drought-responsive genes possible (Salava et al., 2021; Krishna et al., 2022; Taheri et al., 2022). Furthermore, the induced loss of function of *SlARF4*, *SlLBD40* and *SlGID1a* genes by CRISPR/Cas9 technology has been found to enhance tomato drought tolerance (Illouz-Eliaz et al., 2020; Liu et al., 2020; Chen et al., 2021).

It has been previously reported that foliar application of salicylic acid (SA) to tomato plants under water deficit conditions can increase stomatal conductance, CO_2 assimilation, and water use efficiency (WUE), mitigating the oxidative stress caused by ROS over-production (Aires et al., 2022). One of the key enzymes in SA metabolism is DOWNY MILDEW RESISTANT 6 (DMR6), which catalyzes the formation of 2,5-dihydroxybenzoic acid through the hydroxylation of SA at the C5 position of its phenyl ring (Zhang et al., 2017). Inactivation of *DMR6* results in increased SA acid levels (Zeilmaker et al., 2015; Paula De Toledo Thomazella et al., 2021). Tomato *SlDMR6-1* mutants (characterized by high accumulation of SA) showed broad-spectrum disease-resistance phenotype against several pathogens, suggesting that disease resistance is associated with activation of plant immune responses mediated by SA (Paula De Toledo Thomazella et al., 2021).

Here we explored drought tolerance of *Sldmr6-1* tomato mutants obtained through CRISPR/Cas9. T_1/T_2 lines, characterized by the absence of any transgene and the disabling of *SlDMR6-1* locus in homozygosity were selected (Figure 5 and 6). At first, we assessed the agronomic performances of *Sldmr6-1* mutants and wild-type plants growing under well-watered conditions. No clear phenotypic differences and pleiotropic effects were observed (Figure 7), in agreement with previously reported results (Kieu et al., 2021; Paula De Toledo Thomazella et al., 2021).

After, we examined the impact of the *Sldmr6-1* mutation on 7 days of water deprivation, and while the WT plants exhibited severe wilting and chlorosis, *Sldmr6-1* mutants showed relatively turgid and green leaves (Figure 8). Under water restriction, plants' strategy of modulating gas exchange by reducing the stomatal conductance and transpiration results in a lower assimilation of CO_2. Ecophysiological traits measured during the period of water stress showed that *Sldmr6-1* adopted a water saving behaviour reducing A, E and Gs and supporting a proper photosynthetic metabolism, since no difference in Ci and an increase in WUE were observed.

The regulation of stomatal closure and maintenance of high soil Relative Water Content (RWC) is an important strategy for water conservation under drought stress. In our study *Sldmr6-1* lines maintained higher soil RWC than control plants during the whole imposed water stress of 1-week (Figure 9), presumably by reinforcing stomatal closure or preventing stomata opening.

Drought avoidance (referred to as dehydration avoidance in recent literature) occurs when plants increase their WUE by reducing transpiration and avoiding dehydration during periods of drought stress (Kooyers, 2015). The lower transpiration detected in our *Sldmr6-1* mutants suggests that their improved performance under deficit conditions was due to the drought avoidance mechanism, as previously observed in other tomato mutants (Shohat et al., 2021; Seymour & Gibernau, 2008).

Water deficit causes the production of a large amount of reactive oxygen species (ROS) within plant cells, which provoke oxidative damage, especially in plants adopting drought prevention strategies by reducing transpiration, which in turn increases the dangers associated with heating the leaves (Bleau & Spoel, 2021). It has been previously demonstrated that

the increased activity of enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione S-transferase (GST) can contribute to the enhancement of drought resistance in tomato (Chen et al., 2021; Liang et al., 2022). Our data highlight that, following drought stress, *Sldmr6-1* mutants up-regulated the transcription of *SlGST* and *SlPOD* (Figure 11), two key anti-oxidant genes significantly upregulated when tomato plants were exposed to abiotic stress (Khan et al., 2015). The increased antioxidant activities in *Sldmr6-1* mutants might lead to a less severe oxidative damage under drought stress. The successful coupling between the drought prevention strategy and an efficient ROS scavenging activity allowed stomatal control of photosynthesis, which did not cause metabolic imbalances causing negative feedbacks on stomatal regulation. This is clearly evident from the overlapping of the Ci trends calculated on the basis of gas exchange data in both edited and WT plants and leading to the maintenance of high WUE values in the edited plants.

In response to water stress, a crosstalk between jasmonate acid (JA), SA, and abscissc acid (ABA) in tomato has been underlined (Muñoz-Espinoza et al., 2015). In salt-stressed tomato, SA modulated the expression of the genes involved in ABA accumulation and promoted the ABA transport to the shoot (Horváth et al., 2015). A cross-talk between SA and ABA signalling is thus important for the regulation of plant growth and reproduction under combined abiotic and biotic stresses (Berens et al., 2019).

Drought avoidance is mainly regulated by ABA which induces stomatal closure by regulating the expression of many stress-responsive genes. The accumulation of ABA is modulated by a balance between its biosynthesis (catalysed by 9-cisepoxycarotenoid dioxygenase enzymes) and catabolism (catalysed by 8'-hydroxylases). For its synthesis, three *SlNCED* genes have

been characterized in tomato, while for ABA catabolism, the *SlCYP707.A1*, *A2*, *A3*, and *A4* genes play a pivotal role (Liang et al., 2022). The contribution of *SlNCED1* or *SlCYP707.A2* in ABA accumulation has been demonstrated through over-expression and silencing approaches (Sun, et al., 2012; Chatfield et al., 2000; Sun et al., 2012).

Our qPCR analyses demonstrated that the knock-out (KO) of *SlDMR6-1* prompted the downregulation of *SlCYP707.A2* upon stress application in contrast with WT plants. Contrary to what observed in *SlDMR6-1* KO lines, *SlCYP707.A3* was up-regulated in the WT (Figure 11). The results suggested that *Sldmr6-1* mutation increased endogenous ABA level by suppressing ABA degradation, thereby positively affecting the stress resistance mechanisms of plants.

On the basis of our physiological data, we can state that a high SA content in *SlDMR6-1* KO lines might reduce ABA degradation, leading to drought avoidance through stomatal closure and reduced water loss in response to drought stress.

Although the KO mutation of *SlDMR6-1* has been demonstrated to confer a broad-spectrum disease-resistance phenotype in tomato (Paula De Toledo Thomazella et al., 2021), the potential resistance to *Phytophthora infestans* (the causal agent of Late Blight) has never been tested. Late blight is a serious disease that may affect tomatoes and can destroy an entire unprotected tomato crop within 7–10 days of infection. Our results showed, for the first time, a reduced susceptibility to late blight in *Sldmr6-1* tomato edited lines (Figure 12) in agreement with what was observed in potato (Kieu et al., 2021).

Manipulating stomatal closure is helpful for plants to respond to pathogens and/or drought. We can hypothesize that stomatal closure in our *Sldmr6-1* mutants, responsible for drought avoidance, represents a barrier stopping

P. infestans, which is specialized to grow sporangiophores through opened stomata for secondary inoculum production (Yang et al., 2021).

Even if the CRISPR/Cas9 approach induces, at target loci, random mutations that are functionally equivalent to spontaneously occurring ones, it is not always easy to predict the functional equivalence between natural and induced mutations. It has been suggested that most untargeted variations in edited lines are induced by somaclonal variation during *in vitro* culture, inheritance from the maternal plants and pre-existing variation across the germline (Sturme et al., 2022*).* Whole genome sequencing (WGS) can be a valuable tool to assess the substantial equivalence of edited lines with their WT equivalent, because it provides comprehensive information about genomic variations, such as indels, SNPs and other structural differences. Several studies employed WGS analysis of WT and CRISPR/Cas9-edited plants to investigate the specificity of genome editing, and observed that off-target mutations occur at a much lower level than background mutations, due to pre-existing/inherent genetic or/and somaclonal variations (Tang et al., 2018; Li et al., 2019; Wang et al., 2021; Sturme et al., 2022; Li et al., 2022*).* In agreement with previous observations, targeted deep sequencing of *SlDMR6-1* KO line at putative 53 off-target loci once again confirmed the lack of significant mutated off-targets (Table 1). The average SNP number across not edited and edited (52.4 SNPs per Mb vs 53.9 SNPs per Mb) was comparable, as was the average mutation rate (0.0052% for unedited plants, 0.0054% for edited plants) (Table 2).

Our results confirm the high specificity of CRISPR/Cas9 in tomato, which represents one of the “cleanest” tools in our hands to introduce targeted mutations. Our edited line does not carry any foreign DNA sequences but

only carries an insertion, completely indistinguishable from spontaneously occurring mutations.

CONCLUSIONS

The drought-avoidance mechanism observed in our *Sldmr6-1* mutants might be related to a successful coupling between the drought prevention strategy and an efficient ROS scavenging activity allowing stomatal control of photosynthesis. These results suggested that the drought avoidance in our *Sldmr6-1* lines could be correlated with the activation of antioxidant genes, leading to a more efficient ROS scavenging that could prevent the damage associated with leaf heating, likely danger when transpiration is reduced. On the other hand, the *SlDMR6-1* KO lines might reduce ABA degradation, leading to drought avoidance through stomatal closure and reduced water loss in response to drought stress. In addition, our results add *P. infestans* to the list of pathogens to which *SlDMR6-1* gene KO can confer resistance (*P. syringae pv. tomato, X. gardneri, X. perforans, P. neolycopersici, P.capsici;* Paula De Toledo Thomazella et al., 2021).

Our results might be extended to other crops, being *DMR6* orthologs interesting targets for improving multi-stress tolerance through genome editing tools.

SUPPLEMENTARY FIGURES

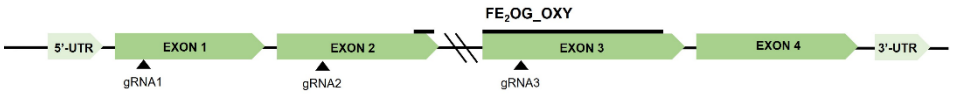


Figure S1: Position of gRNAs' target sites on *SlDMR6-1*.

Figure S2: Comparative images of WT, T_2_6 and T_2_7 *Sldmr6-1* lines.

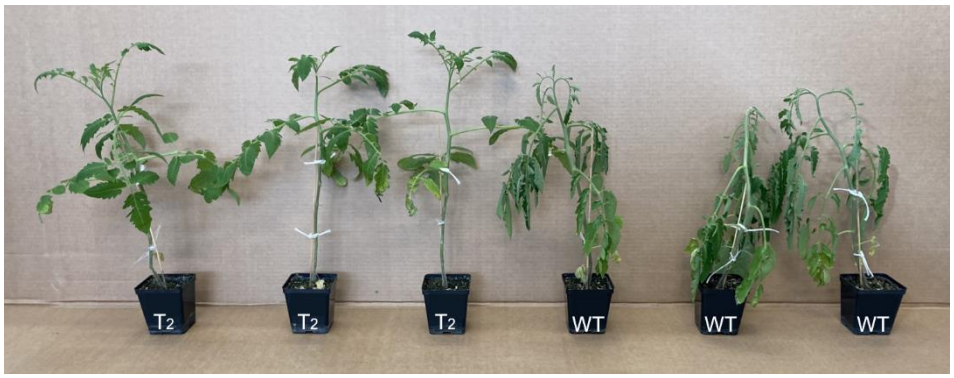


Figure S3: T2_6 *Sldmr6-1* plants and WT plants under drought stress.

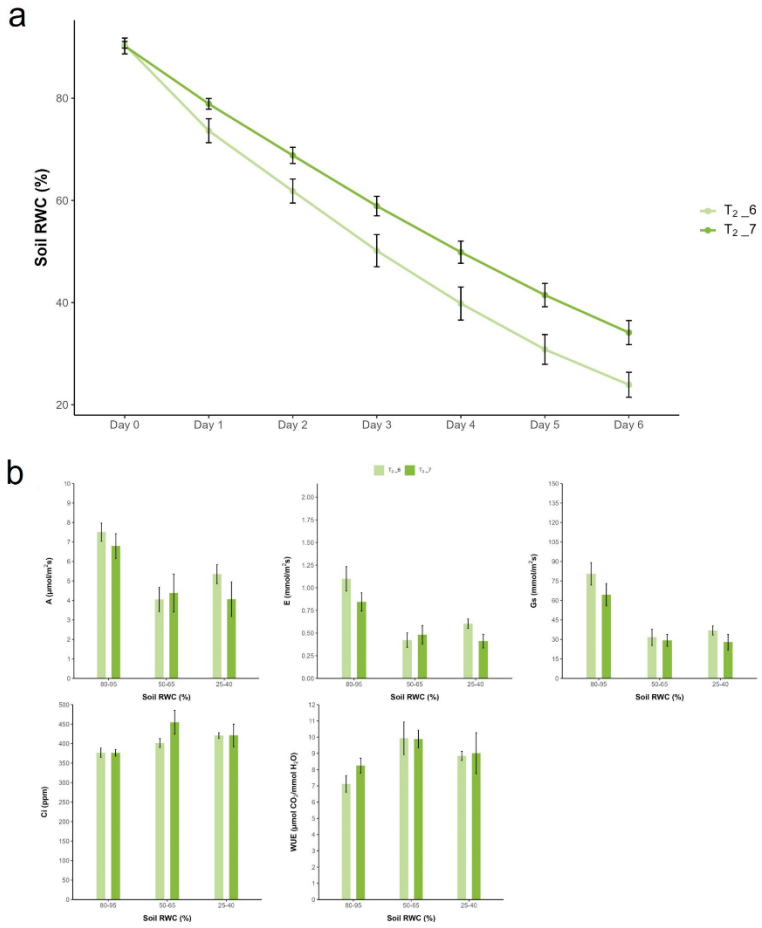


Figure S4: Comparative ecophysiological and RWC measurement of T_2_6 and T_2_7

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DATA STATEMENT

Sequencing data used in this study are openly available in the NCBI database (PRJNA846963).

Supporting data and information can be retrieved at this link: https://drive.google.com/drive/folders/1TlSewUCYY5QRF0oz2X4Lw-Tjs8xqQA1O?usp=drive_link.

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CHAPTER II

frontiers in Plant Science

ORIGINAL RESEARCH
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Simultaneous CRISPR/Cas9 Editing of Three PPO Genes Reduces Fruit Flesh Browning in *Solanum melongena* L.

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ABSTRACT

Polyphenol oxidases (PPOs) catalyse the oxidization of polyphenols, which in turn causes the browning of the eggplant berry flesh after cutting. This has a negative impact on fruit quality for both industrial transformation and fresh consumption. Ten *PPO* genes (named *SmelPPO1-10*) were identified in eggplant thanks to the recent availability of a high-quality genome sequence. A CRISPR/Cas9-based mutagenesis approach was applied to knock-out three target *PPO* genes (*SmelPPO4*, *SmelPPO5* and *SmelPPO6)*, which showed high transcript levels in the fruit after cutting. An optimized transformation protocol for eggplant cotyledons was used to obtain plants in which Cas9 is directed to a

conserved region shared by the three *PPO* genes. The successful editing of the *SmelPPO4*, *SmelPPO5* and *SmelPPO6* loci of *in vitro* regenerated plantlets was confirmed by Illumina deep sequencing of amplicons of the target sites. Besides, deep sequencing of amplicons of the potential off-target loci identified *in silico* proved the absence of detectable non-specific mutations. The induced mutations were stably inherited in the T_1 and T_2 progeny and were associated with a reduced PPO activity and browning of the berry flesh after cutting. Our results provide the first example of the use of the CRISPR/Cas9 system in eggplant for biotechnological applications and open the way to the development of eggplant genotypes with low flesh browning which maintain a high polyphenol content in the berries.

INTRODUCTION

The polyphenol oxidases (PPOs) are a group of enzymes catalyzing the oxidation of phenolic compounds into highly reactive quinones (García-Fortea et al., 2020; Mishra et al., 2013; Plazas et al., 2013; Prohens et al., 2007). The physiological role of PPOs in plants has not been fully clarified yet, but a defense role against pathogens and pests has been postulated because of their increased localized activity in response to cutting and wounding. The relationship between PPO expression or activation and pathogen infections was proved in tomato by either silencing (Thipyapong et al., 2004) or over-expressing PPO genes (L. Li & Steffens, 2002). PPOs oxidize polyphenols to toxic quinones which bind to aminoacids in the insect gut, exerting an antifeeding role. Previous studies have associated PPO activity with resistance to various types of insects (Mahanil et al., 2008).

In recent years, PPOs have been largely investigated for their involvement in the browning process, a colour reaction caused by the oxidation of phenolic compounds during postharvest processing and storage. Enzymatic browning is a two-step reaction, consisting of the oxidation of a monophenol to a o-diphenol (cresolase/monophenolase activity), which is further oxidized to yield a o-quinone (catecholase/diphenolase activity). O-quinones can then undergo condensation or polymerization reactions, producing the dark pigments melanins. Fruit cutting causes cellular disruption and damages membrane integrity, allowing the PPOs sequestered in the plastid to come into contact with the hydroxycinnamic acid derivatives, which are their substrates. Extensive browning of cut fruit and vegetable surface compromises food quality and usually impairs the properties of the product, representing a major economic problem both for the food industry (e.g. the industrial manipulation and preservation of these products) and for consumers (in the case of fresh and ready-to-eat fresh cut fruit and vegetables). Since PPO activity is influenced by factors such as pH, temperature and oxygen, the browning process is limited in the food industry through the use of chemical and/or physical agents, with a negative impact on nutritional and organoleptic properties. Browning negatively affects the commercial value of many key agricultural productions, including potato, lettuce, cereals, banana, cucumber, grape and eggplant (Taranto et al., 2017).

Eggplant (*Solanum melongena* L.) berries are characterized by a remarkable content in phenolic compounds, represented mainly by chlorogenic acid (5-O-caffeoylquinic acid). Chlorogenic acid plays important therapeutic roles due to its antioxidant, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory and anti-microbial properties (Naveed et al., 2018). In eggplant, a correlation between the

concentration of phenolics (mainly chlorogenic acid) and browning has been detected in the fruit flesh, although additional morphological and physiological factors may be involved in browning phenomena (Kaushik et al., 2017). Furthermore, in commercial varieties, the selection for berries with a reduced degree of browning in the flesh has resulted in the indirect selection of accessions with lower concentrations of phenolics (Prohens et al., 2007).

(Shetty et al., 2011) identified six genes encoding PPOs in eggplant and, on the basis of both protein sequence similarity and organ-specific patterns of expression, they proposed the distinction of eggplant PPOs in two clades: A and B, with clade A encompassing genes expressed mostly in roots, while clade B genes are involved in defense mechanisms. This categorization was further extended to the rest of Solanaceae PPOs (Taranto et al., 2017).

The development of new technologies to disable genes coding for PPOs represents the most promising strategy to avoid undesired browning in plant-derived products, as it would allow to positively select genotypes enriched in beneficial phenolic compounds, while reducing the need for physical and chemical treatments in the food industry. The positive impact on the storability of these foods, in addition, would help reduce waste.

Several examples are available on the adoption of RNA silencing strategies to down-regulate PPO genes in order to reduce the enzymatic browning in potato tubers (Bachem et al., 1994; Chi et al., 2014; Coetzer et al., 2001; Llorente et al., 2011; Rommens et al., 2006). By using artificial micro-RNAs (amiRNAs) all *StuPPO* genes have been silenced individually or in combination, identifying *StuPPO2* as the main contributor to PPO activity (Chi et al., 2014). A few notable examples exist of commercially available genetically modified plants in which PPOs have

been silenced, such as the Arctic Apple® and the Innate® potato. The emergent CRISPR/Cas9 technology has proved extremely efficient in gene editing and is expected to play a key role in crop breeding. This technology makes it possible to induce point mutations in one or multiple target sequences simultaneously, as well as to introduce new genetic variants through homology directed recombination (HDR), or to modulate transcription and chromatin structure at selected target loci (Doudna & Charpentier, 2014). While this technique has been successfully applied to some Solanaceae species, such as tomato and potato, including the knock out of the *StuPPO2* gene in the potato tetraploid cultivar Desiree (González et al., 2020), no examples of genome editing in eggplant have been reported in literature so far (Van Eck, 2018).

In this study, thanks to the recent availability of a high quality, annotated and anchored eggplant genome sequence (https://solgenomics.net/organism/Solanum_melongena/genome; Barchi et al., 2019), we report the homology-based characterization, functional domain identification and phylogenetic analysis, of 10 PPO (*SmelPPO1-PPO10*) genes in eggplant. On the basis of their expression in the fruit after cutting, *SmelPPO4*, *SmelPPO5* and *SmelPPO6* were selected for the generation of knock-out mutants using the CRISPR/Cas9 technology. Regenerated T_0, T_1 and T_2 lines were screened for induced mutations in the target genes as well as in potential off-target loci. In addition, PPO activity and the degree of browning in the flesh of eggplant berries were analyzed in our knock-out T_1 and T_2 edited lines.

MATERIALS AND METHODS

Mining of PPO in the eggplant genome and phylogenetic analysis

The six eggplant PPO aminoacidic sequences previously reported (Shetty et al., 2011) were used for a BlastP search of the eggplant proteome (https://solgenomics.net/organism/Solanum_melongena/genome) with an E-value threshold of $1e^{-5}$. The polypeptide sequences of eggplant PPOs, together with those of six tomato and nine potato PPOs (Supplementary File 1), were used for a multiple alignment (Clustal Omega; https://www.ebi.ac.uk/Tools/msa/clustalo/). A phylogenetic analysis was performed with the MEGA X software. An unrooted phylogenetic tree was generated, applying the Neighbor-Joining (NJ) algorithm. The statistical significance of individual nodes was assessed by bootstrap analysis with 1,000 replicates, and the evolutionary distances were calculated using the p-distance method with default parameters.

qPCR analysis

To identify *PPO* genes involved in the browning phenotype their corresponding mRNA levels were analyzed in the flesh of fruits of the 'Black Beauty' variety harvested at the commercial ripening stage (Mennella et al., 2012) after cutting them transversally with a sharp knife. One gram of frozen fruit flesh was ground in liquid nitrogen to a fine powder and RNA was extracted using the "Spectrum plant total RNA kit" (Sigma-Aldrich, St. Louis, USA). RNA was extracted in three biological replicates from commercial grade ripe fruit 1 cm-wide slices exposed to air for 0 min (t_0) and 30 min (t_{30}).

cDNA was synthesized from 1 µg of RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, USA) as directed by the manufacturer. Using the Primer 3 software (http://bioinfo.ut.ee/primer3),

primers targeting the ten identified eggplant *PPO* genes were designed (Supplementary Table 1). PCR reactions were carried out in three biological replicates using the StepOnePlus Real-Time PCR System (Applied Biosystems). The following PCR program was used: 95°C/10min, followed by 40 cycles of 95°C/15s and 60°C/1min. Data were quantified using the $2^{-\Delta\Delta Ct}$ method based on Ct values of actin and elongation factor as housekeeping genes. Values are expressed as relative mRNA abundance at 30 minutes after cutting compared to time 0 (just after cutting).

Target identification, DNA construct cloning and off-target search

Sequences of the wound-induced *SmelPPO4*, *SmelPPO5* and *SmelPPO6* genes were aligned to find conserved regions, and BlastX and Prosite were used to annotate functional domains. A gRNA (ATGAATGGAAAGCAATCGGA) was designed to target a conserved region of these three genes and assembled into a CRISPR/Cas9 construct carrying the h*Cas9* and the *nptII* gene for kanamycin resistance, using the GoldenBraid (GB) assembly system and following GB software-directed procedures (https://gbcloning.upv.es/). An additional guanine was added at the 5'end in order to improve expression under the U6-26 RNA PolIII promoter (Cong & Zhang, 2015). The h*Cas9* expression is driven by the CaMV 35S promoter, while the gRNA is placed under the control of the *At*U6-26 RNA PolIII promoter. Putative off-target sites were identified with the CasOT software (http://casot.cbi.pku.edu.cn/), using the eggplant genome as reference. Four off-targets (OT1-OT4) were selected based on the number and position of mismatches (Supplementary Table 2); the corresponding loci (a 1 kb region around the putative off-target site) were

inspected, to determine whether they corresponded to functional genes, and considered for sequencing analyses.

Genetic transformation of plants

The final pCambia vector *Tnos:nptII:Pnos-U6-26:gRNA:scaffold-P35S:*h*Cas9:Tnos* was transformed into LBA4404 *Agrobacterium tumefaciens* strain. A pre-culture was set up in a modified MGL liquid medium (tryptone 5 g l^{-1}, yeast extract 2.5 g l^{-1}, NaCl 0.1 g l^{-1}, mannitol 5 g l^{-1}, glutamic acid 1.15 g l^{-1}, KH_2PO_4 0.25 g l^{-1}, $MgSO_4.7H_2O$ 100 g l^{-1}, biotin 1 mg l^{-1}, pH 7) supplemented with 50 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin, and incubated overnight at 28°C. From this, a second culture was set up in TY liquid medium (tryptone 5 g l^{-1}, yeast extract 3 g l^{-1}, $MgSO_4.7H_2O$ 0.5 g l^{-1}, pH 5.8) supplemented with 200 μM acetosyringone and incubated overnight in the dark at 28°C. Before transformation, the optical density of the culture at 600 nm (OD_{600}) was measured and the bacterial culture was diluted to a final OD_{600} of 0.10-0.15 in TY medium supplemented with 200 μM acetosyringone. Explants of about 5 mm in length were cut from the cotyledons of *in vitro* germinated 'Black Beauty' seeds, dipped in the bacterial culture for a minimum of 10 minutes, blotted dry on filter paper and transferred for 48 hours on a co-culture medium (MS basal salt mixture 4.5 g l^{-1}, MES 0.5 g l^{-1}, sucrose 30 g l^{-1}, phytoagar 10 g l^{-1}, Gamborg vitamin mixture 1 ml l^{-1}, trans-zeatin 2 mg l^{-1}, IAA 0.1 mg l^{-1}, acetosyringone 200 μM, pH 5.8), in the dark. For organogenesis and shoot induction, a common basal induction medium was used, as previously described (Muktadir et al., 2016)(MS basal salt mixture 4.5 g l^{-1}, MES 0.5 g l^{-1}, sucrose 30 g l^{-1}, phytoagar 10 g l^{-1}, Gamborg vitamin mixture 1 ml l^{-1}, trans-zeatin 2 mg l^{-1}, IAA 0.1 mg l^{-1}, kanamycin 30 mg l^{-1}, carbenicillin 400 mg l^{-1}, pH 5.8),

with three different conditions: without further additives, with supplementation of ascorbic acid 5 mg l^{-1} and citric acid 5 mg l^{-1}, and with supplementation of polyvinylpyrrolidone (PVP40) 200 mg l^{-1}. Furthermore, for each medium composition, two conditions were tested during the first 3 days of induction: no incubation, or 3 days of incubation in the dark, after which explants were grown in the same conditions as the untreated group (16:8 light:dark cycle, 24°C). Elongation and rooting were performed on the same media for all conditions and explants were moved to a fresh medium every 2-3 weeks. Both media were previously described (Muktadir et al., 2016) and were not supplemented with antioxidants, as no oxidative damage was observed from this stage onwards. The elongation medium was supplemented with kanamycin 30 mg l^{-1} and carbenicillin 400 mg l^{-1}, but did not contain any hormone. Kanamycin was removed from the rooting medium to avoid inhibitory effects on root development, and 0.2 mg l^{-1} indolebutyric acid were added. Fully developed plantlets were then moved to soil and gradually acclimated to *ex vitro* conditions.

Target and off-target sequencing

Genomic DNA was extracted using a CTAB protocol (Doyle & Doyle, 1987) from leaves sampled when plantlets were transferred from *in vitro* growth conditions to soil. The presence of the transgene was assessed by amplifying the h*Cas9* (Supplementary Table 3) gene by using qPCR (in three technical replicates) according to the protocol described in the previous paragraph. DNA was also extracted from T_1 and T_2 progeny plants (Supplementary Data Spreadsheet).

Mutation frequencies at the target and off-target sites were evaluated according to an adapted version of the 16S Metagenomic Sequencing

Library preparation protocol provided by Illumina (16S Sample Preparation Guide). Amplifications were carried out using the KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, Boston, MA). Dual indexing was done using the Nextera XT system (Illumina, San Diego, CA) using 16 i5 indexes (S502-S522) and 24 i7 indexes (N701-N729), enabling the multiplexing of 333 individual libraries. Due to their high sequence identity, a differential amplification of *SmelPPO4* and *SmelPPO6* was obtained with a first specific PCR, using primers designed on flanking non conserved regions, while a second amplification was performed with non-specific primers carrying Illumina adapter sequences (Supplementary Table 3). Amplifications of *SmelPPO5*, OT1, OT2, OT3, OT4 were done directly using primers modified with Illumina adapter sequences (Supplementary Table 3). Products were diluted 1:50 and used as templates to add dual Nextera XT indexes (Supplementary File 2). Finally, indexed amplicons were purified using AmpureBeads (0,7X) and quantified using Qubit 2.0 (Life Technologies, Carlsbad, CA, USA), based on the Qubit dsDNA HS Assay (Life Technologies, Carlsbad, CA, USA). All samples were diluted to 4 nM and pooled in a single tube. Sequencing was performed with an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA) and 150 bp paired-end reads were generated. From reads generated by WGS sequencing, adapters were removed and reads that were less than 50 nucleotides long were discarded using Trimmomatic v0.39 (Bolger et al., 2014). Processed reads were analyzed for CRISPR/Cas9 editing events with CRISPResso2 (http://crispresso2.pinellolab.org (Clement et al., 2019) (Supplementary Table 4). Sequences can be can be downloaded at http://bit.ly/ppo-eggplant-illumina.

PPO activity assay

Fruits of the wild type and edited lines (T_1 and T_2) were collected in eight biological replicates at the commercial ripening stage (Mennella et al., 2012). Flesh slices about 1 cm thick, cut at the midpoint between the blossom and stem ends, were exposed to air for 30 min (t_{30}) before pictures were taken. After the exposition, all fresh tissues were immediately frozen in liquid nitrogen and stored at -80°C for PPO activity measurement of the eight biological replicates. PPO activity analysis was performed according to previously described protocols (Bellés et al., 2006; Plazas et al., 2013) with minor modifications: 1 g of fresh frozen peel tissue was taken and ground in a mortar with liquid nitrogen and 50 mg of polyvinylpolypyrrolidone before being resuspended in 4 ml 0.1 M sodium phosphate buffer pH 6. Samples were sonicated in a water bath for 10' at 20°C, centrifuged at 12,000 rpm for 15' at 4°C and the supernatant was collected. Protein concentration was evaluated using Bradford's dye (Sigma Aldrich) binding assay using bovine serum albumin (Sigma Aldrich) as a standard (Bradford, 1976). PPO activity was measured colorimetrically at room temperature using a spectrophotometer (Beckman Coulter, Brea, CA, USA) to follow the emerging enzymatic reaction. For sample analysis, 145 µl sodium phosphate buffer (0.1 M, pH 6, RT), 15 µl chlorogenic acid (Sigma Aldrich, 35.5 mg ml^{-1}) and 40 µl of protein extract were mixed and absorbance (415 nm) measured every 10 seconds for 25 minutes. A negative control without protein extract was even analysed. One unit of enzyme activity was defined as the increase in 0.1 absorbance unit per minute per milligram of fresh weight (Kaushik et al., 2017).

RESULTS AND DISCUSSION

SmelPPO identification and phylogenetic analysis

In addition to the six sequences previously reported (Shetty et al., 2011), four new loci in the eggplant genome were found to encode polyphenol oxidases and named *SmelPPO7-10* (Table 3).

Locus	Gene name	Chr	Chromosome location	ORF length (bp)	Strand	Size (aa)	Protein domains	Pfam domains
SMEL_008g312510.1.01	*Smel_PPO1*	8	97,412,508: 97,414,307	1,800	-	600	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264
SMEL_008g312500.1.01	*Smel_PPO2*	8	97,401,279: 97,403,066	1,788	-	596	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264
SMEL_008g312430.1.01	*Smel_PPO3*	8	97,284,426: 97,286,198	1,773	+	591	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264
SMEL_008g312420.1.01	*Smel_PPO4*	8	97,238,764: 97,239,741	1,734	+	578	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264
SMEL_008g311990.1.01	*Smel_PPO5*	8	96,314,480: 96,316,243	1,764	-	588	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264
SMEL_008g312010.1.01	*Smel_PPO6*	8	96,395,550: 96,397,448	1,899	-	633	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264
SMEL_008g312490.1.01	*Smel_PPO7*	8	97,397,374: 97,399,167	1,794	-	598	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264
SMEL_008g312460.1.01	*Smel_PPO8*	8	97,349,335: 97,351,020	1,686	-	562	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264
SMEL_008g312520.1.01	*Smel_PPO9*	8	97,429,811: 97,432,277	2,466	-	822	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264
SMEL_000g064350.1.01	*Smel_PPO10*	2	982,270: 984,463	2,193	-	731	PPO1_DWL–DUF_B2219–Tyrosinase	Pfam 12142–Pfam 1243-Pfam 00264

Table 3: Characteristics of PPO encoding genes and of PPO proteins. *SmelPPO1-9* cluster on chromosome 8, while *PPO10*, which was initially located on an unanchored scaffold, is probably located on chromosome 2. All PPOs share the same functional domains (PPO1_DWL and Tyrosinase, and a conserved domain of unknown function, DUF_B2219).

Coding sequences retain extensive structural similarities both within *S. melongena* and with homologs in tomato and potato. The CDS of *PPO*s range in size from 1,686 to 2,466 bp; all genes except *SmelPPO3* and *SmelPPO4* are on the negative strand and, like the *PPO* genes of tomato and potato, eggplant *PPO*s do not possess introns. In all the three *Solanum* species, *PPO* genes cluster on chromosome 8 (Figure 13A), with the exception of one orthologous gene (*SmelPPO10* in eggplant, *StuPPO9* in potato, and *SlPPOG* in tomato), mapping on chromosome 2.

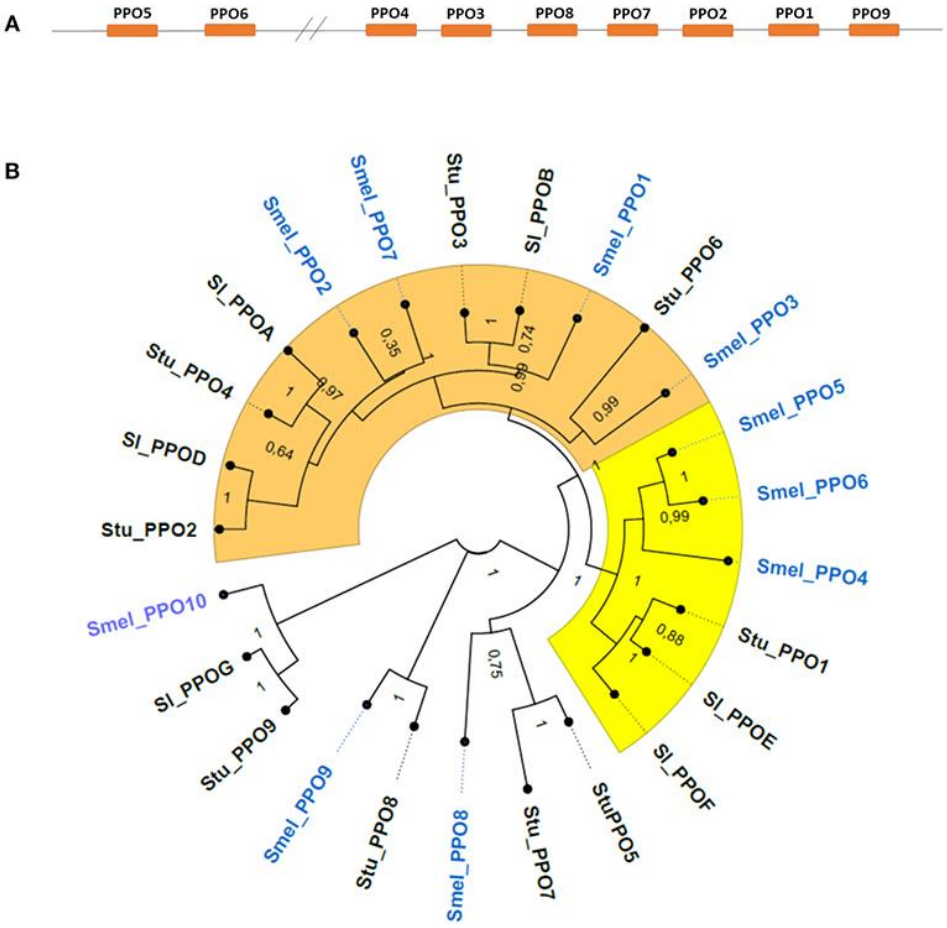


Figure 13: (**A**) Relative position and organization of *PPO1-9* genes on chromosome 8 of *Solanum melongena.* All eggplant *PPO*s except *SmelPPO10* are located on chromosome 8. **(B)** Phylogenetic analysis of PPO proteins. The neighbour-joining trees was constructed by aligning the PPO protein sequences contained in Supplementary File 1. Clade A (orange) and B (yellow) proteins. The number at each node represents the bootstrap percentage value from 1,000 replicates. *Smel = Solanum melongena*, *Sl = Solanum lycopersicum*, *Stu = Solanum tuberosum*.

This suggests that *PPO* genes evolved from tandem duplications and further supports the notion that the structure of this gene family, and possibly its functional specializations, are conserved across *Solanaceae* species.

The *PPO* encoded proteins range in size from 562 to 822 aa (Table 3). All polypeptides possess the same functional domains, namely the central tyrosinase and PPO1_DWL domains, and a C-terminal domain of unknown function (DUF_B2219), characterized by the KFDV conserved motif. In accordance with previous reports (Taranto et al., 2017), we confirmed that in the Solanaceae family two main clusters can be distinguished among PPO proteins (Figure 13B), which correspond to a functional separation between PPOs that are preferentially expressed in roots (tomato *Sl*PPO A-D, potato *Stu*PPO2 and *Stu*PPO4 and eggplant class A proteins, i.e. *Smel*PPO1-3) and PPOs whose expression is associated to defence responses (tomato *Sl*PPO E and F, potato *Stu*PPO1 and eggplant class B proteins, i.e. *Smel*PPO4-6). Among the newly identified proteins, *Smel*PPO7 clusters with class A proteins, *Smel*PPO8 with *Stu*PPO5 and *Smel*PPO9 with *Stu*PPO8. Finally, *Smel*PPO10 clusters with *Stu*PPO9 and *Sl*PPOG.

Transcriptional profiling in response to wounding

Oxidative browning in eggplant is influenced by multiple factors, including total phenolic content, *PPO* expression and also the way in which the plant integrates environmental stimuli to elicit defense responses (Docimo et al., 2016; Mishra et al., 2013; Plazas et al., 2013). The differential spatial and temporal expression patterns of PPOs *in planta* reflect the functional diversity among the *PPO* gene members. In eggplant, the expression of *SmelPPO1-6* genes was higher in young tissues and declined during plant development in mature and reproductive organs (Shetty et al., 2011). In fruits, *PPO* expression was mainly concentrated in the exocarp and in the areas surrounding the seeds in the mesocarp (Shetty

et al., 2011). *PPO* expression is mainly induced by herbivores or by mechanical damage, such as cutting.

The promoters of group B genes (Shetty et al., 2011) are characterized by the presence of several responsive elements for wounding stress and defense response (Thipyapong et al., 1997). The structural similarity of eggplant class B *PPO* genes (*SmelPPO4-5-6*) to wound-induced tomato *SlPPOF* might suggest an analogous pattern of gene regulation (Thipyapong et al., 1997).

In our study we analysed the transcript levels of *PPO* genes in the flesh of full-ripe eggplant berries of the 'Black Beauty' variety 30 minutes after cutting (Figure 14).

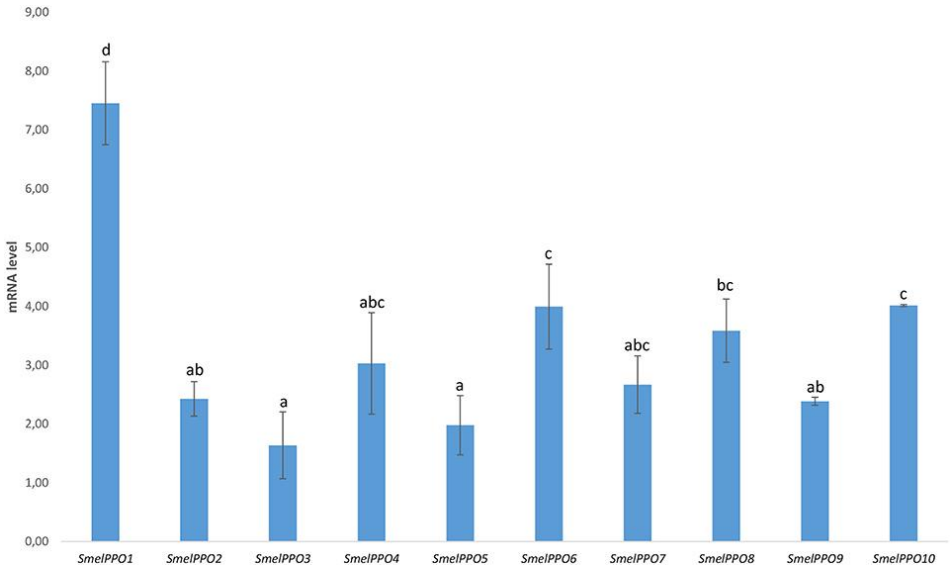


Figure 14: Transcriptional levels of 10 PPO-encoding genes in the Black Beauty variety 30 minutes after fruit cutting. The values are expressed as relative mRNA abundance at 30 minutes after cutting compared to time 0 (just after cutting). Eggplant actin and elongation factor genes were used as the reference genes. Data are means of three biological replicates ± SE. Different letters associated with the set of means indicate a significant difference based on Tukey b test ($P \leq 0.05$).

A strong increase in gene transcription in the flesh was observed for all *PPO*s, and especially for *SmelPPO1* (7.45X), *SmelPPO4* (3.03X), *SmelPPO6* (4.00X), *SmelPPO8* (3.59X) and *SmelPPO10* (4.01X). The simultaneous activation of both A and B classes of *PPO* genes was already observed in the eggplant cultivars AM086 (Docimo et al., 2016) and Arka Shirish (Shetty et al., 2011). Based on this transcriptional profile, we hypothesized that the design of an appropriate editing strategy directed at reducing detrimental oxidative browning in fruit tissues might require simultaneous suppression of several members of this multigene family. In our experiments we targeted class B *PPO* genes (*SmelPPO4*, *SmelPPO5* and *SmelPPO6*) through a CRISPR/Cas9 editing strategy. Due to their extremely high level of similarity, it was possible to design a unique gRNA against the tyrosinase domain of all class B genes.

Plant regeneration

The development of new genome editing technologies in plant breeding has fostered a growing interest for *in vitro* culture and regeneration protocols, which represent a major bottleneck in the application of these techniques in many plant species of agricultural and industrial interest. Due to the difficulties often encountered in eggplant regeneration, with available protocols being mostly inefficient or highly dependent on the genotype, no examples of genome editing in this species has been reported in literature so far. After the first report of the *Agrobacterium*-mediated transformation of eggplant (Guri & Sink, 1988), several examples of genetic transformation have been proposed using seedling explants like the hypocotyl, epicotyl, and node segments and cotyledon segments, leaf disks or roots (García-Fortea et al., 2020; Rotino et al., 2014; Saini & Kaushik, 2019).

In many plant species, the browning of tissues, which leads to toxicity and necrosis, is one of the major causes of unsuccessful *in vitro* organogenesis and regeneration from explants. Browning is associated with the oxidation of phenolics, whose release is caused by cutting and manipulating explants and calli. This problem is particularly relevant in eggplant, whose tissues are rich in phenolic compounds. Among strategies to avoid browning, the most common include the supplementation of culture media with antioxidant or adsorbent compounds (Abdelwahd et al., 2008; Menin et al., 2013).

We tested different strategies to reduce browning during eggplant tissue culture, including the addition of citric and ascorbic acid and PVP supplementation, and we found out that PVP supplementation exerts a positive effect on shoot regeneration. Among a total of 15 rooted shoots, 10 derived from the PVP-supplemented medium, 4 from not supplemented medium, and only 1 from the medium supplemented with ascorbic and citric acids. No differences were found in the phenotype of regenerants from different culture conditions. However, in spite of their notably higher number, the emergence of shoots on the PVP-supplemented medium was slower.

Dark treatments are known to increase adventitious shoot formation in cotyledon, leaf and hypocotyl explants in a number of species, including eggplant (Muktadir et al., 2016). However, we did not observe differences between shoots which underwent the 3-day dark treatment and those which did not. For all regenerating conditions, shoots apt for rooting were recovered in as short as six weeks (Supplementary Figure 1).

Screening for mutations in the T_0 generation

CRISPR/Cas9 induced mutagenesis can be employed to induce the simultaneous knockout of multiple targets within a gene family (Karunarathna et al., 2020; Sashidhar et al., 2020). Targeting a conserved gene family poses some challenges regarding the design of the gRNAs as well as the screening of edited genotypes and off-target effects. This can be particularly problematic for *PPO*s, since different members of this gene family, including the ones implicated in defence response, possess distinct activation patterns and specialized metabolic functions. By identifying a conserved region of *SmelPPO4* and *SmelPPO5*, corresponding to the tyrosinase domain, we designed a gRNA targeting both *SmelPPO4* and *SmelPPO5*, as well as *SmelPPO6* (Figure 15A).

After transformation of the CRISPR/Cas9 constructs in eggplant cotyledons and regeneration, 12 eggplant T_0 individuals (T_0_1-T_0_12) were analysed (Supplementary Data Spreadsheet). The qPCR analysis using *Cas9* gene-specific primers revealed genomic integration of the construct in nine T_0 plants, while T_0_1, T_0_6 and T_0_10 did not possess the transgene. In order to detect mutations in *SmelPPO4-5-6*, we employed targeted deep sequencing of genomic DNA, which allowed us to comprehensively assess the editing efficiency and the types of mutations (Supplementary Data Spreadsheet). Among the 9 transformed plants, the Illumina amplicon sequencing revealed that simultaneous editing of *SmelPPO4, SmelPPO5 and SmelPPO6* genes occurred in 2 lines (T_0_3 and T_0_4) (Figure 15B).

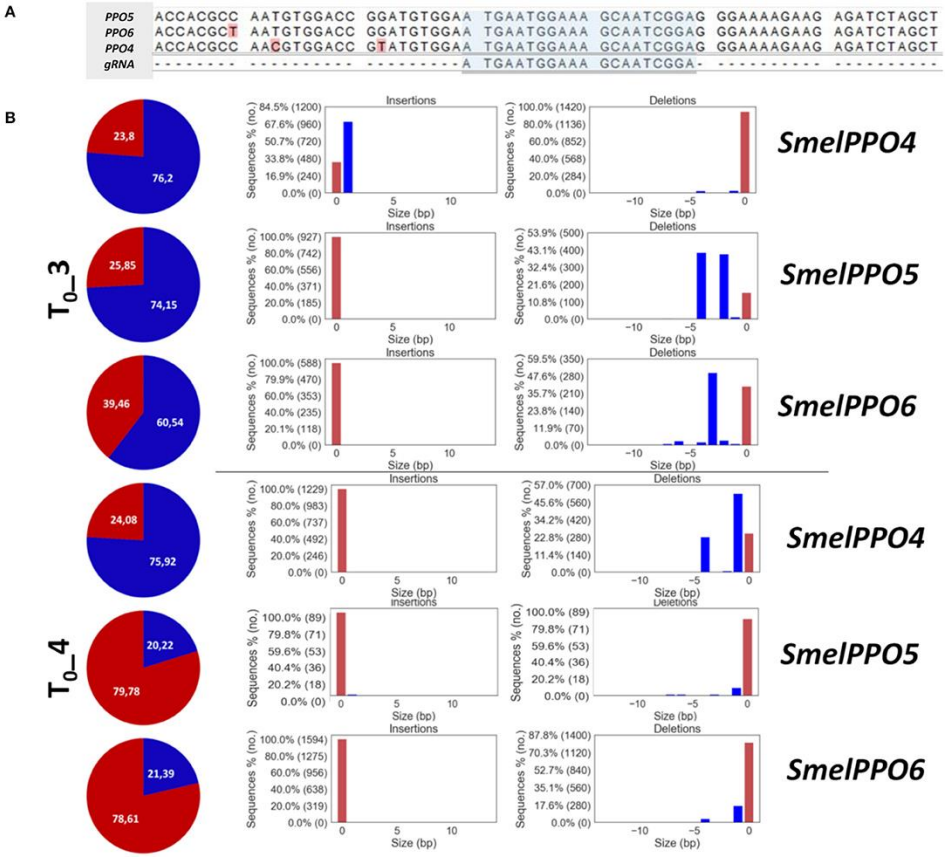


Figure 15: **(A)** Alignment of *SmelPPO4*, *SmelPPO5 SmelPPO6* with selected g*RNA*. **(B)** Genotyping of targeted gene mutations induced by CRISPR/Cas9in the T_0 generation. Quantification of Illumina reads edited at the target locus in T_0_3 and T_0_4. For each line, the percentage of reads carrying mutated (orange) as well as not mutated (blue) target sequence is reported together with the pattern and frequency of targeted gene mutations.

For the remaining lines, T_0_5 and T_0_12 showed editing only at the *SmelPPO5* locus, while T_0_10 at the *SmelPPO4* locus (Supplementary Data Spreadsheet). In most transformants, *SmelPPO5* appears edited to a higher extent than *SmelPPO4* and *SmelPPO6*, with the exceptions of T_0_3, T_0_4 and T_0_10. Notably, in T_0_5 *SmelPPO5* reached an editing efficiency of 50% while the other two loci displayed negligible levels of mutation.

Preferential editing of one member of a family sharing the same gRNA recognition sequence might depend on the transcriptional status of the target sequences. Although after wounding the expression of *SmelPPO5* is induced at lower levels than the ones of *SmelPPO4* and *SmelPPO6*, its transcript abundance seems to be generally higher, as suggested by its Ct values. The transcriptional accessibility of this locus might also reflect on its availability for the Cas9 endonuclease. It is interesting to point out that, in T_0_10, *SmelPPO4* was edited with an efficiency of over 70% although the transgene was not integrated, which highlights that it is possible to retrieve non-transgenic plants derived from edited cells in which the editing machinery presumably acted in a 'transient' fashion.

T_0_3 showed the greatest editing efficiency for all three loci, *i.e.* 76% for *SmelPPO4*, 74% for *SmelPPO5* and 60% for *SmelPPO6.* In T_0_4, a high editing efficiency was also detected, *i.e.*76% for *SmelPPO4*, 20% for *SmelPPO5* and 21% for *SmelPPO6* (Supplementary Data Spreadsheet and Figure 15B), although these values are lower than those of T_0_3. The number of plants edited at all loci was low (22%) and editing efficiencies were also significantly below the ones observed in tomato and potato, presumably as a consequence of low levels of expression of Cas9 and gRNAs (Pan et al., 2016). T_0_3 and T_0_4 had chimeric mutations (with at least 3 different alleles) in all targeted loci and retained a proportion of the wild type allele. The wild type copy of the target gene in chimeric plants could thus continue to mutate either in T_0 or in the following generations if the Cas9 transgene does not segregate. The predominance of this chimeric status in the T_0 resembles the pattern described in tomato (Nonaka et al., 2017; Pan et al., 2016). Chimerism suggests that gene editing occurred after the emergence of differentiated tissues, leading to a heterogeneous mutation pattern within the same plant. Transgene

expression might be influenced by the chromatin status at its insertion locus and, of course, by the choice of promoters. In Arabidopsis, where mutants are obtained through floral dipping and where the expression of Cas9 in the germline is crucial to fix edited alleles, the use of egg cell-specific promoters for Cas9 expression allowed to efficiently obtain non-mosaic T_1 mutants for multiple target genes (Z. P. Wang et al., 2015). In plants regenerated through somatic organogenesis, the use of egg-cell and embryo-specific promoters might also help retrieving T_1 generations with higher levels of homozygous or biallelic mutations (Zheng et al., 2020). Since no previous reports of gene editing dynamics existed for eggplant, and because we predicted the regeneration process to be the limiting factor (both in terms of efficiency and time consumption), we prioritized the use of standard gene editing constructs to maximize Cas9 expression and establish a baseline protocol. Based on this, other variants (e.g. tissue- or species-specific promoters) can be successively factored in to fine-tune the editing outcome.

Previous observations showed that small indels are the predominant mutations introduced in plants by gene editing and that the breakpoint introduced by Cas9 is placed at 3 nucleotides upstream of the PAM (Andersson et al., 2017; Bortesi et al., 2016; Pan et al., 2016). In plants, insertion of one nucleotide or deletion of 1-10 nucleotides are the most common mutations (Pan et al., 2016). The most common mutations in our T_0 eggplant plantlets were represented by a single nucleotide insertion (+G; T_0_3-*SmelPPO4*) and by a deletion of one (T_0_4-*SmelPPO4/6*; T_0_5-*SmelPPO5*), two (T_0_3-*SmelPPO5*), three (T_0_3-*SmelPPO6*) or four (T_0_4-*SmelPPO*5; T_0_10-*SmelPPO4;* T_0_12-*SmelPPO5*) nucleotides (Supplementary Data Spreadsheet).

Analysis of off-target mutations

Only few occurrences of low-frequency off-target mutations induced by CRISPR/Cas9 have been reported in plant species so far (Feng et al., 2013; Hahn & Nekrasov, 2019; Peterson et al., 2016; Wolt et al., 2016) contrary to what observed in human cells (Fu et al., 2013). The risk of off-target effects has been reported as comparable to that of somaclonal variation deriving from plant tissue culture itself (Ma et al., 2015). In order to reduce off-target effects, a strategy based on Cas9/sgRNA ribonucleoprotein complexes has been proposed (Hahn & Nekrasov, 2019). Indeed, only through a whole genome resequencing of the edited lines is it possible to exhaustively evaluate the presence of off-target mutations induced by the selected sgRNAs (Y. Liu et al., 2019). However, other screening methods make it possible to rule out the occurrence of undesired mutations at selected loci, which is reliable particularly if they correspond to transcriptionally active sequences.

One of the major risks related to targeting conserved regions in a gene family is that putative off-targets are most likely other members of the same family which, in the case of PPOs, are also located in close proximity on the genome. This makes it much more difficult to eliminate potentially undesired off-targets by breeding, than it is for non-linked loci. With respect to our gRNA, four putative off-target sequences were identified: one was an intergenic sequence, while three corresponded to other members of the *PPO* family (*SmelPPO2, SmelPPO3* and *SmelPPO7*) (Supplementary Table 2).

In order to confirm that our selected T_0 edited lines (T_0_3 and T_0_4) displayed mutations only in the *SmelPPO4-5-6* loci, we sequenced the candidate off-target loci by applying the same Illumina Amplicon Sequencing Protocol we used for the sequencing of target loci, and which

allowed us to get a deep insight into possible nonspecific editing activity. The total variation at putative off-target sites was compared between edited and wild type plants (Table 4).

SAMPLE	TARGET	READS	NUMBER OF MUTATED READS	% OF MUTATED READS
WT	OT1	17926	549	3,06
	OT2	17855	1354	7,58
	OT3	27671	10758	38,88
	OT4	26977	803	2,98
T0_3	OT1	24196	747	3,09
	OT2	21205	922	4,35
	OT3	30086	3203	10,65
	OT4	34541	822	2,38
T0_4	OT1	20621	591	2,87
	OT2	13231	476	3,60
	OT3	20282	1648	8,13
	OT4	274	8	2,92

Table 4: Quantification of Illumina reads edited at putative off-target *loci* in T_0 generation. For each individual and for each locus the total number of reads is reported, together with the percentage of reads carrying the mutated off-target sequence. The percentage of mutated sequences is reported.

We seldom observed only base substitutions consistent with SNPs or sequencing errors and, even considering those, no increase in total variation was observed between wild type and mutants. Our analyses thus demonstrated the lack of off-target effects, confirming the specificity of Cas9-mediated *PPO* gene editing in eggplant. The presence of mismatches in the seed region between our selected sgRNA and the off-target *SmelPPO*s supports the specificity of our results (Hahn & Nekrasov, 2019), since this 3' terminal region of the target sequence is known to strongly affect recognition by the Cas9 endonuclease.

Segregation of the transgene and of mutated alleles in the T_1 and T_2 progeny

Due to the early finding that in Arabidopsis many somatic mutations were not efficiently inherited, concerns about the heritability of CRISPR/Cas-induced mutations were initially raised (Feng et al., 2013). However, in all other edited monocot and dicot species, T_1 generations with high mutation efficiencies have been obtained, demonstrating the heritability of edited alleles (Gao et al., 2015; Z. Li et al., 2015; Miao et al., 2013; Pan et al., 2016; Svitashev et al., 2015). In our case, 14 T_1 plants of the T_0_4 progeny were examined to investigate the transmission pattern of CRISPR/Cas9-induced mutations. Out of 14 analysed individuals, 4 presented no detectable amplification of *hCas9* and therefore it is reasonable to deduce that the transgene was segregated (Supplementary Data Spreadsheet).

In order to detect the mutation efficiency and patterns at different sites in *SmelPPO4-5-6* genes, we employed targeted deep sequencing. The average editing efficiency was 60% for *SmelPPO4,* 52% for *SmelPPO5* and 52% for *SmelPPO6* (Figure 16A). Focusing on the *SmelPPO4* locus, five were heterozygous mutants, four chimeric, three homozygous and two WT. At the *SmelPPO5* locus, four were homozygous mutants, eight chimeric and two WT. At the *SmelPPO6* locus, three were homozygous mutants, seven chimeric and four WT (Figure 16B; Supplementary data Spreadsheet).

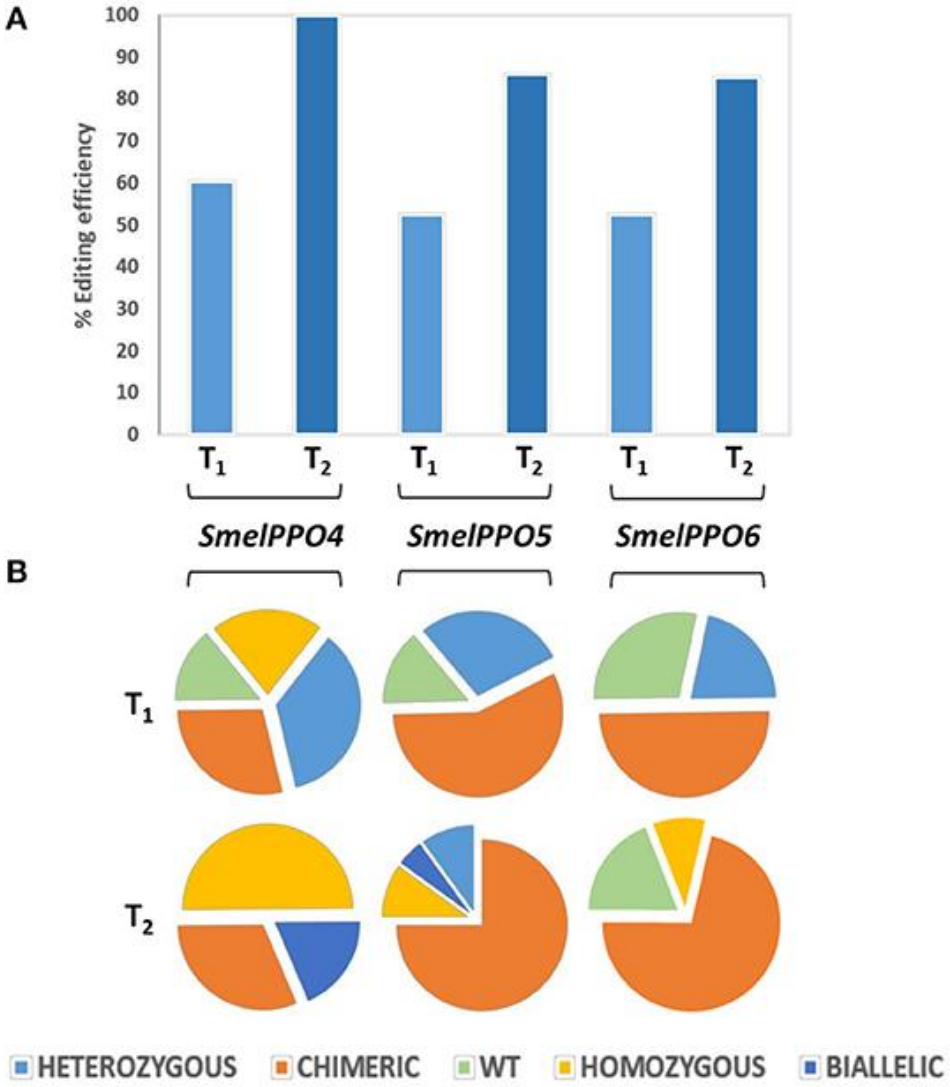


Figure 16: Genotyping of targeted gene mutations induced by CRISPR/Cas9 in the T_1 and T_2 generations. **(A)** Mutagenesis frequencies for all three targeted loci in T_1 and T_2 progenies. **(B)** Zygosity of targeted gene mutations in T_1 and T_2 populations.

The most common mutation at the *SmelPPO4* locus was a single nucleotide deletion, followed by a 4-nucleotide deletion. At the *SmelPPO5* locus different mutations were present: -2/-1/-4/-3. The segregation pattern at the *SmelPPO6* locus (which was less mutated in T_0) was less predictable and a number of new mutations (-2/-3/-7) were found in the T_1 lines. The

highest editing efficiency was highlighted for T_1_4_10: 85% for *SmelPPO4,* 90% for *SmelPPO5* and 90.7% for *SmelPPO6* (Supplementary Data Spreadsheet).

To further investigate the genetic stability of the targeted mutations we screened the T_2 plants derived from selfing T_1_4_8, T_1_4_9 and T_1_4_10 (Supplementary Data Spreadsheet). The presence of a transgene in most of the analysed T_2 plants (19/21) suggested that more than one copy of the transgene was inserted in those T_0 regenerants, which explains that Cas9 can still be active in all T_2 plants.

Compared to the T_1 generation, the mutagenesis frequency (99% for *SmelPPO4,* 85% for *SmelPPO5* and 85% for *SmelPPO6*) as well as the overall proportion of homozygous, biallelic and chimeric assets increased (Figure 16). As expected, all 7 T_2 progeny of T_1_4_11 were homozygous at the *SmelPPO4* locus, indicating that the mutations in the homozygotes were stably passed to the next generation in a Mendelian fashion. As previously observed in other species (Pan et al., 2016), the segregation patterns of the T_1 chimera lines were less predictable and a number of new mutants were obtained due to the probable continued Cas9 activity. Interestingly, T_2_4_10_1 showed homozygous mutations for *SmelPPO4* (-1/-1), *SmelPPO5* (-4/-4) and *SmelPPO6* (-4/-4) (Figure 17).

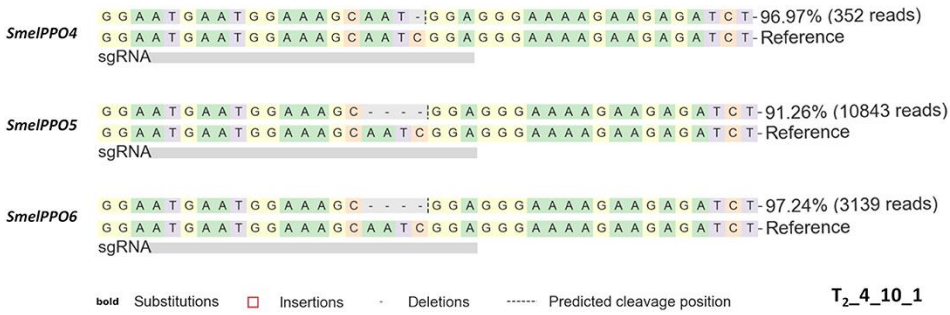


Figure 17: Genotyping of targeted gene mutations induced by CRISPR/Cas9 in the T_2_4_10_1 plant. The dashed lines represent nucleotide deletions. The reported number represents the frequency and the number of reads carrying mutated (edited) target sequence.

It has been previously demonstrated how off-target effects can be further exacerbated in the T_2 progeny as compared to T_0 and T_1 (Q. Zhang et al., 2018). Targeted deep sequencing at putative off-target loci once again demonstrated the lack of significant mutated off-targets in our T_2 progeny, confirming the specificity of Cas9-mediated *PPO* gene editing in eggplant (Supplementary Data Spreadsheet). We observed only base substitutions consistent with SNPs or sequencing errors, with similar frequencies to those observed in the T_0, which did not represent an increase in total variation between wild type and mutant lines.

Enzymatic browning and PPO activity analysis in eggplant berries

We hypothesized that the CRISPR/Cas-mediated knock out of *PPO*s would result in a lowered enzymatic browning, due to the reduced PPO activity. Selected T_1 lines (T_1_4_8, T_1_4_9 and T_1_4_10) carrying mutations in *SmelPPO4-5-6* genes were subjected to phenotypic analysis of enzymatic browning and PPO activity in berries. The lines were grown in a greenhouse and no growth alteration or changes in berry size/weight were observed during plant development when compared to wild type, as

previously observed in potato (Llorente et al., 2011). The berries were cut and exposed to air for browning induction. After 30 minutes, the typical brown discoloration due to phenolic oxidation was detected and it was clearly more evident in wild type plants in comparison to edited lines (Figure 18A). The average PPO activity of T_1_4_8, T_1_4_9 and T_1_4_10 lines was also found to be reduced by 48, 61 and 52%, respectively, compared to the wild type (Figure 18B).

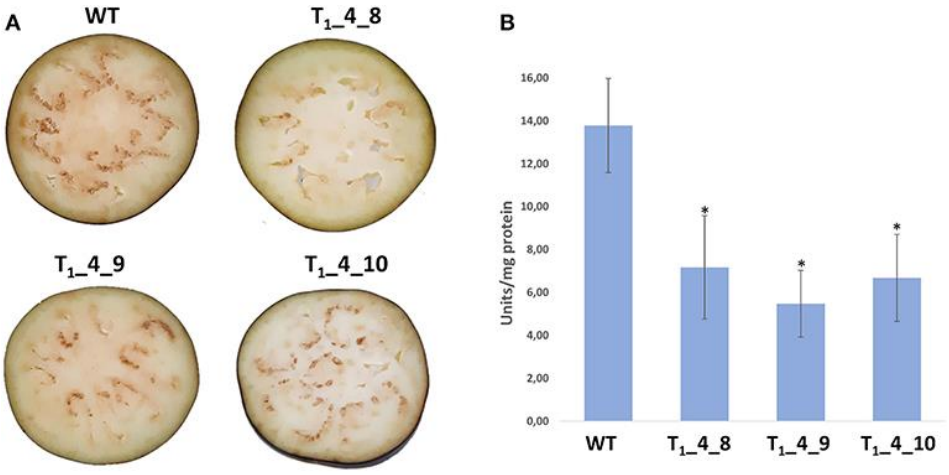


Figure 18: Phenotypical and biochemical changes associated with postcut browning. **(A)** Cut fruits of Wild Type, T_1_4_8, T_1_4_9 and T_1_4_10 showing post-cut browning 30 minutes after cutting. **(B)** Polyphenoloxidase (PPO) activity in fruits of Wild Type, T_1_4_8, T_1_4_9 and T_1_4_10. Data are means of eight biological replicates ± SD. Asterisks indicate a significant difference based on Tukey's HSD test ($P \leq 0.05$).

By comparing the T_2 edited lines with wild type a reduction of PPO activity as well as of browning discoloration upon cutting was highlighted (Supplementary Figure 2).

Several studies applied RNA silencing technologies to down-regulate the expression of *PPO* genes in potato tubers (Bachem et al., 1994; Chi et al., 2014; Llorente et al., 2011; Rommens et al., 2006). In this species, by using

the amiRNA technology (Chi et al., 2014), a reduction in PPO activity of 15–95% was obtained and it was more marked when *StuPPO1* to *4* were simultaneously suppressed. Furthermore, in a more recent study in potato, CRISPR/Cas mutants for the four alleles of the *StuPPO2* gene (which is considered the major contributor to the PPO protein content) displayed a reduction up to 69% and 73% in the PPO activity and enzymatic browning, respectively (González et al., 2020).

We can hypothesize that the partial reduction of PPO activity in eggplant, comparable to the one observed in potato mutants, might be enhanced through the knockout not only of class B PPOs (*SmelPPO4-5-6*), but even of class A PPOs (*SmelPPO1* and *SmelPPO3*). However this approach could provoke downside effects, due to the involvement of the *PPO* multigene family in important cell functions (Jukanti & Bhatt, 2015).

CONCLUSIONS

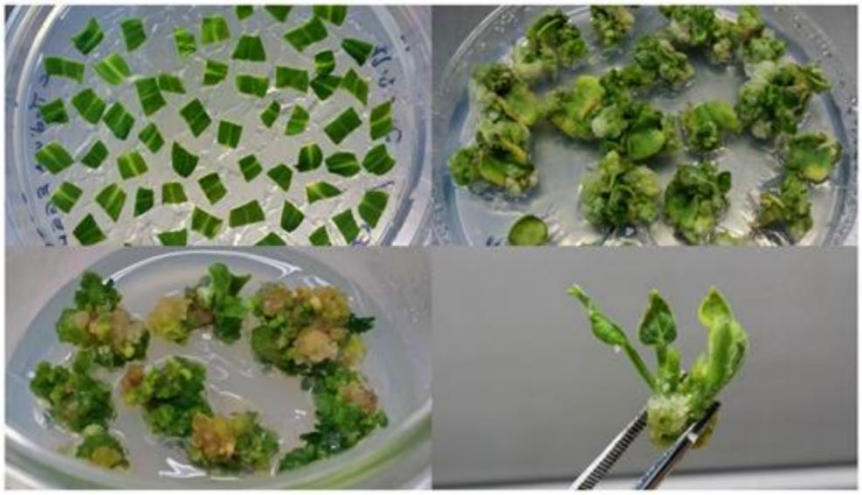
We have established a successful protocol for gene editing in eggplant, adding to the list of Solanaceae species for which CRISPR/Cas9 represents an alluring option for the introduction of specific traits through a biotechnological approach. Our system, based on the use of one guide RNA directed simultaneously at three members of the *PPO* gene family, demonstrated to be specific for the target genes, without detectable off-target effects on other members of the same gene family.

Upon cutting, edited T_1 and T_2 eggplant fruits showed a reduction of the typical brown coloration due to phenolic oxidation. Through our approach it will be possible to develop eggplant varieties that maintain their antioxidant and nutritional properties during harvest and post-harvest procedures, without reducing the content in phenolics, which are beneficial for human health.

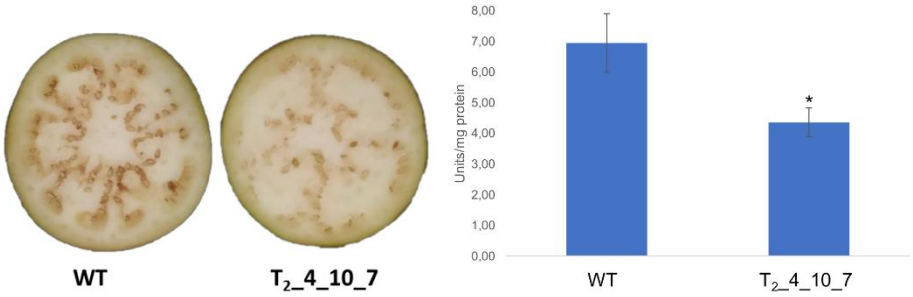
Phenolics provide a substrate to oxidative reactions catalysed by PPOs that, consuming oxygen and producing fungitoxic quinones, play a role in making the medium unfavourable to the further development of pathogens (Taranto et al., 2017); however, contrasting results are reported in literature, as *PPO* silenced lines of potato were found to acquire higher resistance to *P. infestans* (Llorente et al., 2014). Our future studies will be thus focused in assessing the relationship between PPO knock-out and pathogen response in our mutant eggplant lines.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.607161/full#supplementary-material



Supplementary Figure 1: Regeneration of Black Beauty explants transformed with *Tnos:nptII:Pnos-U6-26:gRNA:scaffold-P35S:*h*Cas9:Tnos*.



Supplementary Figure 2. (A) Cut fruits of Wild Type and T_2_4_10_7 showing post-cut browning 30 minutes after cutting. (**B)** Polyphenoloxidase (PPO) activity in fruits of Wild Type and T_2_4_10_7. Data are means of eight biological replicates ± SD. Asterisks indicate a significant difference based on Tukey's HSD test ($P \leq 0.05$).

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CHAPTER III

Setting up of Virus-Induced Genome Editing (VIGE) protocol in eggplant

ABSTRACT

Up to date, in eggplant only two CRISPR/Cas9 editing events are reported both relying on *A. tumefaciens* transformation followed by *in vitro* regeneration. Unfortunately, available protocols are mostly inefficient, highly dependent on the genotype and time-consuming, and thus new cutting-edge solutions need to be established.

Over the past several years, there has been a growing interest in using plant viruses as vectors to deliver gene-editing reagents. A possible approach is Virus-Induced Genome Editing (VIGE) based on delivering gene editing elements to plant's tissue using viral constructs.

In our work we propose an *in vitro*-free protocol for VIGE in eggplant based on TRV construct. To maximize viral expression and persistence inside the plant tissue, we optimized VIGS approach previously developed in eggplant by targeting a reporter gene (*SmelChl*). We compared two infection methods (agro-infection and *Nicotiana benthamiana* sap inoculum) in cotyledon tissues. Our results highlighted that sap inoculum on cotyledons is the best suiting approach to be used for VIGE.

Three VIGE constructs have been developed, with sgRNA targeting eggplant *Chl* fused to: (a) isoleucine tRNA (b) Flowering Locus T and (c) truncated version of Flowering Locus T. 20% and 10% of the plants infected with the first two constructs showed the expected phenotype, no changes were observed upon the infection with the third construct. Infected plantlets were checked for mutations in the target region and sequencing analysis highlighted editing efficiency spanning from 3 to 36%.

INTRODUCTION

CRISPR/Cas9 is at current time the most solid technique amongst genome editing tools. This system can be used in several plants species for a plethora of purposes. Above all, it represents a fundamental instrument to elucidate and validate gene function (Putcha et al, 2022). Even if different protocols for CRISPR/Cas9-based genome editing have been already developed, the application of this technology is not a routine in most cases. Its widespread use is prevented by the need of genetic transformation tools mediated by *Agrobacterium tumefaciens* or biolistic methods. Both the techniques have important limitations, such as the recalcitrance of many plant species to *Agrobacterium* transformation and the lack of an *in vitro* regeneration protocol for the species and the genotype under research (Lacroix et al, 2019).

Overexpression of morphogenic regulators like *Baby boom*, *Wuschel* (Lowe et al, 2016) or *GRF* and its cofactor *GIF1* (Debernardi et al, 2020), have been explored as a tool for expanding the range of plant species and genotypes regenerable *in vitro* (Mookkan et al, 2017; Lowe et al, 2018). In any case, the regeneration process itself presents different flaws: it can be a source of genetic and epigenetic differences caused by *in vitro* conditions (Phillips et al, 1994; Kaeppler et al 2000) and represents a time and resources consuming step that requires expertise and facility to be carried out.

To get rid of integrated transgenes like Cas9, sexual segregation is needed. Transient expression of Cas9 machinery, like protoplast transfection with ribonucleoprotein (Park et al, 2019) or delivery into whole plants using nanoparticles (Demirer et al., 2019; Kwak et al., 2019), avoids the introgression of any transgene but still requires *in vitro* regeneration steps to obtain edited plants.

The possibility to use viral constructs to deliver gene-editing elements into plants' tissue, Virus-Induced Gene Editing (VIGE), had been proposed as a solution to avoid any *in vitro* regeneration step (Dinesh-Kumar et al, 2020; Atkins et al, 2020). Different viral constructs have been already used for VIGE (summarized in Zhang et al, 2022). Depending on the cargo capacity of the viral construct used, this technique can be applied following two main strategies: 1) using the virus for the expression of one or more gRNAs into a plant expressing Cas and 2) using the virus for the expression of both gRNAs and Cas machinery. To recover edited seeds, editing element (sgRNAs/Cas) or the virus particles must be able to move into meristems and therefore into sexual organs. Because viruses rarely move into the meristems, an attractive solution could be to induce the editing element expressed in somatic tissues to move as protein or RNA into meristems itself. An interesting possibility is to fuse the sequence of the sgRNA to that of the Flowering Locus T (*FT*). *FT* mRNA is known to move into shoot meristems in both *Nicotiana benthamiana* and *Arabidopsis thaliana* (Jackson et al, 2012; Notaguchi et al, 2015). It is also known that *FT* mRNA bound to viral RNA makes the latter to spread easier into both shoot meristems and systemic cells (Li et al, 2009). From study on RNA moving by floematic stream (Thieme et al, 2015) and viral moving RNA (Takeda et al, 2016) it has been possible to identify conserved structures, called t-RNA like structure (TLs), that facilitate movement of such RNAs (Zhang et al, 2016). The usage of sgRNAs bound to *FT* RNA and tRNA has been successfully implemented in both *Nicotiana benthamiana* (Ellison et al, 2020) and *Arabidopsis thaliana* (Nagalakshimi et al, 2022) plants harboring highly-expressed *Cas9*, allowing the production of edited seedlings. A Tobacco Rattle Virus (TRV) based construct was used in both these transformations. Efficient

heritable gene editing was also obtained with a vector derived from potato virus X (PVX) in *N. benthamiana* using the mobile FT fragment (Uranga et al., 2021a).

TRV is a well-studied RNA-positive strand virus belonging to *Tobravirus* genus. TRV viral genome is bipartite into two genomic parts, named TRV1 and TRV2, required for infection (MacFarlane et al, 1999). TRV was first engineered for VIGS (Ratcliff et al, 2001) and its use for this purpose is well established, mainly in *Solanaceae* species (Mysore et al, 2022). TRV possesses different features making it suitable to VIGE: it infects a wide range of host species, viral RNA genome doesn't integrate in plant's genome, its small genome size can be easily engineered (Ali et al, 2015) and induce mild to no symptoms (Ratcliff et al, 2001). Moreover, TRV is able to move into meristems, even if with low efficiency (Ratcliff et al, 2001). For these reasons, TRV has been used for VIGE in different *Solanaceae*, like *Nicotiana benthamiana* (Ali et al, 2015; Ellison et al, 2020), *Nicotiana attenuata* (Oh et al, 2021) and *Arabidopsis thaliana* (Nagalakshimi et al, 2022).

In eggplant (*Solanum melongena* L.), belonging to *Solanaceae* family, genome editing is not well developed. Up to date, only two events of Cas9-mediated genome editing targeting PPO family have been carried out (Maioli et al, 2020; Kodackattumannil et al.,2023). Even if an *in vitro* protocol has been developed, low *in vitro* regeneration rates and editing efficiency prevent its application.

In eggplant TRV has been already used for VIGS purpose (Liu et al, 2012; Yan et al, 2012; Xi'ou et al, 2015; Wang et al, 2017) showing a good movement and persistence in plant's tissues. Liu et et, 2012 reported a persistency of the virus, highlighted by silencing of reporter genes, up to three months. In eggplant different methods have been used to infect

plants, such as agro infection (Liu et al 2002a, Liu et al 2012) or high-pressure spray (Liu et al, 2002, Liu et al, 2012). An interesting alternative method, never applied in eggplant, is to inoculate viral particles into the plant using sap collected from a *N. benthamiana* previously infected with TRV vectors (Brigneti et al, 2004).

In our work we propose an *in vitro*-free protocol for VIGE in eggplant based on TRV construct. To maximize viral expression and persistence inside the plant tissue, we first optimized VIGS approach previously developed in eggplant by targeting a reporter gene (*SmelChl*). The optimized protocol has been applied taking advantage of three VIGE constructs with sgRNA targeting eggplant *Chl* fused to: (a) isoleucine tRNA (b) a modified *FT* (*mFT*) and (c) a modified and truncated version of *FT* (*mtFT*).

MATERIALS AND METHODS

Optimization of VIGS protocol in eggplant

VIGS: Plasmids preparation

In order to compare our results with previous published protocol, target gene and sequence used for gene silencing was chosen from Liu et al, 2012: H subunit of the magnesium chelatase enzyme (*SmelChl_H*; gene ID SMEL4.1_11g022140.1.01). Silencing of this gene confers to the leaves a yellow phenotype due to chlorophyll depletion.

TRV-2 silencing plasmid was therefore prepared according to Liu et al, 2002. Target sequences were bought as oligonucleotides (O1; Supplementary table 4). 3'-XbaI and a 5'-SacI restriction sites were added by PCR using P1 and P2 primer on O1 (Supplementary table 4). PCR products were then purified using "DNA Clean & Concentrator" (ZymoResearch, Irvine, USA). Purified amplicons and plasmid pYL156

(TRV-2, addgene #148969) were digested using XbaI (NEB, Ipswich, USA) and SacI (NEB, Ipswich, USA). Digested fragments and plasmids were purified from gel using Zymoclean Gel DNA Recovery Kit (ZymoResearch, California, USA) and quantified by Nanodrop. pYL156-*Chl_H* was generated by ligation reaction between digested pYL156 and amplicon. A specific software (NEBioCalculator) was used to calculate 3:1 vector/insert molar ratio. All the ligation product was transformed into *Escherichia coli* strain DH10b by heat shock and plated on LB agar + kanamycin 50mg/l and incubated at 37°C overnight. Positive colonies were cultured overnight in LB + kanamycin 50mg/l at 37°C. From the liquid culture a glycerol-stock was prepared (1600 µl bacteria + 400 µl glycerol 100%) and the plasmids were harvested using ZymoPURE Plasmid Miniprep Kit (ZymoResearch, California, USA). Plasmids were Sanger-sequenced using P1-P2 on pYL156-*SmelChl_H*. pYL156-*SmelChl_H*, pYL156 and pYL192 (TRV-1, addgene #148968) were transformed into *A. tumefaciens* strain GV3101 by heat shock and plated in LB agar + kanamycin 50 mg/l + gentamycin 25 mg/l + rifampicin 25 mg/l at 28°C overnight.

VIGS: Plant material

Seeds of eggplant cv Black Beauty and of *N. benthamiana* were sown in soil (van Egmond universele potgrond) and grown in a climate chamber (temperature 25°C, RH 60%, 16 h light: 8 h dark photoperiod cycle, light intensity of 300 µmol m^{-2} s^{-1} PPFD). Plants for inoculation on cotyledons were ready after 19 days. For *N. benthamiana* 4 weeks old plants were used for agro infection.

VIGS: Inocula preparation

Three *A. tumefaciens* inocula were prepared (pYL156-*SmelChl_H*, pYL156 and pYL192). VIGS inocula were prepared in 3 days. On the first day *A. tumefaciens* glycerol-stocks were streaked on LB agar + kanamycin 50 mg/l + gentamycin 25 mg/l + rifampicin 25 mg/l and incubated at 28°C overnight. Grown bacteria were collected with a tip and inoculated in LB + kanamycin 50 mg/l + gentamycin 25 mg/l + rifampicin 25 mg/l at 28°C overnight. Liquid cultures were then centrifuged at 3000 rpm for 20', resuspended in infiltration medium (Supplementary table 5) at OD=1 and incubated for 5h at room temperature in the dark.

So, a bacterial solution was prepared mixing 1:1 *A. tumefaciens* pYL192 (containing TRV1) with pYL156_*SmelChl_H*.

VIGS: Eggplant cotyledon inoculation

Cotyledons from 19 old days plants were infiltrated by a needless 1ml syringe using the bacterial solutions prepared as described in Material and methods (VIGS *Inocula preparation*). 8 plants were infiltrated using pYL192-pYL156_*SmelChl_H* and 2 plant was infiltrated using pYL156 as a control.

VIGS: *N. benthamiana* sap inoculum preparation

Leaves of 4-weeks old plants were infiltrated by a needless 1ml syringe using the bacterial solution prepared as described in Material and methods (VIGS *Inocula preparation*). After 5 days, those leaves were crushed in a mortar, added of 0,5-1ml of sodium phosphate 100 mM and filtered through a double layer of cheesecloth.

This procedure was followed to produce two different infected sap: (1) pYL192-pYL156_*SmelChl_H* and (2) pYL156 (as a control).

VIGS: eggplant sap inoculum in cotyledons

Sap inoculum was rubbed using a hard sponge on the back of cotyledons from 19 old days plants. 8 plants were infected using pYL192-pYL156_*SmelChl_H* and 2 plants were infected using pYL156 as a control.

VIGS: evaluation of gene silencing

After 4 weeks plants were checked for silencing phenotype and pictures were taken. RNA was extracted from all the treated plants using GeneJET RNA Purification Kit (Thermo scientific, Massachusetts, USA) following instructions. cDNA was synthesized from 1 µg of RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, USA). For the evaluation of gene silencing RT-qPCR were performed on cDNAs by the amplification of three targets: elongation factor (*SmelEF*) as plant housekeeping gene, TRV2 and *SmelChl_H*. Primers are reported in Supplementary table 3. PCR reactions were carried out by using the StepOnePlus Real-Time PCR System (Thermo Fisher, Waltham, USA). The following PCR program was used: 95°C/10', followed by 40 cycles of 95°C/15'' and 60°C/1'. Transcript levels were quantified through the $2^{-\Delta\Delta Ct}$ method.

Optimization of VIGE protocol in eggplant

VIGE: sgRNA design and plasmid preparation

One sgRNA for target *SmelChl_H* (T1) was designed using the online tool CRISPR-P2.0 (hzau.edu.cn). Sequence is reported in Supplementary table 3. Three TRV-2 plasmids containing the same sgRNA for *SmelChl_H* were made, according to the procedure described in Material and methods (VIGS *Plasmids preparation*). XbaI and SacI digested and purified

plasmids and sgRNA were ligated. Three different plasmids were obtained: (1) SPDK3888_*SmelChl_H* (sgRNA-tRNA^{ileu}), (2) SPDK3895_*SmelChl_H* (sgRNA-*mFT*) and (3) SPDK3905_*SmelChl_H* (sgRNA-*mtFT*). All the plasmids were transformed by heat shock method into *A. tumefaciens* strain GV3101.

VIGE: plant material

Eggplant and *N. benthamiana* were sown and grown following procedures described in Material and methods (VIGS *Plant material*).

VIGE: inocula preparation

According to the protocol described in methods (VIGS *N. benthamiana sap inoculum preparation*) five inocula were prepared: (1) SPDK3888_*SmelChl_H* (2) SPDK3895_*SmelChl_H*, (3) SPDK3905_*SmelChl_H*, (4) pYL192 and (5) pYL156.

Four bacterial solutions were thus prepared by mixing 1:1 *A. tumefaciens* pYL192 with: (1) SPDK3888_*SmelChl_H* (2) SPDK3895_*SmelChl_H*, (3) SPDK3905_*SmelChl_H*.

VIGE: infection

According to the data gathered during VIGS protocol optimization, the infection for VIGE was performed using *N. benthamiana* infected sap rubbed on eggplant's cotyledons. Procedures are described previously. For each inoculum 20 eggplant plantlets were infected while 5 plantlets were infected with YL156 as control.

VIGE: molecular screening

One month after the infection, the plants showing the expected edited phenotype (yellow leaves) were photographed. DNA was extracted from these plants using E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek, Norcross, USA). Editing efficiency was evaluated by amplification of the target genomic region flanking the editing site (P9-P10; Supplementary table 4). DNA was amplified using KAPA HI-FI Taq (Kapa Biosystems, Boston, USA). After purification with AMPure XP beads (Beckman Coulter, Brea, USA), PCR products were sequenced by Sanger method. Chromatograms were analyzed using Synthego ICE online tool (https://ice.synthego.com/#/).

RESULTS

With the aim of optimizing the movement and the persistence of TRV in eggplant a VIGS experiment was set up. To determine if endogenous gene silencing could be elicited by TRV-mediated VIGS, we inserted *SmelChl_H* gene into a pTRV2 vector. Two different infection methods were compared: agro infection and sap-inoculation.

Virus induced gene silencing in eggplant through agro infection method

8 plantlets were infected by agro infection on cotyledons with a mixture of Agrobacteria solution containing TRV1 and TRV2-*SmelChl_H*. 2 plantlets were infected as controls using TRV2.

4 weeks after the infection, only one plant treated with TRV2-*SmelChl_H* showed silencing phenotype (yellow leaves caused by decreased chlorophyll content) (Table 5; Figure 19). A5 plant required one additional week (5 weeks) to show silencing phenotype.

Plant	Silencing phenotype
A1	✓
A2	✗
A3	✗
A4	✗
A5	✓
A6	✗
A7	✗
A8	✗
Ctr1	✗
Ctr2	✗

Table 5: phenotypic evaluation of TRV agro-inoculated plants

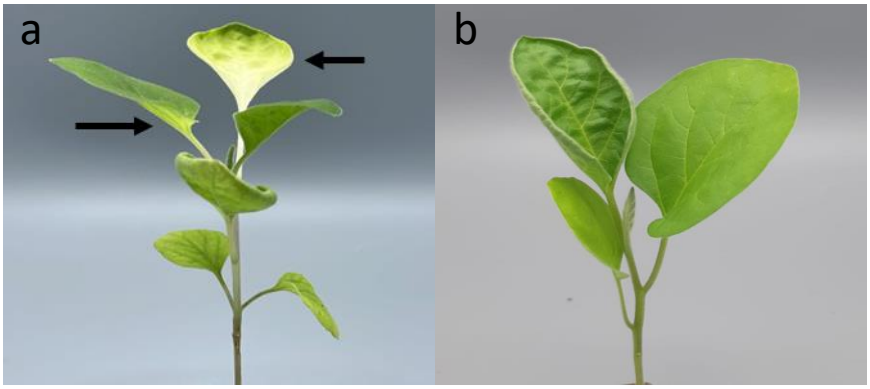

Figure 19: a) A1 plant, showing *SmelChl_H* silencing phenotype (yellow leaves), b) control plant.

To monitor the silencing level of *SmelChl_H* and the presence of TRV, qPCR analyses were performed (Figure 20). As expected, the expression of TRV2 was only detected in the two plants showing silencing phenotype, A1 and A5 (although at much lower level). Target analysis on *SmelChl_H* showed very variable results, with only two plants presenting a clear reduction of transcript levels of the target gene (A1: -67%; A5: -87%).

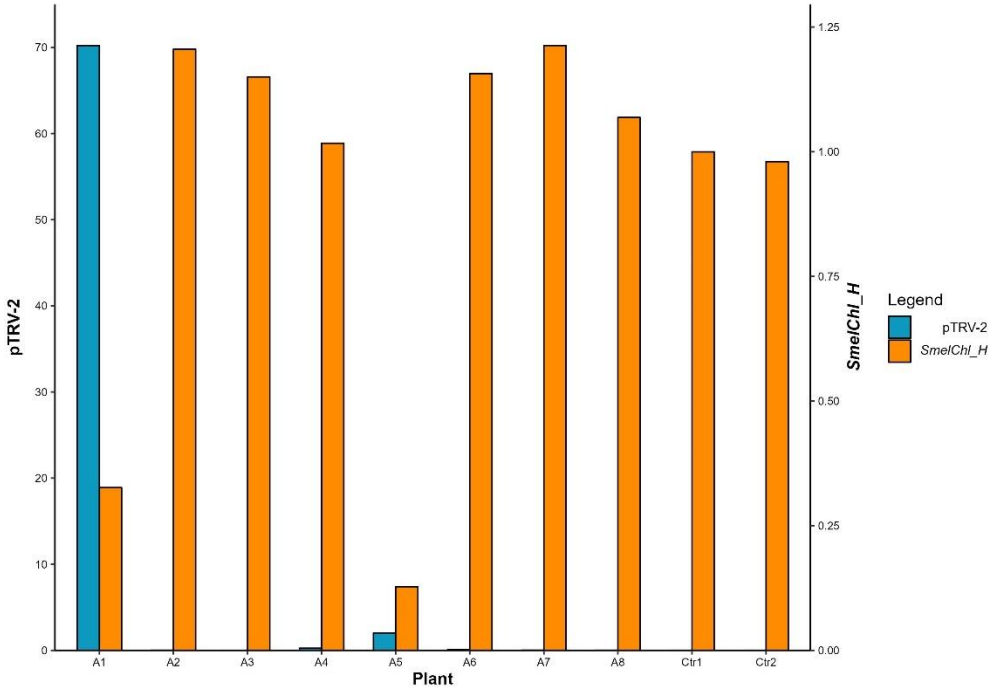

Figure 20: pTRV2 and *SmelChl_H* expression in agro infected plants

Virus induced gene silencing in eggplant through sap inoculation method

8 plantlets were infected by rubbing the cotyledons using sap collected from *N. benthamiana* leaves inoculated with a mixture of Agrobacteria solution containing TRV1 and TRV2-*SmelChl_H*. 2 plantlets were infected as controls using sap from a *N. benthamiana* agro inoculated with TRV2.

After 3 weeks, all the inoculated eggplant plantlets showed yellow leaves caused by decreased chlorophyll content (Table 6; Figure 21).

Plant	Silencing phenotype
S1	✓
S2	✓
S3	✓
S4	✓
S5	✓
S6	✓
S7	✓
S8	✓
Ctr1	✗
Ctr2	✗

Table 6: phenotypic evaluation of sap-inoculated plants.

Figure 21: a) b) c) plants showing *SmelChl_H* silencing phenotype (yellow leaves); d) control plant.

TRV2 was detectable in all the analyzed samples although at variable level, the highest expression was underlined for plant S8 (Figure 22). *SmelChl_H* expression showed a lower level than the control in all the inoculated plants. Average *SmelChl_H* transcript levels were reduced of 60% compared with control, spanning from S5 (reduction of 43%) to S4 (reduction of 75%).

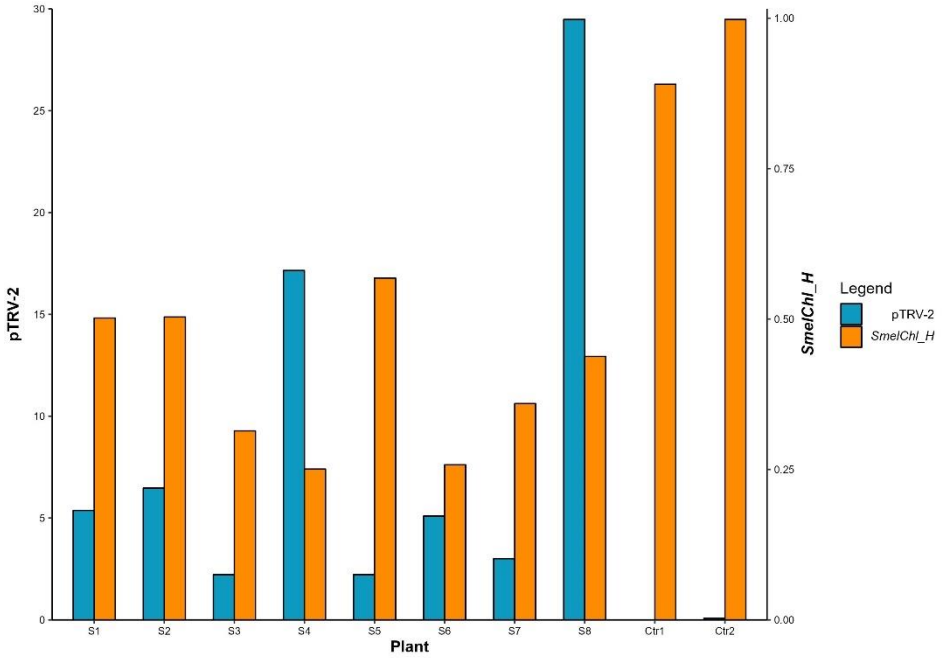

Figure 22: pTRV2 and *SmelChl_H* expression in sap infected plants

Setting up of VIGE protocol in eggplant

VIGE was performed using three pTRV2 constructs harboring the same sgRNA, targeting *SmelChl_H*, fused to three mobile elements ($tRNA^{ileu}$, *mFT*, *mtFT*). Mobile elements have been demonstrated to improve sgRNA mobility into meristems and therefore increase the chance to obtain edited seeds (Ellison et al, 2020; Nagalakshimi et al, 2022).

Because sap inoculum resulted to be the best infiltration method, it was used for VIGE.

For each construct, 20 eggplants were infected on cotyledons using sap collected from *N. benthamiana* agro-infiltrated with (1) SPDK3888_*SmelChl_H* (2) SPDK3895_*SmelChl_H*, (3) SPDK3905_*SmelChl_H*, (4) pYL156.

4 weeks after inoculation, plantlets showed leaf yellowing mainly in proximity of veins (Figure 23). Compared to silencing phenotype observed upon VIGS (Figure 19, Figure 21), VIGE-infected leaves presented much patchier yellow spots.

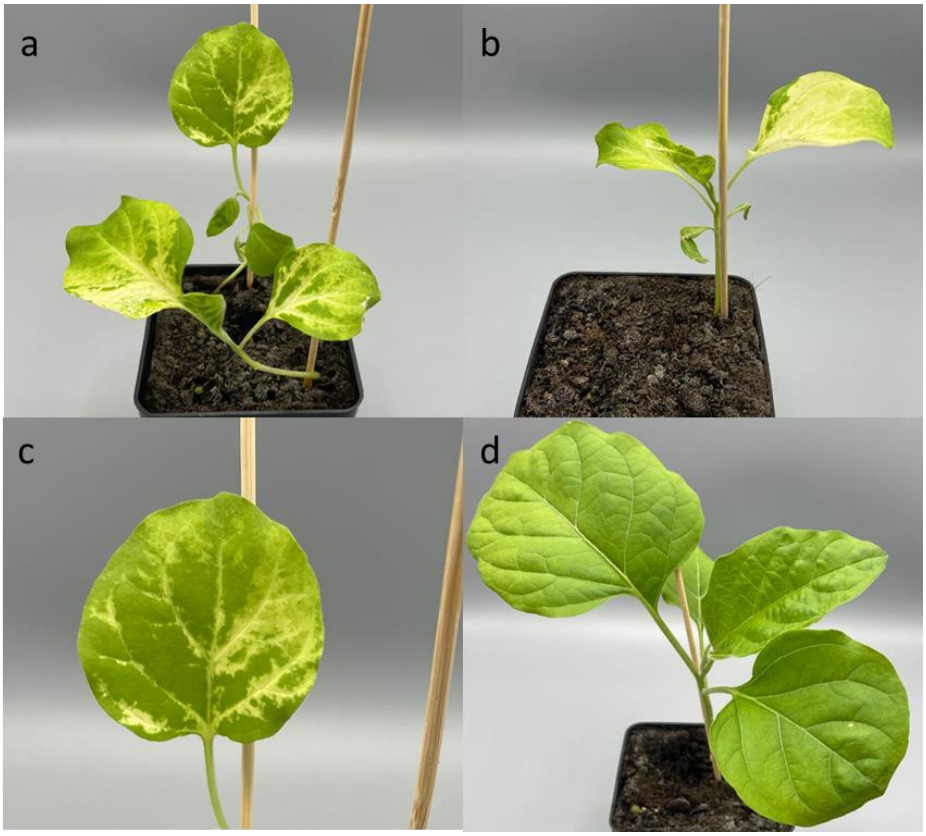

Figure 23: a) b) VIGE-infected eggplant plantlets; c) a leaf close-up; (d) control plant

Plants showing altered phenotype were sequenced through Sanger method and chromatograms were analyzed using Synthego online tool (Figure 24).

Figure 24: example of Synthego output (plant V6)

To check the relation between the editing efficiency and expected phenotype even some plants not showing any abnormal phenotype were sequenced. The average VIGE efficiency of the three constructs was variable: 20% for SPDK3888_*SmelChl_H* (4 plants out of 20), 10% for SPDK3895_*SmelChl_H* (2 plants out of 20) and 0% for SPDK3905_*SmelChl_H* (Table 7).

Plant	Construct	Phenotype	Editing Efficiency	R^2	Wt	+1	-1	-2	-3	-6	-7
V1	SPDK3888_*SmChl_H*	✓	13	0,99	86	5	6	2			
V2	SPDK3888_*SmChl_H*	✓	16	0,99	83	12	2	3			
V3	SPDK3888_*SmChl_H*	✓	16	0,99	83	12	3	2			
V4	SPDK3888_*SmChl_H*	✓	0	1	100						
V5	SPDK3888_*SmChl_H*	✗	0	1	100						
V6	SPDK3895_*SmChl_H*	✓	36	0,96	60	15	9	5	4	1	2
V7	SPDK3895_*SmChl_H*	✓	3	0,99	96	3					
V8	SPDK3895_*SmChl_H*	✗	0	1	100						
V9	SPDK3895_*SmChl_H*	✗	0	1	100						
V10	SPDK3905_*SmChl_H*	✗	0	1	100						
V11	SPDK3905_*SmChl_H*	✗	0	1	100						
V12	SPDK3905_*SmChl_H*	✗	0	1	100						
Ctr	TRV2	✗	0	1	100						

Table 7: Genotyping of targeted gene mutations induced by VIGE on *SmelChl_H*.

As expected, indels were detected only in plants showing altered phenotype with editing efficiency spanning from 3% to 36%. The most common mutation obtained was the insertion of a single nucleotide, followed by the deletion of a single nucleotide. Deletion of a higher number of nucleotides (up to 7) were also detected but at a lower rate. Notably, in V6 an editing efficiency of 36% was observed.

Surprisingly, plant V4 presented no edited alleles on target gene even if some phenotype was recognizable.

DISCUSSION

To overcome both *in vitro* regeneration bottleneck and transgene integration, an intriguing solution might be represented by the usage of viral constructs to deliver CRISPR/Cas machinery (Cas and sgRNA) into meristems in order to obtain edited seeds (Dinesh-Kumar et al, 2020; Atkin et al, 2020). This transgene-free and *in vitro* free transgenerational gene editing could allow an improvement of gene editing in eggplant.

There are several viral-based constructs used throughout different species for Virus-Induced Genome Editing (VIGE). Since in eggplant no examples of VIGE were reported so far, we selected Tobacco Rattle Virus (TRV) previously used for VIGS in this plant species (Liu et al, 2012; Zhen et al, 2015; Yang et al, 2020). Even if VIGS and VIGE are two techniques with different expected outcomes, the optimization of VIGS protocol is a pivotal step for VIGE setting up.

Inoculation methods for VIGS and VIGE are the same and a wide range of options can be considered. In eggplant only few inoculation methods have been used: agro-infection, high-pressure spray (Liu et al, 2012) and vacuum infiltration of sprouts (Yan et al, 2012). An interesting alternative method could be represented by the inoculation of infected sap (rich in

viral particles) collected from infiltrated *N. benthamiana*'s leaves (Senthil-Kumar et al, 2014). This could avoid the bacterial solution used in agro infection and its related optimization of methods for inoculation of specific plant (bacterial OD used, *A. tumefaciens* strains etc). Sap inoculum has been observed to induce less necrosis on infection site than agro infection (Brigneti et al, 2004).

In eggplant VIGS has been applied in leaf (Liu et al, 2012; Yang et al, 2020) and fruit (Zhen et al, 2015; Wang et al, 2018) tissues. VIGS on cotyledon has been demonstrated to be a better option than leaves for different plant species, such as *Solanum rostratum* (Meng et al, 2016), cotton (Gao et al, 2012), tomato (Velazques et al, 2007), pepper (Zhou et al, 2021) and *N. benthamiana* (Rahman et al, 2021).

In order to optimize the movement, replication and persistency of TRV particles in eggplant's tissue, we optimized VIGS approach previously developed in eggplant by targeting a reporter gene (*SmelChl_H*). *SmelChl_H* gene encodes for H subunit of the magnesium chelatase enzyme, a key enzyme involved in chlorophyll biosynthesis. Its silencing confers an easily recognizable phenotype, making the leaves yellow due to chlorophyl depletion. *Chl_H* silencing has already been performed in different species, like *Pisum sativum* (Wu et al., 2022), *Nicotiana benthamiana* (Hiriart et al., 2003) and eggplant (Liu et al, 2012).

Based on literature data we infected eggplant cotyledons through agro infection and sap inoculum, that represent a complete novelty in eggplant. Our results were directly compared with Liu et al, 2012, whose constructs were used in our research too. In Liu et al, 2012 four reporter genes were silenced through 2 methods: agro infection and high-pressure spray. Out of the four silenced reporter genes (*SmelChl_H*, *SmelPDS*, *SmelSu*, *SmelCla1*), *SmelChl_H* resulted the most interesting one on the basis of

faster and long-lasting phenotype appearance and silencing efficiency and has been targeted in our experiments.

Results obtained through VIGS showed important differences between the two used delivery methods. Agro infection on 8 plants allowed to recover only 2 plant (25%) showing silenced phenotype (yellow leaves, Table5, Figure 19) and *SmelChl_H* silencing (of 67% ad 87%, Figure 20). In plants showing no phenotype, no virus or negligible presence was underlined. Sap inoculum' results were instead far more interesting. 100% of the treated plants showed the presence of TRV and the expected phenotype (Table 6, Figure 21). Gene silencing was also highlighted to be highly variable ranging from -43% to -75% (average -60%, Figure 22). In Liu et al, 2012, 78% of plants showed *SmelChl_H* silencing phenotype after high-pressure spray on leaves. On the basis of this comparison silencing efficiency appeared to be higher using sap on cotyledons than agro infection using high-pressure spray on leaves. *SmelChl_H* transcript levels were not evaluated in Liu et al, 2012. Wang et al, 2017 reported an 80% reduction of *SmelChl_H* transcript following agro infection of eggplants leaves. While it appears to be higher than the average silencing evaluated in our research (60%), no data about the number of plants showing phenotype are reported.

Sap inoculum on cotyledon's tissue appeared to be an effective approach to be performed with minimal equipment required and was adopted for our VIGE experiment. Persistency of silencing (3 months) observed in our experiment is comparable to data reported by Liu et al, 2012.

With the aim of obtaining a transgene-free line by an *in vitro* free method, we followed a VIGE approach described in *N. benthamiana* (Ellison et al, 2020) and in *Arabidopsis thaliana* (Nagalakshmi et al, 2022). Both these approaches were based on Cas9-expressing lines infected with TRV

harboring sgRNA fused to mobile elements (ME). ME are a class of RNA that able to travel into floematic stream. Previous studies highlighted that *Flowering Locus T* (FT) mRNA (Jackson et al, 2012; Notaguchi et al, 2015) and tRNA-like viral particles (Thieme et al, 2015; Takeda et al, 2016) are able to move into floematic stream. For these reasons Ellison et al, 2020 and Nagalakshmi et al, 2022 fused their sgRNAs with FT RNA (in three versions: full, modified, modified and truncated) and different RNAs ($tRNA^{ileu}$, $tRNA^{met}$). In addition to somatic editing, heritable gene editing has even been obtained.

The VIGE approach chosen for eggplant comprised the usage of the same kind of TRV construct and three different ME (*mFT*, *mtFT*, $tRNA^{ileu}$) and a sgRNA targeting *SmelChl_H*.

While 20% of the plants treated with sgRNA-$tRNA^{ileu}$ showed yellow phenotype, only 10% of the eggplant infected with sgRNA-mFT showed modified leaf color. No modified phenotype was underlined with sgRNA-*mtFT* (Figure 23, Table 7). It is noteworthy that induced phenotype, if any, was far less spread on the tissue than the one induced by VIGS probably due to a chimeric zygosity status. In Arabidopsis about 3%, 8% and 22% of the plants showed photobleached regions (upon *pds* editing) after leaf infiltration, agro-pricking methods and agro-flooding (Nagalakshmi et al., 2022).

In our work, target sequencing showed an editing efficiency of *SmelChl_H* spanning from 13% to 16% (for sgRNA-$tRNA^{ileu}$) and from 3% to 36% (for sgRNA-*mtFT*), while no editing was observed for sgRNA-*mFT* (Table 7). Our plants present much lower editing efficiency than what observed in *N. benthamiana* and *A. thaliana* (Ellison et al, 2020; Nagalakshmi et al, 2022). In *N. benthamiana*, TRV-based VIGE allowed to recover an average somatic editing of 61% using sgRNA_*NbPDS-FT*, 87% using

sgRNA_*NbPDS-mFT*, 88% using sgRNA_*NbPDS-mtFT*, 73% with unmodified sgRNA-*NbPDS*, but no statistical differences were observed among the different constructs. Addition of mobile elements increased strongly the hereditability of editing nin the progenies (from 10% for not modified sgRNA to around 80% for modified sgRNAs) (Ellison et al, 2020). In *Arabidopsis* the editing efficiency was 60%–99% in the completely photobleached regions, 20%–70% in the mosaic regions, and 0%–26% in the green regions. On the contrary our results are comparable to those observed in cotton, where the mutation efficiencies of *GhCLA1*-sgRNA1, *GhCLA1*-sgRNA2 and *GhPDS*-sgRNA were 30.01-51.14%, 16.85-42.46%, and 25.74-52.68%, respectively (Lei et al, 2022).

Molecular analysis performed in edited plant obtained through stable transformation (Maioli et al, 2020) demonstrated that -1 and -2 are the most common induced mutations. In VIGE, the most common mutation was the insertion of a single nucleotide, followed by the deletion of one or two bases. Average editing efficiency from stable transformation trial was 16,1%, while in VIGE was 16,8%. Even if results are strongly comparable, editing efficiency still needs to be improved. It can be hypothesized that the low efficiency detected in our VIGE experiment might be related with the low level of Cas9 protein expression, that need to be quantified through a Western Blot analysis. Another option could be related to behavior of TRV: even if it can infect different *Solanaceae*, eggplant is not one of the main host for this virus.

CONCLUSIONS

Although the strategy of fusing sgRNA with FT and $tRNA^{ileu}$ allowed efficiency somatic editing, we still have to demonstrate if this could also

generate heritable gene-edited offspring. Once seeds will be obtained, they will be sown and plants checked for phenotype and editing efficiency.

CRISPR-Cas technologies require the expression of large Cas nucleases, whose sizes were generally considered above the cargo limit of viral vectors. A higher cargo capacity was highlighted for Sonchus Yellow Net virus (SYNV), that still requires *in vitro* regeneration of infected tissue (Ma et al, 2020). In a recent work a vector from Tomato Spotted Wilt Virus (TSWV, family *Tospoviridae*) was engineered to express SpCas9, LbCas12, or Cas-derived base editors along with multiple guide RNAs (Liu et al, 2023). Since TSWV displays one of the largest host ranges among plant viruses, it will be interesting to test this viral system even in eggplant. Another alternative could be represented by Cas12e and Cas12j, formerly known as CasX and CasΦ, small enough to be used for VIGE using TRV vectors.

DATA STATEMENT

Supporting data and information can be retrieved at this link: https://drive.google.com/drive/folders/1TlSewUCYY5QRF0oz2X4Lw-Tjs8xqQA1O?usp=drive_link.

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CONCLUSIONS AND FUTURE PERSPECTIVES

The development of novel genome editing tools in *Solanaceae* has the potential to significantly enhance comprehension of gene functions and revolutionize plant breeding strategies. CRISPR/Cas9 has been successfully established in Solanaceous crops and has proven its value in applied research directed at understanding the genotype-phenotype relationship. Genome editing in tomato is well consolidated with array of genome editing tools already optimized.

In warm semi-arid areas, crop plants are exposed to a multitude of stressors and may be challenged by more than one stress factor at the same time, and therefore need to adapt to different combinations of stresses. One of the greatest challenges for agricultural science is to develop cultivars resistant to biotic and abiotic stresses to enhance crop resilience.

Chapter I focuses on *SlDMR6-1* tomato mutants generated through the CRISPR/Cas9 technique. We demonstrated for the first time that *SlDMR6-1* gene knock-out could contribute to the development of new varieties resistant to drought stress through a water saving strategy. The drought-avoidance mechanism might be related to a successful coupling between an efficient ROS scavenging activity and the drought prevention strategy allowing stomatal control of photosynthesis.

All the data generated in this study makes gene editing of *DMR6-1* a highly reliable and valuable target for tomato breeding. An interesting future perspective could be to test the drought avoidance of *Sldmr6-1* lines in field-trial condition or by using High-Throughput Phenotyping platform available in our Department. Since KO mutation of *SlDMR6-1* has been demonstrated to confer a broad-spectrum disease-resistance phenotype in tomato, it would be challenging to test the effect of combined abiotic and biotic stresses on *dmr6-1* mutants.

Protocols for *in vitro* culture and regeneration represent a major bottleneck in the application of genome editing techniques in many plant species of agricultural and industrial interest. The difficulties encountered in *in vitro* regeneration hampered the application of genome editing tools in eggplant. The **Chapter II** reports the first example of CRISPR/Cas9 genome editing in eggplant. Our editing system, based on the use of one guide RNA directed simultaneously at three members of the PPO gene family (*PPO4*, *5* and *6*), demonstrated to be specific for the target genes, without any off-target effects. The induced mutations were associated with a reduced PPO activity (of 50%) and browning of the berry flesh after cutting. Our study represents a first step towards the development of eggplant varieties maintaining the nutritional properties during harvest and post-harvest procedures, without the utilization of potentially harmful browning controlling agents. This could result in benefits for eggplant processing industry, and finally the consumer.

Despite its fundamental importance genome editing still needs faster, easier and more efficient protocols in many plant species. To overcome both *in vitro* regeneration bottleneck and transgene integration, an *in vitro* free Virus Induced Genome Editing (VIGE) protocol for eggplant based on viral constructs able to deliver CRISPR/Cas machinery was set up and described in **Chapter III**. The experimental results showed that the TRV-mediated VIGE system can effectively achieve targeted editing of eggplant endogenous genes but we still have to demonstrate if this could also achieve heritable gene editing. Moreover our study provides an accurate and rapid validation tool for screening effective sgRNAs in eggplant.

Although functional, CRISPR/Cas9 approaches based on both stable transformation and VIGE protocol in eggplant have been found to be characterized by low editing efficiency. It would be interesting to test the

efficiency of different Cas protein, like the super compact Cas12e and Cas12j, formerly known as CasX and CasΦ. These proteins could present a higher editing efficiency in eggplant and could be small enough to be used for VIGE using TRV vectors, potentially leading to an *in vitro* and transgene free method. Recent work by Yang and colleagues (doi.org/10.1038/s41587-022-01585-8) described a transgene-free and heritable strategy for the incorporation of targeted mutagenesis in plants through grafting. Their study shows the mobility of Cas9 and single guide RNA (sgRNA) transcripts when fused with tRNA-like sequence (TLS) motifs across the grafted junction from transgenic rootstock to wild-type scion. This breakthrough strategy could be an ambitious alternative to be tested even in eggplant.

When discussing the regulation of genome edited plants, it is important to understand if the frequency of unintended DNA mutations deriving from NHEJ-mediated repair, as it happens in CRISPR/Cas9 events, differs from the other conventional breeding methods. The European Commission recently planned (Proposal for a REGULATION OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on plants obtained by certain new genomic techniques and their food and feed) to separate plants obtained through New Genomic Techniques (NGTs) into two categories: those "comparable to naturally occurring or conventional plants" ('category 1 NGT plants') and those with "more complex modifications" ('category 2 NGT plants'). Thus plants and products belonging to 'Category 1 NGT', such as those obtained through targeted mutagenesis or cisgenesis, should not be subject to the rules and requirements of the Union GMO legislation. 'Category 1 plants' must differ from the recipient or parent plant by no more than 20 genetic modifications, among whose: i) substitution or insertion of no more than 20 nucleotides; ii) deletion of

any number of nucleotides. On the basis of our Whole Genome sequencing analysis we can state that CRISPR/Cas9 represents the "cleanest" tool in our hands to introduce targeted mutations, since our *dmr6-1* tomato edited line carries an insertion that makes it completely indistinguishable from spontaneous mutants. While our *dmr6-1* tomato edited line can be considered a Category 1 NGT plant, our *ppo* eggplant edited lines not due to the presence of the transgene (Cas9) within the genome. Italian Parliament recently approved field trials of plants obtained in the laboratory belonging to category 1 NGT plants. We hope that our *dmr6-1* tomato edited lines might be tested soon in field trial.

PUBLICATIONS

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