

University of Naples Federico II

Department of Agricultural Sciences

PhD in Food Science

XXXVIII Cycle

Improving tomato fruit quality by editing genes of strigolactone and ascorbic acid pathway

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2024-2025

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SOMMARIO

La definizione generale di “qualità del frutto” racchiude diversi aspetti che, nel loro insieme, influenzano l'apprezzamento del consumatore verso un prodotto alimentare e la sua idoneità al mercato. Per il pomodoro (*Solanum lycopersicum* L.), l'ortaggio più diffuso e consumato al mondo, il profilo nutrizionale del frutto è fondamentale per definirne la qualità complessiva, e il suo miglioramento ha suscitato notevole interesse nella ricerca agricola negli ultimi anni.

In questa tesi di dottorato è stata esplorata la qualità nutrizionale del pomodoro in piante in cui sono stati mutati diversi geni appartenenti al pathway degli strigolattoni (SL) e al pathway dell'acido ascorbico (AsA) usando la tecnologia CRISPR/Cas9.

Nel **Capitolo 2** di questa tesi è riportata la caratterizzazione di una collezione di piante di pomodoro modificate per i quattro geni principali coinvolti nella biosintesi degli SLs, ovvero *SID27*, *SICCD7*, *SICCD8* e *SIMax1*. Queste piante mutate non accumulano SLs, risultando quindi resistenti alla colonizzazione da parte di piante parassite (*Phelipanche ramosa* L. e *P. aegyptiaca* L.). In diverse specie, incluso pomodoro, è stato consolidato come il knockout (KO) parziale o completo dei geni della biosintesi degli SL abbia effetti sullo sviluppo morfologico-vegetativo della pianta mutata. In questa tesi, ci si è focalizzati sul confronto di questi geni con il controllo non-editato (Wild-Type, WT) per tratti fiorali e relativi al frutto. Questi studi hanno confermato la presenza di cambiamenti del fenotipo nelle linee mutate *Slccd7*, *Slccd8* e *Slmax1*. Tuttavia, la linea *Sld27* KO è risultata simile al genotipo controllo, anche per i tratti legati al peso e alle dimensioni del frutto. Inoltre, poiché gli SL sono composti derivati dai carotenoidi, l'accumulo di questi metaboliti è stato analizzato nei frutti delle linee KO, non mostrando tuttavia variazioni sostanziali. Al contrario, importanti variazioni sono state riscontrate nel profilo di accumulo di alcuni composti volatili (VOCs), metaboliti essenziali che influenzano l'aroma e la qualità complessiva del frutto di pomodoro. Il maggiore impatto è stato osservato nei VOC derivati da fenilpropanoidi, aminoacidi ramificati e fenilalanina. Anche in questo caso, le linee *Sld27* hanno mostrato solo alterazioni minori nella produzione di VOC e, al contrario, cambiamenti significativi sono stati dimostrati nei mutanti per i geni più a valle nella via biosintetica come *Slccd8* e *Slmax1*. In particolare, lo pseudoionone, un metabolita derivato dagli apocarotenoidi (ApoC) e positivamente associato all'aroma del pomodoro, è risultato moderatamente aumentato nelle *Sld27*. Nel complesso, questi risultati hanno mostrato un effetto gene-specifico mai descritto prima per i geni implicati nella biosintesi degli SL e hanno evidenziato che la mutazione del gene *SID27* non causa effetti deleteri sul fenotipo delle piante editate.

Il gene *SID27* codifica per l'unica β -carotene isomerasi nota finora in pianta; tuttavia, due ulteriori β -carotene isomerasi – note come *D27-Like 1* e *D27-Like 2* – sono state recentemente descritte in riso e in *Arabidopsis*. È stato ipotizzato che i geni *D27* e *D27-Likes* siano coinvolti nella biosintesi degli SL e nell'interazione tra SL e l'acido abscissico (ABA), un ulteriore fitormone che regola finemente la fisiologia e lo sviluppo della pianta. Ciononostante, le funzioni di questi geni rimangono ancora poco chiare. La linea di ricerca sviluppata nel **Capitolo 3** di questa tesi si è concentrata sulla

caratterizzazione funzionale dei geni appartenenti alla famiglia genica del gene *D27* in pomodoro. In particolare, è stata descritta l'identificazione dei geni *SID27-Like 1* e *-Like 2* nel genoma del pomodoro mediante analisi filogenetiche *in silico*, e la generazione di linee KO CRISPR/Cas9 per gli stessi geni. Gli effetti di queste mutazioni sul fenotipo, sulla fisiologia e sulla composizione di metaboliti secondari sono stati valutati nei mutanti *SID27-Like 1* e *-Like 2* ottenuti, e nelle linee *SID27* precedentemente prodotte. Come precedentemente descritto in letteratura, il fenotipo delle linee in cui i geni *SID27-Like1* e *-Like 2* sono stati inattivati, è risultato indistinguibile da quello delle linee non modificate (WT). L'ipotesi di un ruolo di questi geni nell'interazione SL-ABA è stata esplorata analizzando l'accumulo di ABA nelle foglie, il quale è risultato aumentato in tutti i mutanti editati. Coerentemente, la conduttanza stomatica (gs) è risultata generalmente ridotta in tutte le linee *Sld27* e *Sld27-Like 1* e *-Like 2*. L'analisi dei metaboliti secondari nelle foglie delle piante mutate ha mostrato come l'accumulo dei metaboliti non polari rimane perlopiù invariato; al contrario i composti polari hanno mostrato variazioni rilevanti. In particolare è stato evidenziato un accumulo di AsA nelle linee KO *Sld27* e *Sld27-Like 1*, e di acido chinico (QA) in tutte le linee *Sld27* e *Sld27-Likes*. Altri polifenoli, come la naringenina calcone (ChNar), l'iperoside (quercetina-3-galattoside; Hyp) e il kaempferolo-3-O-glucoside (K-3-G), sono risultati moderatamente diminuiti, principalmente nelle linee *Sld27*. Nel complesso, le linee CRISPR/Cas9 KO per i geni *SID27-Like 1* e *-Like 2* generate nel corso di questa tesi, rappresentano materiali unici e di grande valore per futuri studi volti a stabilire il ruolo delle *SID27* β -carotene isomerasi nella biosintesi dell'ABA e nella composizione dei metaboliti dei frutti.

Nel **Capitolo 4** di questa tesi, la tecnica CRISPR/Cas9 è stata utilizzata al fine di inattivare due ascorbato ossidasi (*SIAO* e *SILAC*) attive nel pathway di rigenerazione dell'AsA, ed aumentare il contenuto di questo metabolita essenziale nei frutti di pomodoro. Il fenotipo delle piante mutate è risultato simile al fenotipo delle piante non mutate. Il contenuto di solidi solubili (SSC, °Brix), è invece risultato moderatamente aumentato nelle linee editate per i geni *SIAO* e *SILAC*. I risultati più interessanti, tuttavia, sono stati ottenuti relativamente al contenuto di AsA ridotto in radice, foglia e frutto. Le linee KO, infatti, hanno mostrato un accumulo fino al 60,5% di AsA ridotto nei frutti a piena maturazione (mature red, MR). Inoltre, è stato osservato un aumento nell'accumulo di questo metabolita nelle radici dei mutanti *Siao* e nelle foglie dei mutanti *Sllac* suggerendo una diversa espressione dei geni *SIAO* e *SILAC* nelle radici e nelle foglie delle piante di pomodoro.

Nel complesso, questa tesi ha fornito risultati notevoli nell'ampliare le conoscenze sugli effetti prodotti dalla manipolazione di due diverse vie biosintetiche sulla qualità del frutto di pomodoro, con la produzione di prezioso materiale genetico, che rappresenta una ricca risorsa per studi futuri.

SUMMARY

Fruit quality is a general term that encompasses several aspects that, taken together, influence consumer appreciation of a food product and its market suitability. For tomato (*Solanum lycopersicum* L.), the most widespread and consumed vegetable in the world, the fruit nutritional profile is crucial for defining its overall quality, and in recent years its improvement has gained considerable interest in agricultural research.

In this thesis, tomato nutritional quality was explored in plants employing the CRISPR/Cas9-mediated inactivation of various genes from two different pathways: the strigolactone (SL) and the ascorbic acid (AsA) pathways.

In the **Chapter 2** of this thesis, the characterization of a repertoire of tomato edited plants for the four core genes involved in SLs biosynthesis, namely *SID27*, *SICCD7*, *SICCD8* and *SIMax1* is described. These edited plants did not accumulate SLs being, therefore, resistant to parasitic weeds (*Phelipanche ramosa* L. and *P. aegyptiaca* L.) colonization. It was established in various species that the partial or complete knockout (KO) of SL-biosynthesis genes has deep effects on the plant phenotype. Therefore, morphology-, flower-, and fruit-related traits were assessed in these KO-edited plants and in the non-edited plants. This comparison confirmed the effects on the phenotype of the mutations in the *Slccd7*, *Slccd8* and *Slmax1* genes. On the contrary, the *Sld27* KO line was largely comparable to the non-edited wild-type (WT) line, also for fruit-related traits as fruit weight and size. Since SLs are carotenoid-derived compounds, the composition in carotenoids was analysed in fruits from the KO lines. The most significant variations were found in the production of VOCs, metabolites essential for determining aroma quality, another fundamental aspect affecting the overall quality of tomato fruit. The most affected VOCs are the derivatives of phenylpropanoids, branched-chain amino acids and phenylalanine. Also in this case, the *Sld27* lines showed only minor alterations in VOC production and, conversely, significant changes emerged in mutants for genes downstream in the biosynthetic pathway, such as *Slccd8* and *Slmax1*. In particular, pseudoionone – an apocarotenoid (ApoC)-derived metabolite positively associated to tomato flavour – was found to be moderately increased in the line *Sld27*. Altogether, these results displayed a gene-specific effect, never reported before for the genes involved in the SL biosynthesis, and highlighted the *Sld27* as a promising line due to its WT-like phenotype. The gene *D27* has been the only gene known for encoding a β -carotene isomerase so far. However, two additional *D27*-Likes (*D27*-Like 1 and *D27*-Like 2) genes were recently described in the rice and Arabidopsis genome. It has been hypothesised that the *D27* and *D27*-like genes are involved in SL biosynthesis and have been proposed as key mediators of the SL-ABA interaction, which finely tunes plant physiology and development. Nonetheless, the physiological functions of these genes are far from being clear. The research line developed in **Chapter 3** was focused on the functional characterization of the members of the *SID27* gene family. Herein it was described the identification of the *SID27*-Like 1 and -Like 2 genes in the genome of tomato, and the generation of CRISPR/Cas9 KO lines. The effects of these mutations on

the plant phenotype, physiology and secondary metabolite composition, were assessed on the complete panel of mutants, therefore including the KO lines for the already known *Sld27* gene. In according to the literature, the plant phenotype of the *Sld27*-Likes KO lines was undistinguishable from those of the non-edited lines. The hypothesis that these genes have a role in the SL-ABA interaction was explored analysing the accumulation of ABA that resulted augmented in the leaves of all the edited lines. Consistently, the stomatal conductance (gs) was generally reduced in all the *Sld27* and *Sld27*-Likes KO lines. Polar and non-polar secondary metabolites accumulation were further analysed in leaves. Non-polar metabolites accumulation was generally unchanged, whereas the accumulation of polar compounds exhibited relevant variations. AsA levels, in particular, increased in the *Sld27* and *Sld27*-Like 1 KOs, and quinic acid (QA) accumulation resulted higher in all the edited lines. Other polyphenols – as naringenin chalcon (ChNar), hyperoside (quercetin-3-galactoside; Hyp) and kaempferol-3-O-glucoside (K-3-G) – were moderately decreased mostly in the *Sld27* lines.

Chapter 4 describes how the mutation of two ascorbate oxidase genes, which are active in the AsA recycling pathway, increases the fruit content of this essential metabolite. In particular, the role of the *S/OAO* and the *S/LAC* genes encoding for two ascorbate oxidases was studied through the characterization of KO lines produced by CRISPR/Cas9-mediated editing. Minor changes were reported for the plant phenotype of the mutated lines. The soluble solids content (SSC, °Brix) was higher in the *S/OAO* KO1 and KO2 lines and in *S/LAC* KO line. Interestingly, the KO lines were found to accumulate up to 60.5% more reduced AsA in mature red (MR) fruits compared to the non-edited lines. Moreover, this metabolite was higher in the roots of the *S/OAO* lines and in the leaves of the *S/LAC* line. These results support the previously predicted function of *S/LAC* as a potential additional ascorbate oxidase. Furthermore, the accumulation pattern observed for the reduced AsA in leaves and roots suggest a differential expression these genes, with *S/OAO* being mainly expressed in root and *S/LAC* in leaves.

Altogether, this thesis has provided remarkable outcomes in expanding the knowledge on the effects produced by the manipulation of two different biosynthesis pathway on the tomato fruit quality, with the generation of valuable genetic materials, representing a rich resource for future studies.

CHAPTER 1 – General introduction

1.1 Importance and diffusion of tomato

Tomato (*Solanum lycopersicum*, L. 1753) belongs to the Solanaceae botanical family, that includes more than 3000 species and some of the most popular and widespread vegetables crops, such as potato (*S. tuberosum*, L.), eggplant (*S. melongena*, L.) and pepper (*Capsicum annuum*, L.). Being native of the Andean region – as part of Chile, Boliva, Ecuador, Colombia and Peru, tomato reached Europe during the colonization of the New World in the 15th century, and is currently the most diffused vegetable throughout the world, after potato (Bai & Lindhout, 2007). Tomato is cultivated for its fruits, regarded as a berry with a fleshy mesocarp (Davies et al., 1981). In 2023, the tomato global production reached 192 million tonnes distributed on 5 million hectares (FAOSTAT 2024). China and India supply more than half of the entire production, while Europe's contribution is also significant, accounting for around 16.1%, with Italy as the second largest producer in the world (FAOSTAT, 2022).

While in the Mediterranean area tomato is commonly consumed fresh, processed-tomato products are the most widespread. The transformation process, in fact – as canning and drying – facilitates its storage, still preserving most of its nutritional properties (Vats et al., 2022). Due to the feasibility of cultivation and distribution on the market, tomato has rapidly become a primary crop and a staple food of global importance, significantly contributing to the dietary intake of vegetables (Vats et al., 2022).

1.2 Tomato fruit quality: an overview

Since its impact on the human diet and its relevance as a source of nutrients, the aspect of tomato quality is crucial.

Tomato quality is a complex and dynamic parameter defined by extrinsic and intrinsic properties of the berries. The visual and sensory traits are related to the early perception of the fruit by the market and the consumers, and include the integrity, the morphology (size, shape) and the general appearance (colour, firmness, aroma) of the fruit (Fernqvist, 2014). These quality traits vary depending on the intended use of the fruits, particularly if they are for fresh consumption or for the processing (Davies et al., 1981). The nutritional profile, conversely, is commonly referred to as an intrinsic property, as it could be only assessed through analytical processes. Nonetheless, the composition in primary and secondary metabolites significantly influences the extrinsic properties of the fruit, as the colour, the flavour and the aroma (Fernqvist, 2014).

The tomato nutritional quality depends largely on the accumulation of primary metabolites. Primary metabolites are in fact the most abundant compounds, being also referred to as macronutrients (Ali et al., 2020; Quinet et al., 2019). The content in primary metabolites is defined on the dry matter, which generally accounts for the 5-10% of the total weight, and is typically classified on the basis of the alcohol-solubility of its constituents (Davies et al., 1981). Most of the soluble fraction – being around 75% of the dry matter – is composed by reducing sugars, with fructose and glucose being the most represented, whereas the amount of sucrose is negligible. Organic acids – as citric and

malic acid – along with dicarboxylic amino acids (glutamic and aspartic acids), and most minerals give an important contribution to the composition of the soluble fraction. The insoluble fraction includes proteins and fibres (pectic substances, cellulose, and hemicellulose). Lipids can be found both in the soluble and insoluble fractions and encompasses around 2% of the total dry matter. Overall, these metabolites have a main role in determining the fruit flavour (soluble fraction) and consistency (insoluble fraction) (Ali et al., 2020; Davies et al., 1981).

Normally less abundant than the primary metabolites, secondary metabolites are micronutrients produced by the plants upon external stimuli and stresses, or for further biological necessities, as attracting pollinators (Kim et al., 2014). The tomato fruits are characterized by a variegated repertoire of secondary metabolites, including non-polar (lipophilic), polar (hydrophilic) and volatile compounds. Carotenoids (vitamin A, vit. A) and tocopherols (vitamin E, vit. E) for the non-polar fraction, together with ascorbic acid (vitamin C, vit. C) and polyphenols for the polar fraction, are the major contributors to the nutraceutical quality of tomato fruits, being essentials as vitamin precursors and antioxidants. The volatile compounds (VOCs) are instead responsible for the flavour and aroma of the berries (Ali et al., 2020; Davies et al., 1981; Paolo et al., 2018).

1.2.1 Carotenoids and volatiles compounds (VOCs)

Among the tomato non-polar secondary metabolites, carotenoids are the most abundant (Inbaraj & Chen, 2008). Structurally, carotenoids are polyene isoprenoids, and therefore belong to the terpenoid class. In particular, they consist of 40-carbon backbone (tetraterpenoids) linear or cyclic molecules, originating by the repetition of eight residues of the C₅ isoprene structural unit (Inbaraj & Chen, 2008). Due to the presence of conjugated double-bonds along the molecule, carotenoids can function as pigments, thus absorbing and reflecting light (Inbaraj & Chen, 2008; Martí et al., 2016). These compounds are synthesised in all the above-ground organs of the plant by chloroplast; where they localize in the thylakoid membranes associated with the light-harvesting complexes, where they have a major role in the photosynthesis by both extending the range of photosynthetically active radiation (PAR) and having photoprotective effect. During plant development, carotenoids tend to accumulate in other plant organs as flowers and fruits, in specialized plastids – namely the chromoplasts (Fraser et al., 1994; Young, 1991). The total carotenoid content in tomato fruits is 7-23 mg/100 g of fresh weight (FW), depending on the genotype, the ripening stage and the cultivation conditions (Inbaraj & Chen, 2008; Martí et al., 2016). In the ripe berries, lycopene accounts for the 60-90% of total carotenoids, being responsible for their deep-red colour (Wu et al., 2022). Moreover, lycopene is the precursor of the second most abundant carotenoid (up to 1.2 mg/100 g FW) namely β -carotene, associated with the orange colour of the fruit (Martí et al., 2016). Other coloured carotenoids are the yellow xanthophylls lutein, violaxanthin and neoxanthin, mostly found in leaves and flowers in traces (Davies et al., 1981; Martí et al., 2016). The colourless phytoene and phytofluene, biosynthesis intermediates of the previously mentioned carotenoids, can also be present in tomato in concentrations of around 2.9 and 1.6 mg/100 g FW respectively (Martí et al.,

2016). As mentioned above, carotenoids are among the health-beneficial compounds present in the tomato fruits. These molecules are in fact potent antioxidants due to their scavenging activity of the reactive oxygen species (ROS), primarily mediated by the singlet molecular oxygen ($^1\text{O}_2$) quenching. Carotenoids (viz. lycopene, β -carotene, lutein), in fact, preserve the cells from photooxidation damages, having anti-inflammatory effects and being associated with a reduced cancer risk (Rowles & Erdman, 2020). An additional reason beyond the importance of carotenoid as essential nutrients relies on the role of β -carotene as provitamin A, which is converted in vit. A and in its active forms (i.e.: retinol) upon its uptake with the diet. Retinol is crucial in the vision process, being part of the light-sensitive pigment rhodopsin present in the eye cells. However, the processes in which vit. A is involved are multiple and include the regeneration and integrity of the skin and mucous membrane cells and the responsiveness of the immune system, in addition to its aforementioned antioxidant properties, which overall make vit. A important for the prevention of neurodegenerative diseases (Beecher, 1998; Vats et al., 2022).

The aspect of quality related to the fruit aroma and flavour is primarily determined by VOCs accumulation. Usually present at nanomolar or picomolar concentrations, VOCs are generally low-molecular weight compounds derived by the degradation of diverse biosynthesis precursors: carotenoids, amino acids, fatty acids, phenolics and phenylpropanoids. The precursors by which they originate represent the most common way to cluster VOCs (Distefano et al., 2022). Over 400 VOCs have been characterised in tomato, although only 30 are present in appreciable concentrations and 21 have been found to significantly participate in the constitution of the perceived tomato aroma. According to their chemical structure, VOCs are heterogeneous compounds belonging to the chemical classes of aldehydes, ketones, alcohols, esters and nitrogen- and sulphur-containing compounds (Martina et al., 2021).

1.2.1.1 Strigolactones: at the crossroads between carotenoids and VOCs

Beyond the multiple functions as active photosynthetic pigments and major antioxidants described so far, carotenoids are also fundamental as substrate for carotenoid-derived compounds, as in the case of the apocarotenoid phytohormones strigolactones (SLs) (Al-Babili & Bouwmeester, 2015; B. Wang et al., 2017). Strigolactones were initially recognized as chemical signals released in the rhizosphere by the plant roots that induce seed germination of the parasitic weed *Striga* spp.– hence the name “strigolactones” (Cook et al., 1966). Later on, it was shown that these molecules are required for symbiotic associations with arbuscular mycorrhizal fungi (AMF) (Akiyama & Hayashi, 2006). The function of plant growth-regulators was attributed to SLs upon they were recognised responsible for the highly-branched phenotype of the pea (*Pisum sativum*, L.) and petunia (*Petunia hybrida* Hort.) mutants *rms1* (Beveridge et al., 1997) and *dad1* (Napoli, 1996), respectively, that were found to be affected in the biosynthesis of SLs. Further mutants impaired in SLs biosynthesis were identified and studied, generally being characterised by an altered plant phenotype (reviewed in Cuccurullo et al., 2022; Seto & Yamaguchi, 2014). Following mounting evidences, SLs were

recognized as phytohormones with multiple roles in regulating both shoot and root architecture and development, along with several physiological processes related to the plant response to abiotic and biotic stresses and in the crosstalk with other plant hormones (Al-Babili & Bouwmeester, 2015; Dun et al., 2023; Omoarelojie et al., 2019; B. Wang et al., 2017).

As mentioned above, SLs originates from carotenoids (**Figure 1**).

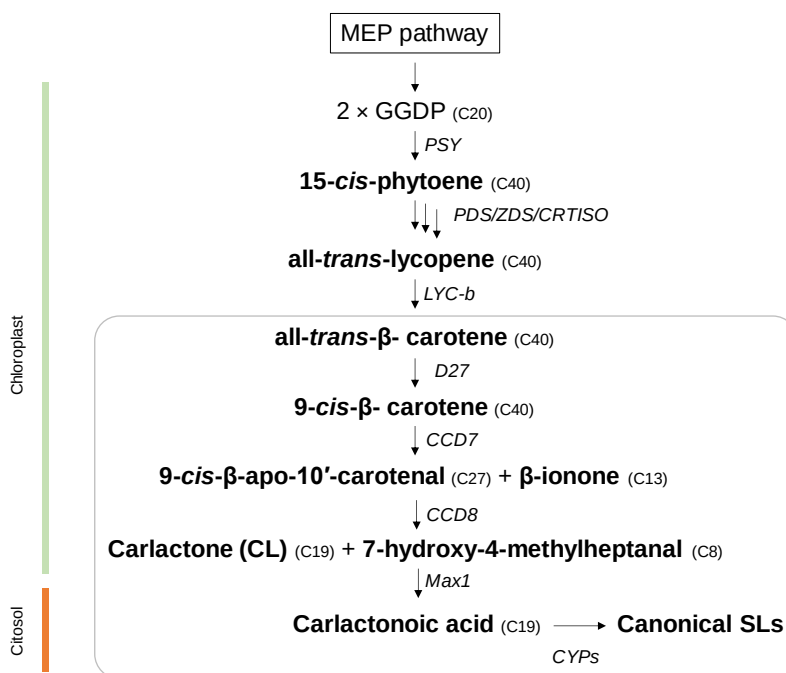


Figure 1 Strigolactones (SLs) biosynthesis originates from the carotenoid pathway.

Their biosynthesis involves four main enzymatic reactions largely conserved across species, and initiates with a 9-cis isomerization of the β-carotene, carried-out by the iron-containing β-carotene isomerase *Dwarf 27* (D27) (Lin et al., 2009; Waters et al., 2012). The 9-cis-β-carotene (C₄₀) is sequentially cleaved by the two following *Carotenoid Cleavage Dioxygenase* (CCDs), namely CCD7 (Booker et al., 2004; Koltai et al., 2010) and CCD8 (Kohlen et al., 2012; Simons et al., 2007; Snowden et al., 2005; Sorefan et al., 2003). The result of the first cleavage from CCD7 is the apocarotenoid 9-cis-β-apo-10'-carotenal (C₂₇) and the VOC β-ionone (C₁₃); the 9-cis-β-apo-10'-carotenal subsequently acts as substrate for CCD8, and by its breakdown are generated the C₁₉ carlactone (CL) and a C₈ by-product, the ω-OH-(4-CH₃) heptanal (7-hydroxy-4-methylheptanal). Carlactone is exported from the plastid – where the previously reactions take place – to the cytosol, and is converted in carlactonoic acid (CLA) by the cytochrome P450 *More Axillary Growth* (Max1), also referred as CYP711A (Booker et al., 2005; Zhang et al., 2014, 2018). Carlactonoic acid is the actual precursor of the variety of existing SLs (Wakabayashi et al., 2019): to date over 35 SLs have been identified, generally being present in a species-specific combination (Q. Zhou et al., 2025). However, the genetic bases of such variability downstream of Max1 are still only partially understood. In tomato two cytochrome P450 were identified as responsible for the biosynthesis of orobanchol (CYP722C; Wakabayashi et al., 2019) and solanacol (CYP712G1; Wang et al., 2022). Orobanchol

and solanacol are the most abundant SLs in tomato, yet additional orobanchol-like SLs were characterised, namely 7-oxoorobanchol, and the recently identified 6,7-didehydroorobanchol, phelipanchol, and epiphelipanchol (Wakabayashi et al., 2022), along with the didehydroorobanchol isomers (DDHs) although its structure is still not defined. The aforementioned SLs are included in the structural class of canonical SLs, consisting of a tricyclic lactone ABC-ring bonded via an enol-ether bridge to a methylbutenolide D-ring. The A and B moieties carry the functional groups and are therefore highly variable, while the D ring is conserved due to its importance in the SLs biological activity, being in fact preserved in non-canonical SLs, which completely or partially lack of the ABC-ring (Omoarelojie et al., 2019).

The biosynthesis of SLs mainly occurs in roots and the transport of the SL cue is shootward (acropetal), as demonstrated by grafting experiments in which a WT rootstock recover the branched-phenotype of the SL deficient scion (Beveridge, 2006; Kohlen et al., 2011; B. Wang et al., 2017). The better characterised SL transporter are the PLEIOTROPIC DRUG RESISTANCE (PDR) which belongs to the family of the ATP BINDING CASSETTE (ABC). PDR1 has been the most extensively studied, with functional analyses on *pdr1*-depleted mutants in *Petunia hybrida* and *P. axillaris* providing the first studies demonstrating the role of this transporter in SL exudations from the root to the rhizosphere, and also its partial involvement in SL movement from the root to the shoot (Banasiak et al., 2020; Sasse et al., 2015; Shiratake et al., 2019). Novel insights in this area were provided from the recent work of (Ban et al., 2025), in which was described the *PDR1* close homolog – namely *PDR2* – in tomato. Despite their genetic similarity, slight differences were described in the two independent KO mutants. Noteworthy, the *Slpdr2* showed a reduced exudation to the rhizosphere, while retaining a certain level of upward SL transport.

The plant response to SL signalling relies on the D14/Max2/SXML transduction cascade, which regulates the expression of SL-dependent genes by removing transcriptional repressors (B. Wang et al., 2017). Strigolactones bind to the DWARF14 (D14/DAD2) α/β hydrolase, inducing its conformational change (Arite et al., 2009; Hamiaux et al., 2012). The thus formed complex interacts with the F-box protein MORE AXILLARY GROWTH 2 (MAX2/RMS4/D3; (Ishikawa et al., 2005; Stirnberg et al., 2002, 2007), and induces the ubiquitin-mediated degradation of the SUPPRESSOR OF MAX2 1-LIKE (SMXL6/7/8 in *Arabidopsis thaliana*; D53 in rice) repressor protein, therefore releasing the relative genes from the inhibition (Jiang et al., 2013; F. Zhou et al., 2013).

The connection between SLs and carotenoid biosynthesis has gained particular attention in recent years mainly in the light of possible interactions with the additional apocarotenoid phytohormone, namely abscisic acid (ABA) (Omoarelojie et al., 2019). There is currently growing evidence produced by studies on various species with either an impaired or a depleted SLs content that, in fact, shows SL-ABA cross-linking (Korek & Marzec, 2023; Tolnai et al., 2023); although there are still numerous missing key-points that remain to be elucidated. Additionally, the tight relation between carotenoids and SLs, along with the presence along the SLs pathway of β -carotene

isomerization, apocarotenoids and VOCs formation, suggest stimulating interrogations about the potential impact of the inactivation of the SLs core biosynthesis genes on the plant and fruit metabolites profile.

1.2.2 Ascorbic acid (AsA)

Tomato is also a source of hydrophilic secondary metabolites, and vit. C (L- ascorbic acid, AsA) is the most represented, with 6 to 23 mg/100 g FW of this compound contained in the berries of the cultivated varieties (Davies et al., 1981; Mellidou et al., 2021). Ascorbic acid is a dibasic acid, and its structure consists of a 5-carbon atoms heterocyclic lactone ring bonded to an enediol group. Depending on the pH, AsA can be found as monoanion – which is the most common form at neutral pH – or dianion (Paciolla et al., 2019). Considering the nutritional aspect, vit. C is an essential phytonutrient. Humans, indeed, have lost the ability to synthesize it, making it necessary to supplement it with the diet, with average requirements of 90 mg/day for men and 80 mg/day for women (Gęgotek & Skrzydlewska, 2022; Mellidou et al., 2021). Vitamin C covers several functions in the organism (Alberts et al., 2025). Due to its antioxidant properties, it intervenes in maintaining the redox cellular homeostasis, regulating the ROS levels generated in highly-oxidative processes as the mitochondrial respiration and by the activity of oxidising enzymes such as NADPH oxidases (NOXs) or xanthine oxidase (XO) (Alberts et al., 2025; Gęgotek & Skrzydlewska, 2022). Remarkably, vit. C contributes in the tocopherols (vit. E), glutathione and polyphenols regeneration, enhancing their antioxidant potential, and additionally stimulates the biosynthesis of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase (Gęgotek & Skrzydlewska, 2022; Mellidou et al., 2021; Raiola et al., 2015). Overall, vit. C is required for a proper functioning of the human organisms, protecting DNA, proteins and lipids from oxidative stresses, and its chronic deficiency leads to severe disturbs as muscular weakness, compromised immuno-defence, and scurvy (Alberts et al., 2025; Gęgotek & Skrzydlewska, 2022; Mellidou et al., 2021).

1.2.2.1 Biosynthesis and metabolism of AsA

Plants are among the various organisms capable of autonomously producing AsA, contrarily to few other living beings, including primates and humans, in which a mutation during the evolutionary process has inactivated the last gene essential for AsA biosynthesis (Fenech et al., 2019).

The AsA biosynthesis derives from sugars, and currently four different AsA-generating pathways have been proposed (**Figure 2**) in plants: the d-mannose/l-galactose (d-Man/l-Gal; Smirnoff & Wheeler, 2024; Wheeler et al., 1998) being the prominent and the only fully characterised pathway, and the d-galacturonate, L-gulose, and myo-inositol being the alternative pathways (reviewed in Quiñones et al., 2024). The first three are isomerization reactions, and starts with the conversion operated by the phosphoglucose isomerase (PGI; Maruta et al., 2008) of the D-glucose-6-phosphate precursor into D-fructose-6-phosphate. This latter intermediate is in turn transformed into D-mannose-6-phosphate and then into D-mannose-1-phosphate by the phosphomannose isomerase (PMI; Maruta et al., 2008) and the phosphomannomutase (PMM; Qian et al., 2007) respectively.

Subsequently, the nucleotidyl transferase GDP-D-mannose pyrophosphorylase (GMP; Conklin et al., 1999) adds a guanosine monophosphate residue to the D-mannose-1-phosphate, generating GDP-D-mannose. This latter compound acts as substrate for the GDP-D-mannose-3',5'-epimerase (GME; Wolucka et al., 2001), which catalyse the GDP-L-galactose formation through the route of the d-Man/l-Gal – also acknowledged as the Smirnoff–Wheeler (SW) route after their discoverers (Smirnoff & Wheeler, 2024; Wheeler et al., 1998). In the SW pathway, the GDP-L-galactose is processed by the consecutive activity of GDP-L-galactose-phosphorylase (GGP; Dowdle et al., 2007; Laing et al., 2007), L-galactose-1-phosphate phosphatase (GPP; Conklin et al., 2006; Laing et al., 2004) and the L-galactono-1,4-lactone dehydrogenase (L-GalDH; Gatzek et al., 2002), which respectively releases L-galactose-1-phosphate, L-galactose and L-galactono-1,4-lactone. The terminal step of AsA biosynthesis involves the transfer of the L-galactono-1,4-lactone from the cytosol to the mitochondria, where this compound is reduced into L-ascorbic acid by the intervention of the L-galactono-1,4-lactone dehydrogenase (GLDH; Mapson & Breslow, 1958; Pineau et al., 2008). The maintenance of adequate AsA levels in the plant is crucial. In this perspective, it was postulated the existence of alternative pathways for AsA biosynthesis, intended at compensating for possible deficiencies in AsA supply through the main SW pathway or in response to stressful conditions (Quiñones et al., 2024). Various studies have confirmed this assumption owever, several aspects of these pathways are still to be elucidated, including their effective participation in controlling AsA levels, their regulation, and the involved enzymes (Quiñones et al., 2024). The alternative L-gulose pathway (Wolucka & Van Montagu, 2003) encompasses the formation of GDP-L-gulose in place of GDP-L-galactose by a side reaction carried-out by the GME enzyme from the SW route. The GDP-L-gulose is subsequently converted into L-gulono-1,4-lactone, which is subsequently oxidised into L-ascorbate by a L-gulono-1,4-lactone oxidase GulLO (Lisko et al., 2013). The two further AsA alternative pathways originate from *myo*-inositol and from D-galacturonate. The *myo*-inositol route (Lorence et al., 2004) is hypothesized to reconnect towards the L-gulose pathway, and thus to the L-ascorbate biosynthesis, at the step that involve the compound L-gulono-1,4-lactone which is formed from the *myo*-inositol precursor following various enzymatic steps only partially elucidated. The D-galacturonate pathway (Agius et al., 2003) is instead related to the pectin metabolism. In this AsA biosynthesis additional route, a D-galacturonate reductase (GalUR; Agius et al., 2003) uses D-galacturonate to generate the L-galactonic acid; this latter is used from an aldolactonase to form the L-galactono-1,4-lactone, which is then channelled to the final step of the SW pathway for the generation of L-ascorbate.

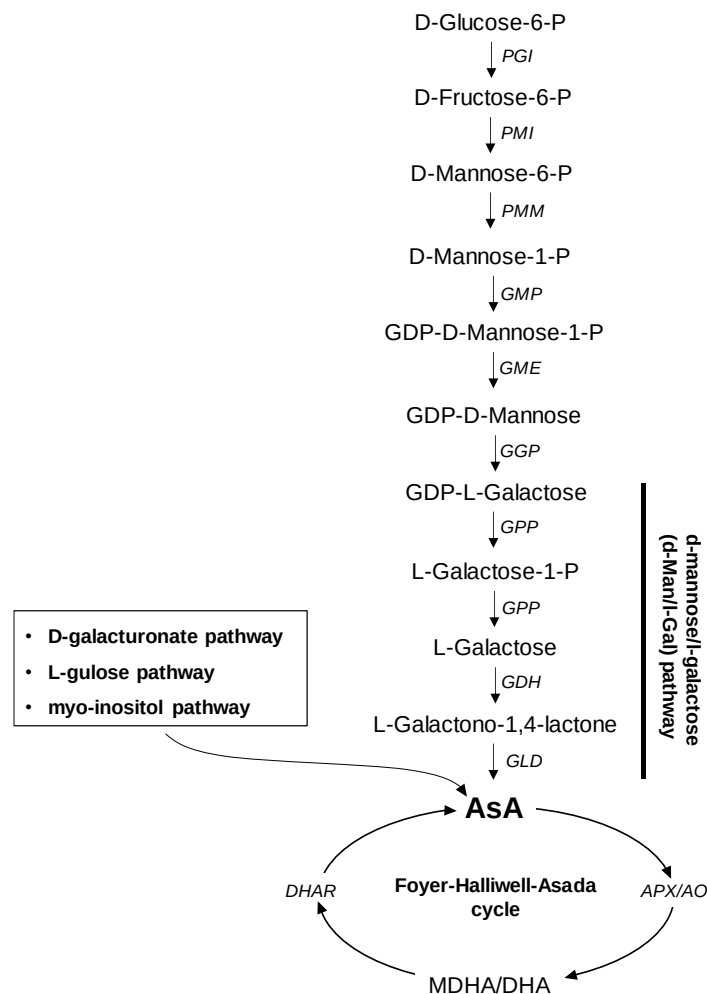


Figure 2 Schematic representation of the ascorbic acid (AsA) biosynthesis (d-mannose/l-galactose, d-galacturonate, L-gulose, and myo-inositol), and recycling (Foyer-Halliwell-Asada cycle) pathways.

Besides to its *de novo* biosynthesis, the maintenance of a stable AsA pool is also ensured from its recycling, by which it is possible to regenerate reduced AsA following its oxidation through the Foyer-Halliwell-Asada cycle (Asada, 1999; Foyer & Halliwell, 1976; **Figure 2**). The monodehydroascorbate (MDHA) and the dehydroascorbate (DHA) are the two oxidised forms of AsA. Monodehydroascorbate is an unstable radical, which is formed consequently to the single deprotonation of AsA, as in the case of the AsA-mediated detoxification of H_2O_2 operated by the Ascorbate peroxidase (APX; Davey et al., 2000). The oxidation of AsA into MDHA upon an oxidative stress could also occur *via* Ascorbate Oxidase (AO; Hoegger et al., 2006). This enzyme is active in the apoplast, where it removes the radical oxygen species, therefore maintaining the redox homeostasis (Mellidou & Kanellis, 2024). In general, MDHA is short-lived, and is rapidly reduced into ascorbate by ferredoxin or monodehydroascorbate reductase (MDAR; Tanaka et al., 2021); otherwise, it can be dismutated by a non-enzymatic reaction into DHA and AsA. Dehydroascorbate is the fully-oxidised form of AsA, in which DHA is recycled back by the glutathione (GSH)-dependent dehydroascorbate reductase (DHAR) (Davey et al., 2000).

Ascorbic acid is finally degraded, although the mechanism underlying the degradation process is still partially unclear. Some of the hypothesized degradation pathways involve the definitive oxidation of DHA or its hydrolysis, the latter producing 2,3-diketogulonic acid (Fenech et al., 2019).

Given the established dietary importance of this phytonutrient, agricultural research has devoted several efforts aimed at increasing AsA levels particularly in food-crops such as tomato, frequently observing further benefits related to the plant response to abiotic stresses (following paragraphs). For this purpose, the manipulation of the tomato AsA recycling pathway represents an intriguing approach (**see Chapter 4**).

1.3 Genetic improvement approaches to increase the carotenoids and AsA in tomato

The genetic improvement of tomato can be traced back to the domestication process, which has originally occurred in its native area with the appearance of the *S. lycopersicum* var. *cerasiforme*. This is considered the first domesticated tomato variety and is probably among those that reached Europe, where tomato domestication began systematic during the 18th and 19th centuries. Since then, a wide range of varieties, landraces and hybrids characterized by an equally broad panel of different colour, shape and size of the berries have been developed (Bai & Lindhout, 2007).

Along with population growth and the increasing demand for tomato and tomato-derived products in recent decades, great efforts have been made to select high-yielding commercial varieties. However, as a main drawback, much of the variability for both the resistance against biotic and abiotic stresses and the nutritional quality of the fruits, which is typical of wild and traditional varieties, has been lost. In more recent times, owing to the rising consumer concerns about the unsatisfactory taste of the fruits and their increasing awareness for a healthy diet, agricultural research has devolved renewed interest to improve tomato fruit quality, in a process known as biofortification (Hirschi, 2009; Raiola et al., 2015; Vats et al., 2022). Among the several targets important for tomato biofortification, enhancing the carotenoids and AsA content in the berries are among those that have received the most attention, due to the nutraceutical significance of this phytochemicals (Mellidou et al., 2021; Meng et al. 2022). Noteworthy results have been obtained in this sense by both classical breeding and genetic engineering, and some exemplificative cases are described below.

Breeding-for-biofortification reached remarkable achievements in the development of high-carotenoids and -AsA tomato lines. Tomato possesses an important reservoir of variability distributed among its 12 wild relatives and the numerous landraces (Bai & Lindhout, 2007; Passam et al., 2007). This variability is frequently made more easily accessible by the production of experimental crossing populations (viz. Recombinant Inbred Lines, RILs; Near Isogenic Lines, NILs), in which entire chromosomal regions from wild species and landraces are introgressed usually in a cultivated tomato genetic background by crossing. A largely studied population is represented by the NILs collection developed in the cv. Ailsa Craig in which over 150 variants were introgressed (consult the C.M. Rick Tomato Genetics Resource Centre-TGRC, <http://tgrc.ucdavis.edu> for the complete list of

accessions) (Darby, 1978; Smith and Ritchie, 1983). In this collection are included lines carrying valuable alleles that were exploited for the investigation and improvement of the tomato fruit quality particularly for the pigments and carotenoids content. In a further example, NILs showing a 70.3% higher level of lycopene in the fruits were developed from a *S. lycopersicum* × *S. pimpinellifolium* (LA2093) RIL population (Kinkade & Foolad, 2013). The breeding approach also provided relevant materials regarding tomato fruits with higher AsA content (Mellidou et al., 2021). In the work of Rigano and colleagues, the positive fruit-quality traits brought by the IL7-3 and IL12-4 introgression lines from a *S. lycopersicum* × *S. pennellii* population were combined by gene pyramiding. In the novel-developed F₃ pyramided lines, AsA was increased by up to 130% compared to the control, together with the total phenols and soluble solids (Rigano et al., 2014). Beyond the fruit quality improvement itself, the identification of novel genes and QTLs represent the further fundamental outcome of modern breeding. Since the last decades of the 20th century, DNA-based selection methods have been developed, allowing the generation of genetic maps increasingly dense in molecular markers and, ultimately, to the establishment of the molecular-marker assisted selection (MAS). Moreover, upon the release of the entire tomato genome sequence (cv. 'Heinz 1706') by the Tomato Genome Consortium in 2012, breeding moved into the genomic Era. Currently, the tools provided by the multi-omics – the genomic itself, along with the transcriptomics, metabolomics, etc – enables to deciphering of the complex genetic bases underlying tomato fruit quality, as comprehensively reviewed in (Causse et al., 2022; Martina et al., 2021; S. Wang et al., 2023). Having information about the specific genes and/or major QTLs paves the way for the more straightforward genetic engineering, which overcomes the main limitations imposed by the breeding programs, above all the long times required for constituting the experimental-breeding populations, the self-incompatibility barriers and the side-effects associated with the selection of undesired detrimental traits along with that of interest (linkage drag).

Genetic engineering encompasses multiple techniques which involve the direct manipulation of the genetic information at the DNA or RNA level, thus having a huge range of application. The use of genetic engineering to intervene on a specific pathway in order to accumulate metabolites or to study the effects of targeted modifications on overall metabolism is referred to as metabolic engineering. These objectives are generally reached by enhancing the expression of key-biosynthesis genes, intervening on limiting-steps along the pathway or modulating the regulatory processes (Raiola et al., 2014; Wai et al., 2020). To date, the metabolic engineering approach has been widely used for the tomato fruit quality improvement, with significant efforts specifically developed to enrich the carotenoids and AsA content in the berries. Transgenesis and RNA interference (RNA-i) are among the most used strategies in this research-field. Transgenesis uses the recombinant DNA technology to introduce exogenous DNA sequences into a recipient organism (viz. cloning). The origin of the exogenous DNA sequence distinguishes the *sensu stricto* transgenesis – in which the exogenous DNA derives from distant or sexually incompatible organisms – from the cisgenesis – when the

transferred DNA is from a genetically close species. In plant transgenesis, *Agrobacterium tumefaciens* is among the most versatile systems for inducing genetic modifications, since its natural ability of transferring small DNA segments (viz. T-DNA) into plant cells. The more direct way to boost phytonutrients by transgenesis consist in overexpressing (OE) the genes involved in their biosynthesis (Wai et al., 2020). In one of the earliest pioneering attempts, β -carotene-enriched fruits were obtained by cloning the *phytoene desaturase* (*crtI*) gene from the bacteria *Erwinia uredovora* in tomato plants. *crtI* mediates the phytoene to lycopene catalysis in a one-step reaction, contrarily to the four-steps reaction occurring in plants; interestingly, in the *crtI* transformants the PDS and ζ -Carotene desaturase (*ZDS*) were upregulated, ultimately inducing increased β -carotene rather than lycopene (Römer et al., 2000). In the work from Ronen et al., 2000, the genomic sequence of the *Lycopene cyclase B* (*Lyc B*) – which converts the lycopene into β -carotene – was cloned from *Solanum pennellii* and expressed in the tomato cultivar M82. Whereas in the wild type (WT) the *LycB* gene is downregulated during the ripening process, in the transgenic lines the biosynthesis of β -carotene was retained, and this compound accumulated in the placenta and columella along with lycopene. The overexpression of biosynthetic genes was similarly carried out by Cronje et al., 2012 to improve the fruit-AsA content. The constitutive promoter 35S was used to drive the expression of yeast-derived *GDP-mannose pyrophosphorylase* (*GMPase*) and the *arabinono-1,4-lactone oxidase* (*ALO*), to increase AsA content in both fruits and leaves. Intervening on a rate-limiting step, representing main bottlenecks in a biosynthesis pathway, is also possible through transgenesis. Yazdani and colleagues adopted (Yazdani et al., 2019) a pull-approach to generate transgenic tomato plants ectopically overexpressing two *Arabidopsis* variants of the *ORANGE* (*OR*) gene, *AtORWT* and *AtORHis* respectively. The *OR* gene regulates chromoplast biogenesis, and in *OE AtORHis* plants, earlier chromoplast formation in young fruits was found, along with carotenoid accumulation at all stages of fruit development. In AsA biosynthesis, GGP is the first enzyme of the d-Man/I-Gal route to be involved in ascorbate production, and it is also considered as the major rate-limiting step. Cloning the GGP1 under the fruit-specific *Polygalacturonase* (*PG*) promoter led to elevated AsA levels in ripening tomato berries, along with further changes in the fruit transcriptome, development and metabolites profile (Koukounaras et al., 2022). Transcription factors (TF) can be further targeted for biofortification purposes. In a recent work, a B-box (BBX) zinc-finger TF was characterized in tomato. The *SIBBX20* gene is a positive regulator of the *PSY* expression. The *SIBBX20-OE* lines showed dark green leaves and immature fruits. Fruits at the three developmental stages – mature green (MG), breaker (BR) and red ripe (RR) – had an increased content of carotenoids and a higher level of lutein, α -carotene and β -carotene compared to the WT (Xiong et al., 2019). Through the OE of the GOLDEN2-LIKE (GLK) TFs – GLK1 and GLK2 – both carotenoids and AsA levels have been improved in ripe tomatoes, suggesting a potential combinatorial strategy for the biofortification of these compounds (Nguyen et al., 2014).

RNA interference was the first widely used technique to induce the inactivation of a gene rather than its overexpression (Jagtap et al., 2011; Koeppe et al., 2023). It is based on a natural cellular defence process that eukaryotes use for counteracting viral infections. The RNAi pathway is triggered by the presence of double-stranded RNA (dsRNA), which generally originates from viral replication or transposable elements. The enzyme Dicer – namely an RNase III – recognizes dsRNA and cleaves it into 20-25 nucleotide long fragments, called small interfering RNAs (siRNAs); siRNAs are then recruited from the RNA-induced silencing complex (RISC) which uses them as probes for the selective degradation of the corresponding mRNA molecules. In plant genetic engineering, the potential of this system relies on the possibility to determine the transcriptional suppression of specific genes. The two more common methods for RNAi involve the host-induced gene silencing (HIGS) and the virus-induced gene silencing (VIGS). In the first case, the host constitutively express the dsRNA through the stable integration of exogenous DNA (transgene-mediated), in the latter the dsRNA introduction is mediated by a viral vector and is transiently expressed (Jagtap et al., 2011; Koeppe et al., 2023). The host-induced gene silencing has been successfully used for biofortification purposes of carotenoids and AsA. In the work from Sun et al. (2012) the transcription of the *9-cis-epoxycarotenoid dioxygenase 1 (NCED1)* gene was suppressed in the fruit by RNAi. *NCED1* leads to the biosynthesis of ABA from carotenoids, thus representing a way towards their degradation. In the *NCED* RNAi tomato lines, was observed the up-regulation of various genes of the carotenoid biosynthesis. Consistently, the total carotenoids are increased by 30–45% in the pulp, as well as lycopene (up to +48%) and β -carotene (up to +98%) (Sun et al., 2012). In a further application, tomato plants were found to accumulate AsA and showed reduced sensitivity to oxidative stress due to RNAi silencing of the *Ascorbic acid Mannose pathway Regulator 1 (AMR1)* gene, encoding for an F-box family protein that tunes the ubiquitination, and thus the transcriptional regulation of the AsA biosynthesis genes along the d-Man/L-Gal pathway (Ye et al., 2025).

In recent years, genetic engineering has been revolutionised by the discovery of an innovative genetic engineering technique: the gene-editing mediated by the CRISPR-Cas9 system.

1.3.1 The CRISPR/Cas9 technology: molecular basis of the most diffused genome-editing system

Genetic improvement techniques, such as classical breeding or traditional genetic engineering, suffer from an intrinsic limited specificity towards the traits that are intended to be modified. At the beginning of 2000's, the earliest gene-editing platforms based on programmable nucleases were established. Zinc Finger Nucleases (ZFNs; Kim et al., 1996) and Transcription Activator-Like Effector Nucleases (TALENs; Javaid et al., 2022), were the first molecular tools used to precisely target a desired region to be edited across the entire genome. However, these systems are laborious to set-up and rely on complex biology, which has negatively impacted their applicability.

The CRISPR/Cas binary module was originally described as part of the adaptive immune system implemented by Bacteria and Archaea to counter infections caused by bacteriophages ((Jansen et

al., 2002; Mojica et al., 2000). It consists of a ribonucleoprotein complex: the CRISPR – which stands for Clustered Regularly Interspaced Short Palindromic Repeats and constitutes the ribonucleic moiety – and the Cas – namely CRISPR-associated protein, an RNA-guided effector endonuclease (Shabbir et al., 2019). The CRISPR-based immunity consists of three sequential steps which ultimately induce the development of an immune “memory” in the attacked organism. Upon the infection, short DNA fragments from the viral invader called spacers, were recognised and integrated into the CRISPR region of the bacterial genome, in a process named adaptation or spacer acquisition (Shabbir et al., 2019). Importantly, the spacers are characterised by the presence of a 2 to 6 base pairs downstream motif, the Proto-Spacer Adjacent Motif (PAM), which is fundamental for the further CRISPR-mediated inactivation. In a second stage, the CRISPR region was entirely transcribed by the RNA polymerase as pre-CRISPR RNA (pre-crRNA); the array of pre-crRNA was subsequently processed into single crRNAs by the Cas proteins. The crRNAs – also known as guide RNAs – are active in the recognition of the foreign DNA or RNA through base complementarity, driving the interference process, which is concluded by the cleavage operated by the Cas endonuclease (Brouns et al., 2008). CRISPR/Cas is categorized into two classes (I and II) and six types (from I to VI) based on the specific properties of the Cas proteins active in the interference process. Class 1 includes the types I, III and IV. Essentially, the class 1 encompasses multi-component Cas proteins and protein-subunits in which the DNA-binding, crRNA processing and the nuclease activity are separated. Types II, V and VI contrarily belong to the class 2, which depends on a single multifunctional protein. Types II is the most common within the class 2, whilst the types V and VI are rarer. The representative effector in the type II is the Cas9 (Makarova et al., 2018). The type II CRISPR is characterised by the presence of an additional element, the trans-activating crRNA (tracrRNA). TracrRNA is located within the CRISPR locus and is fundamental in both crRNA biogenesis and correct interaction with Cas9. TracrRNA associates to the pre-crRNA forming a dsRNA molecule, which is then identified and processed by the non-Cas RNase III enzyme. The resulting tracrRNA:crRNA product constitutes the guideRNA (gRNA) recruited by the Cas9. Within the gRNA, the crRNA part ensures target site-recognition by the Cas9, whereas the tracrRNA acts as a scaffold for the stabilization of the ribonucleoprotein complex. In the Cas9, the cleavage of the target DNA is devolved to the two domains HNH and RuvC and HNH, which are active in cutting the crRNA-complementary and non-complementary strand respectively (reviewed in Shabbir et al., 2019). In a 2012 publication in the Science journal, the use of the CRISPR/Cas9 for genome editing purposes was first reported (Jinek et al., 2012). and two of the paper authors – Jennifer Doudna and Emmanuelle Charpentier – were awarded with the Nobel Prize in Chemistry eight years later (Doudna & Charpentier, 2020). The CRISPR/Cas9 immediately appeared as the most feasible and powerful molecular biology tool available for genome-editing, reporting a huge outbreak of applications in all the fields of research in biology, including plant genetic improvement.

The CRISPR/Cas9 system can be, in fact, programmed to target any desired site in a genome and generate double-strand breaks (DSBs) in the DNA. These DSBs undergo the eukaryotic cell's DNA repair mechanisms. In the Non-Homologous End Joining (NHEJ), the DNA strands are joined back together through an error prone repair pathway that can introduce random insertions/deletions (indels). If the DSB occurs in the coding sequence (CDS) of a gene, the INDELS may cause a frameshift in the reading frame, ultimately determining its inactivation. Alternatively, the DNA damages can be fixed-up through the Homology-Directed Repair (HDR) route, which is a high-fidelity process, even though it requires a DNA template. In CRISPR/Cas9-mediated gene editing, both the NHEJ and DHR pathways can be exploited to manipulate genes, achieving respectively the inactivation (*i.e.*, knock-out, KO) of the target gene or its precise modification based on a provided exogenous template (Javaid et al., 2022). Among these two approaches, the most practicable is that based on the NHEJ (**Figure 3**), that have been largely used in agricultural research for biofortification purposes of carotenoids and AsA.

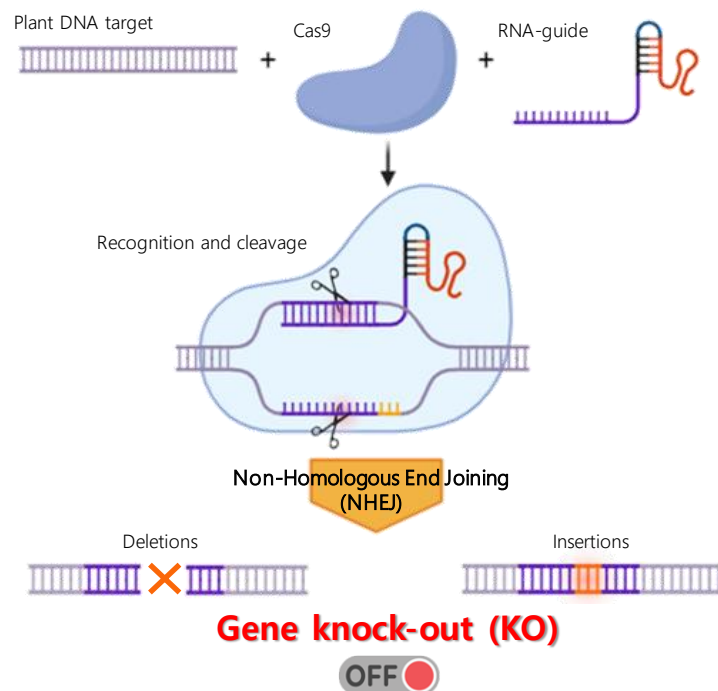


Figure 3 Graphical representation of gene editing mediated by the CRISPR/Cas9-induced Non-Homologous End Joining (NHEJ). Adapted from Doudna & Charpentier, 2020.

1.3.2 Applications of the CRISPR/Cas9 system for carotenoids and ascorbic acid biofortification in tomato

Blocking the degradation of rate limiting enzymes could possibly lead to carotenoids accumulation, as in the research from Wang and collaborators (2020). In this study, an E3 ubiquitin ligase, *Plastid Protein Sensing RING E3 ligase 1 (PPSR1)* was found to be involved in ubiquitin degradation of the PSY1 precursor protein. The tomato fruits from the CRISPR/Cas9 *ppsr1* plants showed an acceleration in the ripening process and elevated contents of phytoene, lycopene, and β -carotene (P. Wang et al., 2020).

A CRISPR/Cas9 multiplex reaction was used to push the biosynthesis of carotenoid towards lycopene by the KO of five genes of the pathway. The targets included the gene *Stay-green 1* (*SGR1*), whose silencing promotes the *PSY* activity and the lycopene synthesis, and the genes *lycopene cyclases* *LCY-E*, *LCY-B1*, *LCY-B2* that catalyze the cyclization of lycopene into α - and β -carotene, and the Beta-lycopene cyclase (*Blc*) with β -/ ϵ -carotene cyclase functions. In the fruits of the KO lines, lycopene increased by more than 5-fold, highlighting the great potential of the CRISPR system to contemporary generate mutations in various sites (Li et al., 2018). Likewise, diversely colored tomato fruits with modified carotenoids profile fruits were obtained in the BC₁F₂ backcross population by crossing the WT genotype (var. Ailsa Craig) with a CRISPR KO line in which the *PSY*, *SGR1* and *R2R3-MYB transcription factor* (*MYB12*) were simultaneously inactivated (Yang et al., 2023). Important results for tomato biofortification were observed in the *sgr1* edited null lines, with elevated levels of both carotenoids and AsA, and further important metabolites as sugars and tocopherols (J. Y. Kim et al., 2023, 2024). β -carotene and AsA were similarly augmented in tomato berries upon the CRISPR/Cas9-mediated breakdown of the *Ethylene-insensitive 3/ethylene-insensitive 3-likes* (*EIN3/EILs*) gene, which indirectly regulates *LYCB2* and *MYO-INOSITOL OXYGENASE 1* (*SIMIOX1*) involved in the carotenoid and AsA biosynthesis respectively (Chen et al., 2023).

In addition to standard CRISPR/Cas9 for gene knockout, several CRISPR-based gene editing approaches have been implemented to address specific needs. Several variants of Cas9 and further Cas endonucleases have been developed, in addition to the conventional Cas9 from *Staphylococcus pyrogenes*, with maximum precision and a broader range of potential target sequences. One above all is the dead Cas9 (dCas9) used in the base editing to induce point substitutions of nucleotides – and thus amino acids, being the more powerful tool available for genes manipulation (Javaid et al., 2022).

Despite their great potential, most gene-edited products in agriculture are considered genetically modified organisms (GMOs) and therefore face significant limitations for their practical use.

1.3.3 Regulatory framework of the CRISPR research-products in agriculture

The definition “New Genomic Techniques (NGTs)” was established by the European Commission (<https://food.ec.europa.eu/>) in the early 2000's to discriminate the standard GMOs from the products of more-targeted DNA manipulations. NGT, indeed encompasses the products of genome editing, including those generated using the CRISPR system.

At present, the largest part of the CRISPR plants have been generated by transferring the genetic information encoding the CRISPR/Cas9 cassette and the additional preparatory components essential for the entire process, which includes bacterial genes and antibiotic resistance genes. The CRISPR/Cas9 cassette along with the preparatory components constitutes the CRISPR construct, which is moved into the recipient organism using the *Agrobacterium*-mediated transformation. This procedure, as well as the conventional transgenesis and RNAi, involves the DNA exchange among

organisms. However, once the editing has occurred, the CRISPR/Cas9 construct is not anymore necessary, and is therefore eliminated by segregation. Hence, although in the first stages the CRISPR plants are GMOs, usually starting from the second generation (T2) onwards, the edited plants result indistinguishable from the non-transformed WT except at the edited site (He & Zhao, 2020).

The regulatory framework to which CRISPR plants are subject presents the same critical issues and ethical debate encountered by traditional GMOs, raising several concerns about biodiversity and food safety (Ahmad et al., 2023). As for the GMOs, the two dominant perspectives follow the product-based approach and the process-based approach. The first criteria focus on the final crop traits, that if achievable with conventional breeding, renders the edited plants considered safe and amenable to minimal regulations. In the second case, the CRISPR agricultural products are judged on the basis of the method used during the editing process, and are then classified as GMOs, with rigorous restrictions for their release into the environment (Sprink et al., 2016). In general, United States, Chile and Brazil have adopted the product-based approach whereas the European Countries the process-based approach; other Countries have established *ad-hoc* guidelines, e.g., Canada assesses the safety of any crop possessing novel traits regardless to the production method (Ahmad et al., 2023; Menz et al., 2020).

Since the strong regulatory divergence, significant gaps have arisen between Countries, with those taking a product-based approach having accelerated the development of NGT crops compared to Countries that follow a process-based approach, in which the field-use of NGT plants is restricted even for research purposes. In 2023 the European Commission (<https://food.ec.europa.eu/>) proposed new Regulations for NGT plants whose DNA modifications can be reconducted to spontaneous events. These NGT crops are classified as Category 1 and should be subjected to less strict measures in order to adapt to technological advancements in plant breeding (Vighi & De Storme, 2023). Additionally, relevant progress has been made by research plant molecular biology to experiment transgenesis-free CRISPR pipelines, that will possibly represent a possible solution to overcome the current limitations (Vighi & De Storme, 2023).

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Aim of the thesis

Tomato is a widespread crop, and is present in the human diet worldwide, therefore being an important source of phytonutrient intake. Carotenoids and ascorbic acid (AsA) are two essential phytonutrients in which tomato is rich, although decades of breeding-for-yield have reduced their overall content. In recent decades, agricultural research has devolved notable efforts aimed at improving the nutritional quality of tomato, with most recent studies in this sense involving CRISPR/Cas9 gene editing.

In this thesis the strigolactones (SLs) and AsA pathways were investigated by:

- Evaluating the impact on flower- and fruit-related traits, and on secondary metabolite profile (carotenoids and VOCs) of fruits, in a repertoire of CRISPR/Cas9 knockout (KO) plants in which the core genes of SL biosynthesis – *SID27*, *SICCD7*, *SICCD8* and *SIMax1* – were depleted (**Chapter 2**);
- Identifying novel members of the *SID27* β -carotene isomerases family – namely *SID27*-Like 1 and *SID27*-Like 2 – and generating the CRISPR/Cas9 KO lines for these genes, that were further characterised for plant morphological- and physiological- traits, and for the profile in secondary polar and non-polar metabolites (**Chapter 3**);
- Assessing the effects of the inactivation of two different tomato ascorbate oxidase enzymes Ascorbate Oxidase (AO) and Laccase 22/L-ascorbate oxidase (LAC) on plant phenotype and leaf-, root- and fruit-AsA accumulation (**Chapter 4**).

CHAPTER 2 – The inactivation of core genes involved in strigolactone biosynthesis differentially affects flower and fruit traits, and tomato fruit quality

Note: This chapter contains data included in a publication submitted to Plant and Cell Physiology.

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Abstract

Strigolactones (SLs) are tricyclic lactones being initially described as rhizosphere signalling molecules for symbiotic fungi and seeds of parasitic weeds. Subsequently, they have emerged as phytohormones generated by the activity of four core genes: *Dwarf 27 (D27)*, *Carotenoid Cleavage Dioxygenase 7 and 8 (CCD7 and CCD8)*, and *More axillary Growth 1 (Max1)*. Mutants defective for the biosynthesis genes have contributed in uncovering the multitude of roles in which SLs are involved, and they overall possess an altered plant phenotype. In the shoot, SLs regulate the vegetative growth and the plant architecture by inhibiting secondary branching, and the elongation of internodes. However, these molecules were found to further influence flower and fruit development. In this chapter, flower and fruit traits were analysed in a collection of tomato CRISPR/Cas9 lines edited for each of the core genes of SL biosynthesis. Fruit quality was also investigated in all the edited lines, and a major impact of the mutations in the VOC composition was demonstrated. This latter aspect represents an important novelty, since no evidence currently exists about the effect of SL-deficiency on fruit metabolites. Additionally, the *Sld27* mutant was found to be non-significantly affected for the reproductive and fruit-traits considered. This result confirms the potential of *Sld27* as a breeding target for parasitic weed resistance (Nicolia et al., 2025). Overall, the comparison of the lines inactivated for the individual genes have highlighted a gene-specific effect beyond the SL-deficiency condition itself.

2.1 Introduction

Phytohormones are chemical cues that coordinate a multitude of essential processes. Among phytohormones, strigolactones (SLs) are those most recently identified, and are synthesised from β -carotene in four key steps mediated by the enzymes Dwarf 27 (D27), Carotenoid Cleavage Dioxygenase 7 and 8 (CCD7 and CCD8), and More axillary Growth 1 (Max1) (see Chapter 1, par. 1.2.1). Strigolactones are characterized by a dual nature, being both internal plant growth regulators and external rhizosphere-signals (Brewer et al., 2013; Qu et al., 2020). These molecules are, in fact, released by the root apparatus in order to facilitate the symbiosis with arbuscular mycorrhizal fungi (AMF), but they are also perceived by the seeds of parasitic weeds for which they act as both indicators of host proximity and germination triggers (De Cuyper & Goormachtig, 2017). As phytohormones, SLs have multiple roles, influencing plant architecture and development (Brewer et al., 2013). Impaired lines for SLs biosynthesis and perception typically shows a “bushy phenotype”, due to the outgrowth of lateral branches and the reduced shoot height (Cuccurullo et al., 2022; Seto & Yamaguchi, 2014). Strigolactones are in fact involved in the More Axillary branches/Ramosus pathway (MAX/RMS; reviewed in Martín-Trillo et al., 2011), mediating the auxins-induced dormancy of the lateral buds through the upregulation of the BRC1 TCP transcription factor (Braun et al., 2012; F. Wu et al., 2022). However, the higher-branching does not directly influence the plant height as initially supposed, which instead depends on the decreased cell division and cell number in the stem (de Saint Germain et al., 2013).

Although to a lesser extent compared to the vegetative traits, reproductive and productive traits were also investigated in previous studies. In particular, in *CCD7* and *CCD8*-impaired lines it has been demonstrated that flowers and fruits were negatively impacted. In *Lotus japonicus* *CCD7* RNAi lines showed less flowers, pods and seeds (Liu et al., 2013), and smaller flowers were observed in *Petunia hybrida* with alterations in the *CCD8* gene (Snowden et al., 2005). In potato (*Solanum tuberosum*) no floral organs were formed in *StCCD8*-RNAi lines (Pasare et al., 2013). Concerning tomato, a trend comparable to that so far described was reported in homografted (SL-/SL-) *CCD7*-silenced lines, which produced a decreased number of flowers and fruits per plant (Visentin et al., 2024). In Kohlen et al., 2012, the *SlCCD8*-RNAi displayed a reduction in the size of all the flower organs, including the ovaries, which in turn determined smaller fruits and a reduced yield. These results were further confirmed in a recent work on the *sb1* and *sb2* EMS-tomato mutants, respectively depleted for the *Slccd7* and *Slccd8* genes (Karniel et al., 2024). Overall, these studies consistently demonstrate a negative impact of the inactivation of the *CCD7* and *CCD8* genes on reproductive traits. However, not much is known about the effects of the depletion of the two most upstream and downstream genes on the SL biosynthesis pathway, *D27* and *Max1*. For this latter gene, Zhang et al. (2018) highlighted reduced flower length and fruit size in the TILLING mutant for the *Max1* gene in tomato.

Importantly, despite the abovementioned evidence, the aspect of fruit quality in SL-depleted lines is completely unexplored. Strigolactones, in fact, are terpenoids molecules deriving from carotenoids, and therefore an effect on these metabolites or on other related secondary metabolites can be speculated. The current knowledge in this research field is mainly limited to the exogenous application of the synthetic SL analogue, GR24 to various crop products. A general positive impact was observed on the nutritional quality and flavour during pre- and post-harvest (Li et al., 2023, 2024). The treatment of tomato fruits at the mature green stage with GR24 increased soluble solid content and ascorbic acid (AsA), and the concentration of phenolics and flavonoids was boosted as well (Wang et al., 2025).

Recently, a collection of independent knock-out (KO) lines for the core genes of SL biosynthesis were generated in the same tomato genetic background (cv. Ailsa Craig) by Nicolia and co-authors using the CRISPR/Cas9 technology (Nicolia et al., 2025). These lines are transgene free and genetically stable, and showed an impaired SLs content in the root exudates. In this collection, flower and fruit traits were assessed, along with the quality profile (carotenoids and volatiles) of the fruits. These results provided a comprehensive and systematic picture on the contribution of the individual genes involved in SL biosynthesis on both reproductive and fruit quality aspects.

2.2 Materials and methods

2.2.1 Plant materials

The collection of tomato (cv. Ailsa Craig) CRISPR/Cas9 KO lines was provided by the CREA-research centre for horticultural and ornamental crops (CREA-OF; Dr. Alessandro Nicolia) and consists of four groups encompassing two to three lines originating from independent editing events: *Sld27* (*Sld27* KO1, KO2), *Slccd7* (*Slccd7* KO1, KO2, KO3), *Slccd8* (*Slccd8* KO1, KO2, KO3) and *SImax1* (*SImax1* KO1, KO2). It has to be noted that the *Slccd8* KO3 were included only in the calculation of the number of flowers on the third inflorescence (see following paragraphs). Details on individual genotypes are summarized in **Table 1**, and more thorough information about the generation of these plants are reported at the sections “Generation of CRISPR/Cas9 knock-out plants” and in supplementary material and methods of Nicolia et al., 2025. The editing events were characterised by Sanger Sequencing, and all the CRISPR-lines showed at least one frameshift mutation in the Coding Sequence (CDS), which ultimately induce the gene KO. No alterations were detected in the three main off-target sites, selected on the basis of the highest off-score.

Table 1 Summary of the genotype details of the CRISPR/Cas9 knock-out (KO) lines targeting strigolactone (SL) biosynthesis genes included in the present characterization. Inserted or deleted (INDEL) nucleotides (nt) are preceded by the symbol “+” or “-” respectively. The position of the INDEL on the CDS is indicated by numbers (1 = first nt in the ATG start codon). The reversed black triangle specifies the nt position of the insertion in the non-mutated sequence.

Target gene	Line name	INDEL		INDEL position on the CDS	
		Site 1	Site 2	Site 1	Site 2
<i>SID27</i>	<i>Sld27</i> KO1	+ A	- T	1▼2	336
	<i>Sld27</i> KO1	+ A	+ T	1▼2	337▼338
<i>SICCD7</i>	<i>Slccd7</i> KO1, 2	- T			128
	<i>Slccd7</i> KO3	- TG			128, 129
<i>SICCD8</i>	<i>Slccd8</i> KO1	- TCCA			729 to 732
	<i>Slccd8</i> KO2	- TC			729, 730
	<i>Slccd8</i> KO3	- TCCAG			729 to 733
<i>SIMax1</i>	<i>Slmax1</i> KO1	- GGAC			774 to 777
	<i>Slmax1</i> KO2	- T			778

The effectiveness of the editing events was previously evaluated and then confirmed by testing the SL content in root exudates both by mass spectrometry (Ultra-Performance Liquid Chromatography coupled to tandem mass spectrometry, UPLC-MS/MS) and by *in vitro* pathogenicity tests on seeds of parasitic weeds (*Phelipanche ramosa*, *P. aegyptiaca* and *Orobancha minor*; Nicolia et al., 2025). Four replicates of the entire panel of the SL-deficient lines along with the non-edited WT were obtained pre-germinating the seeds in Petri-dishes. 7 days (7dd) seedlings were moved to trays containing a mixture of peat and vermiculite for additional 3 weeks. At ca. 30 dd, four replicates were transplanted in 22 × 22 cm pots with peat and perlite, subsequently distributed in a randomized design in a phytotron, and grown under regular watering and fertilizing conditions.

2.2.2 Detection of flower and fruit traits

The number of flowers was scored on the second and third inflorescence. Flowers were tagged at the anthesis to subsequently calculate the average fruit set (%) and the number of days intercurrent between anthesis and breaker. Five fruits per biological replicate were harvested 7 days after the breaker stage (Br +7), and weight, length and width were recorded. These materials were stored at – 80 °C and subsequently used for the biochemical analysis. Five more fruits *per* biological replicate were collected at the mature green stage (MG) and stored in a controlled environment (temperature 23°C; humidity 60 %). The water loss was determined by weighing the fruits every 3 days over 4 weeks.

2.2.3 Extraction and U/HPLC-PDA analysis of chlorogenic acid, rutin, naringenin and sugars

The extraction of simple sugars (glucose and fructose) was performed on 250 mg of lyophilized powder. The resulting extracts were subjected to analysis on the E-Alliance HPLC system (Waters, Milford, Massachusetts, USA), with data acquired and analyzed using Waters Empower software.

The polyphenols naringenin chalcone (NaCh) and rutin (Rut), and chlorogenic acid (CA) were extracted following the method of de Vos et al., 2007 with minor modifications, and separated using an Ultimate 3000 UPLC system (Thermo Fisher Scientific, Sunnyvale, CA, USA.).

Individual biological replicate was analyzed in duplicate, and authentic and properly diluted standards (Sigma-Aldrich, Saint Louis, MO, USA) were employed for the detection of the peaks related to all the above-mentioned polar metabolites. The compound-content was expressed as fold change relative to the non-edited control.

2.2.4 Extraction and HPLC-PDA analysis of carotenoids and tocopherols

Carotenoids and tocopherols extraction was performed according to the protocols described in Barja et al. (2021) and Ezquerro et al. (2023) with modifications. To 15 mg of the pooled and lyophilized fruit material were added 985 μ L of extraction solvent (hexane:acetone:ethanol, 2:1:1) and 15 μ L of canthaxanthin (Sigma-Aldrich, Saint Louis, MO, USA) as an internal standard (IS). The resulting mixture was homogenized for 1 min in a Tissue Lyser II (Qiagen, Hilden, Germany) and added with 100 μ L of water. An additional minute of homogenization was carried-out. The samples were then centrifuged at 3000 rpm for 3 min at 4°C, and the organic phase transferred in 1.5 ml tube. The extraction was repeated using the same procedure on the residue pellet, followed by a 13.000 rpm centrifuge for 5 min at 4 °C. The two combined organic phases were then dried, resuspended in 150 μ l of acetone, and filtered through a 0.2 μ m nylon membrane. Liquid chromatography was performed using an HPLC Waters Alliance 2695 Separations module, with carotenoids detected by a Waters 996 PDA detector and tocopherols identified using a Waters 2475 Multi λ fluorescence detector. Peak areas were recorded and analyzed using Waters Millennium software (Waters, Milford, Massachusetts, USA). The areas were normalized by the IS, and the data were expressed as fold change relative to the non-edited WT plants.

2.2.5 Extraction and GC/MS analysis of volatile organic compounds

The analysis of the volatile compounds (VOCs) was performed on 500 mg of fresh pericarp powder, placed into 7 ml glass vials. These vials containing the sample were incubated at 37°C for 10 min in a water bath. Following the incubation, 500 ml of a 100 mM EDTA solution (pH 7.5) and 1.1 g of CaCl₂ 2H₂O were added. This preparation was gently mixed and sonicated for 5 min. One ml of the resulting mixture was transferred to a 10 mL screw-cap headspace vial with a silicon/PTFE septum and analyzed within 12 hours. Volatiles from the headspace were extracted using a 65 μ m PDMS/DVB solid-phase microextraction fiber (SUPELCO-MERCK, Darmstadt, Germany) and an automatic CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). The vials were initially

incubated at 50°C for 10 min with agitation at 500 rpm. The fiber was then exposed to the vial headspace for 20 min under the same temperature and agitation conditions. Desorption of the compounds occurred at the injection port of a 6890N gas chromatograph (Agilent Technologies, Santa Clara, California, USA) at 250°C for 1 min in splitless mode. Following desorption, the fiber was conditioned in an SPME fiber conditioning station (CTC Analytics) at 250°C for 5 min under a helium flow. Gas chromatography was performed using a DB-5ms capillary column (60 m, 0.25 mm, 1.00 µm; J&W, Agilent Technologies, Santa Clara, California, USA) with helium as the carrier gas at a constant flow rate of 1.2 mL/min. The GC interface and MS source temperatures were set to 260°C and 230°C respectively. Volatile organic compounds were captured using headspace solid-phase microextraction (HS-SPME) and subsequently separated and identified by gas chromatography coupled with mass spectrometry (GC/MS). The oven programming began at an initial temperature of 40°C, held for 2 min, followed by a temperature increase of 5°C/min until reaching 250°C, with a final hold at 250°C for 5 min. Data acquisition was performed using a 5975B mass spectrometer (Agilent Technologies, Santa Clara, California, USA) across the 35-250 m/z range, with a scan rate of 6.2 scans/s, employing electronic impact ionization at 70 eV. Chromatograms were processed with Enhanced ChemStation E.02.02 software (Agilent Technologies, Santa Clara, California, USA). Compound identification was accomplished by comparing retention times and mass spectra with those of pure standards obtained from Sigma-Aldrich (St. Louis, Missouri, USA) except for 1-nitro-2-phenylethane, which was sourced from Apin Chemicals (Oxon, UK). For quantitation, a specific ion was selected for each compound, and the corresponding peak in the extracted ion chromatogram was integrated. Ions were chosen based on their highest signal-to-noise ratio and specificity to ensure accurate peak integration within the chromatogram. Additionally, a reference sample (fruits from non-edited control plants, wild-type fruits) was prepared by thoroughly mixing equal amounts of each sample. A 500 mg aliquot of the reference admixture was analyzed regularly (one admixture for every six to seven samples) and processed as a regular sample within the injection series. This reference admixture, which contained all identified compounds at intermediate concentrations, was used to normalize for temporal variation and fiber aging. Normalized results, adjusted for these factors, were statistically analyzed and finally expressed as the ratio of compound abundance in each sample relative to the reference admixture (Rambla et al., 2016).

2.2.6 Statistical analysis

Statistical analysis was performed using single factor analysis of variance (ANOVA) and Tukey's HSD with Bonferroni correction for multiple comparisons, using the software package Real Statistic (<https://real-statistics.com/>). Statistical analysis on VOCs was performed by t-test with the software Office 365 Excel (Redmond, Washington, US). Linear Discriminant Analysis (LDA) was conducted in R using the MASS package (<https://cran.r179project.org/web/packages/MASS/index.html>).

2.3 Results

2.3.1 Effects of the mutations in SLs biosynthetic genes on tomato reproductive and fruit traits

In this thesis the effects of SLs on tomato reproductive and fruit traits were analyzed in the edited *Sld27*, *Slccd7*, *Slccd8* and *Slmax1* lines. The effects of the depletion of the SLs biosynthesis genes on reproductive traits were evaluated considering the number of flowers and the average fruit set on all the edited lines (**Figure 1A, B**).

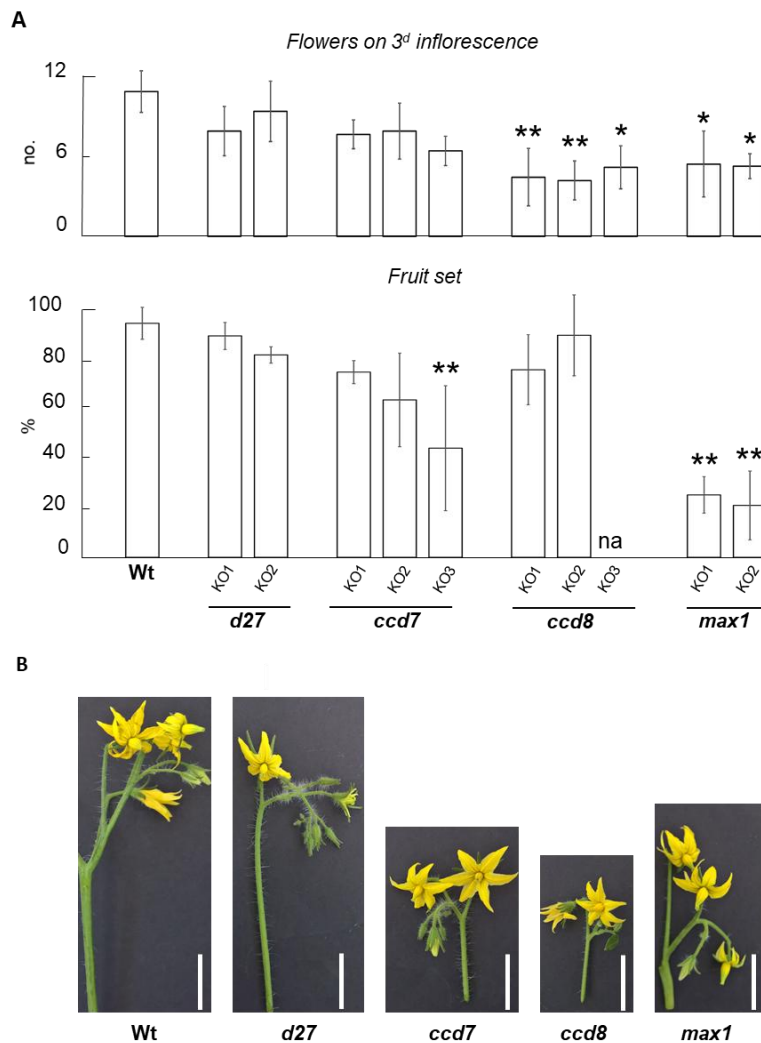


Figure 1. Variation in reproductive traits of CRISPR/Cas9 knock-out (KO) lines targeting strigolactone (SL) biosynthesis genes. **(A)** The bar charts display the mean value $\pm$ SD of flowers on the 3d inflorescence and fruit set, in the KO lines for the four genes. Statistical significance was determined using Tukey's HSD for comparisons with the non-edited control plants (wild type, WT). $n = 4$ for each KO line; statistically significant differences are indicated with asterisks: $p \leq 0.05$ (*); $p \leq 0.01$ (**). na: data not available. **(B)** Representative images of the inflorescence in KO lines compared to WT. Images are shown to scale for accurate visual comparison, with white bars representing 2 cm.

Flower-traits were assessed considering the number of flowers on the third inflorescence set (**Figure 1A**). This trait was found to be severely impaired in the *Slccd8* and *Slmax1* CRISPR KOs, with a general reduction of around 50% compared to the non-transformed lines. For the *Slccd7* lines no

significant differences compared to the WT have emerged, although the means appeared to follow a decreasing trend. The *Sld27* KO lines resulted substantially unaltered for this parameter, as well as for the average of the fruit set (**Figure 1A**), and resulted similar to that of Ailsa Craig. The strongest effects were recorded in both the KO1 and KO2 genotypes of *Slmax1*, in which the average fruit set evidenced a dramatic drop, (up to 73%). In the *Slccd7* and *Slccd8*, no clear changes emerged for this trait, except for the *Slccd7* KO3.

The evaluation of the fruit related traits in the various collection of SL-depleted CRISPR-KOs have included the number of days from anthesis to breaker, the fruit weight, and the size (**Figure 2A, B**).

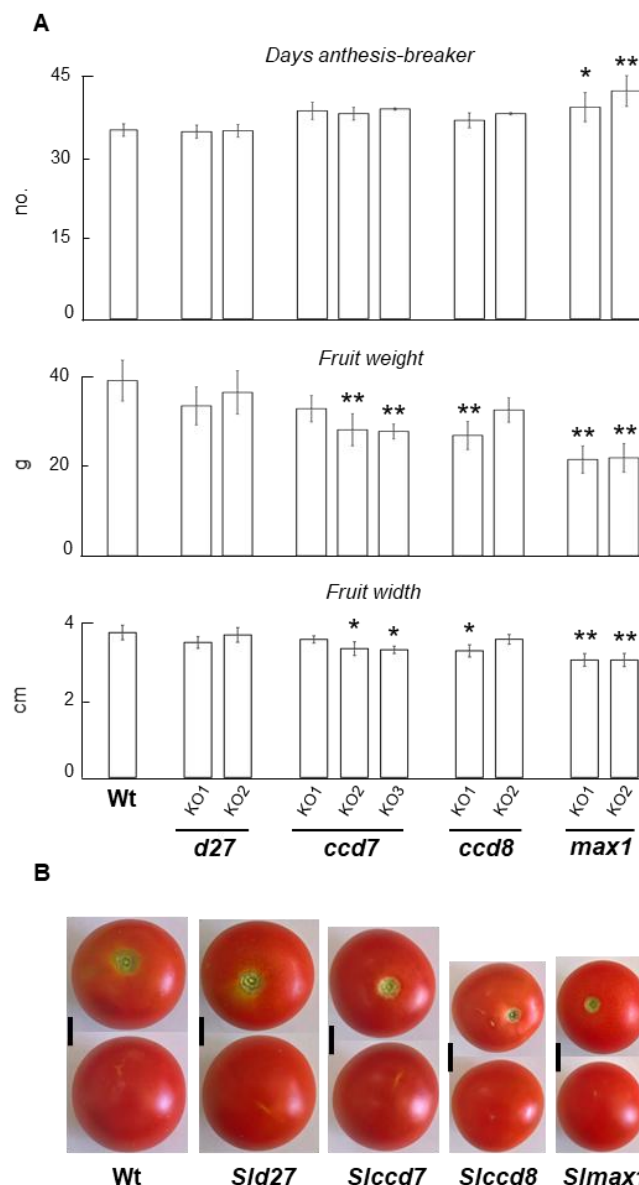


Figure 2. Variation in fruit-related traits of CRISPR/Cas9 knock-out (KO) lines targeting strigolactone biosynthesis genes. **(A)** The bar charts display the mean value $\pm$ SD of days from anthesis to the onset of ripening (breaker), fruit weight and fruit width in the KO lines for the four genes. Statistical significance was determined using Tukey's HSD for comparisons with the non-edited control plants (wild type, WT). $n = 4$ for each KO line; statistically significant differences are indicated with asterisks: $p \leq 0.05$ (*); $p \leq 0.01$ (**). **(B)** Representative images of the fruit in KO lines compared to the non-edited control plant (wild-type, Wt). Images are shown to scale for accurate visual comparison, with vertical black bar representing 1 cm.

The number of days from anthesis to breaker were stable in *Sld27*, whereas *Slmax1* lines emerged as the most severely altered, with *Slmax1* KO2 requiring an additional 7 days on average to reach the onset of ripening (**Figure 2A**).

A general decrease in the fruit weight was reported for the *SlCCDs* and the *Slmax1* KOs (**Figure 2A**). These latter lines exhibited a drop in the fruit weight, with an average of 46% lower than that of WT; the *Slccd7* KO2 and KO3 and *Slccd7* KO1 were negatively affected in this parameter, although to a lesser extent. Fruit size (fruit length and width; **Figure 2A**) mirrored the changes in fruit weight, with the *Slmax1* having the most pronounced reduction in both fruit width (up to 0.72 cm) and length (up to 0.61 cm). The water loss, a fruit quality-parameter which defines the storage properties and the changes in fruit firmness during ripening, did not change in any lines (not shown).

Overall, the *Sld27* genotypes weren't significantly different compared to the non-edited control plants for all the considered traits.

2.3.2 Profiling of fruit metabolites produced by edited tomato lines

Regarding polar metabolites, the amount of sugars (fructose and glucose) did not report substantial variations between the fruit from the SL-depleted lines and from the WT, similarly to the polyphenols chlorogenic acid and rutin (**Table 2**). Conversely, the compound naringenin chalcone appeared generally increased in most of the edited lines of the collection, even though this increase was statistically significant only in the *Slmax1*-KO1.

Table 2 Polar metabolites content (sugars and polyphenols) in fruits of CRISPR/Cas9 knock-out (KO) lines targeting core strigolactone biosynthesis genes. Metabolite levels are expressed as fold changes relative to the non-edited control plants (wild type, WT). Each value represents the average of four biological replicates (n = 4). Fold changes ≤ 0.5 and ≥ 2 are in bold. Statistically significant differences from the single-factor ANOVA coupled with Tukey's HSD for pairwise comparisons are indicated with asterisks: $p \leq 0.05$ (*); $p \leq 0.01$ (**). *nd*: not detectable. Avg., average; SD: standard deviation.

Genotype	Fructose		Glucose		Chlorogenic acid		Rutin		Naringenin chalcone	
	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
<i>Sld27</i> KO1	0.86	0.07	0.86	0.07	1.10	0.08	0.86	0.06	3.57	0.44
<i>Sld27</i> KO2	0.86	0.19	0.82	0.22	1.07	0.18	1.20	0.14	1.83	0.82
<i>Slccd7</i> KO1	0.94	0.07	0.97	0.12	0.60	0.17	0.74	0.39	2.31	0.17
<i>Slccd7</i> KO2	1.05	0.06	1.09	0.05	1.26	0.18	0.75	0.12	3.71	0.28
<i>Slccd7</i> KO3	1.53	0.71	1.41	0.46	<i>nd</i>	-	0.34	0.24	5.89	4.46
<i>Slccd8</i> KO1	0.97	0.12	0.93	0.13	0.93	0.09	1.59	0.15	3.94	0.69
<i>Slccd8</i> KO2	0.88	0.13	0.69	0.48	0.71	0.12	0.60	0.07	3.49	1.79
<i>Slmax1</i> KO1	0.98	0.13	0.99	0.13	1.24	0.26	0.81	0.12	10.29**	2.43
<i>Slmax1</i> KO2	0.87	0.11	0.89	0.17	1.16	0.29	1.68	0.13	3.33	1.46

From the analysis of non-polar metabolites, negligible variations have emerged, with the *S/ccd8* KO1 and KO2, and *S/max1* KO1 showing a deficit in δ -tocopherol, and the sole *S/max1* KO1 having a slightly reduced content in *all-trans* lycopene; however, δ -, γ -tocopherol and lutein followed a decreasing trend, although not relevant at the statistical tests

Table 3 Principal carotenoid content in fruits of CRISPR/Cas9 knock-out (KO) lines targeting core strigolactone biosynthesis genes. Carotenoid levels are expressed as fold changes relative to the non-edited control plants (wild type, WT). Each value represents the average of four biological replicates (n = 4). Statistical significance was assessed using single-factor ANOVA and Tukey's HSD for pairwise comparisons with the WT. Fold changes ≤ 0.5 and ≥ 2 are in bold. Significant differences are indicated with asterisks: p ≤ 0.05 (*), p ≤ 0.01 (**). Avg., average; SD: standard deviation.

Genotype	δ -tocopherol		γ -tocopherol		α -tocopherol		Lutein		Phytoene		β -carotene		cis-B-carotene		cis-Lycopene		All trans-lycopene	
	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
<i>Sld27</i> KO1	0.88	0.09	0.86	0.07	1.02	0.05	0.83	0.13	1.21	0.18	1.03	0.06	1.13	0.41	1.09	0.37	1.01	0.06
<i>Sld27</i> KO2	0.76	0.11	0.76	0.01	0.89	0.12	0.82	0.10	1.23	0.12	0.97	0.23	1.05	0.43	1.06	0.38	0.98	0.26
<i>Slccd7</i> KO1	0.55	0.04	0.64	0.05	0.97	0.08	0.75	0.14	1.03	0.33	0.93	0.19	0.94	0.10	1.15	0.46	1.06	0.16
<i>Slccd7</i> KO2	0.56	0.05	0.65	0.02	1.07	0.04	0.82	0.15	0.97	0.11	0.92	0.08	1.02	0.12	1.21	0.32	1.00	0.08
<i>Slccd7</i> KO3	0.76	0.06	0.73	0.07	0.88	0.02	0.84	0.05	0.84	0.21	0.85	0.15	1.05	0.14	1.16	0.31	0.96	0.04
<i>Slccd8</i> KO1	0.48	0.26	0.79	0.22	1.08	0.11	0.85	0.16	1.11	0.50	0.87	0.20	0.99	0.12	1.07	0.20	0.89	0.11
<i>Slccd8</i> KO2	0.42*	0.08	0.63	0.04	0.99	0.11	0.78	0.07	1.10	0.16	1.11	0.12	1.21	0.23	0.97	0.19	0.92	0.11
<i>Slmax1</i> KO1	0.68	0.17	0.79	0.12	1.07	0.05	0.74	0.21	0.91	0.12	0.94	0.09	1.09	0.15	1.07	0.28	0.90	0.04
<i>Slmax1</i> KO2	0.50	0.29	0.67	0.22	0.70*	0.08	0.72	0.14	0.60	0.17	0.74	0.20	0.89	0.14	0.89	0.22	0.72**	0.06

From the GC/MS, sixty-four VOCs from eight different classes have been detected (**Table 4**). Compounds of these classes resulted relevantly affected by the manipulation of the SLs biosynthesis pathway, showing fold changes of ≤ 0.5 or ≥ 2 . (**Figure 3**).

Table 4 Complete list of the detected volatile compounds (VOCs) in the fruits of CRISPR/Cas9 knock-out (KO) lines for core genes of strigolactone biosynthesis (*Sld27*, *Slcccd7*, *Slcccd8* and *Slmax1*), with indication of the metabolic pathways, retention times (RT) and mass-to-charge ratio (*m/z*). B: Benzenoid/Phenylpropanoid; BCAA: branched-chain amino acid related; ApoC: apocarotenoid; L: fatty acid derivative; Phe: phenylalanine derivative; S: sulfur compound; T: terpenoid; N: not assigned).

Compound	Metabolic pathway	RT	<i>m/z</i>
Benzaldehyde	B	22.57	106.00
Salicylaldehyde		25.66	122.00
Guaiacol		26.84	109.00
Methyl salicylate		30.60	152.00
Ethyl salicylate		32.87	120.00
Eugenol		35.17	164.10
2-methyl-2-propanol	BCAA	6.31	59.00
2-methylpropanal		7.19	72.10
2-methyl-2-butanol		9.71	59.00
3-methylbutanal		10.28	44.10
2-methylbutanal		10.62	57.10
3-methylbutanenitrile		13.08	68.00
3-methylbutanol		13.13	70.10
2-methylbutanol		13.32	56.05
(E)-2-methyl-2-butenal		13.59	84.10
3-methylbutanoic acid		16.552	60.00
1-nitro-3-methylbutane		19.96	71.05
2-isobutylthiazole		25.08	99.10
beta-cyclocitral	Cyclic ApoC 7,8	31.47	137.00
beta-damascenone	Cyclic ApoC 9,10	36.05	68.90
beta-ionone	Cyclic ApoC 9,10	38.74	177.10
6-methyl-5-hepten-2-one	Linear ApoC 5,6	22.86	69.00
Neral	Linear ApoC 7,8	31.62	109.00
Geranial	Linear ApoC 7,8	32.44	41.10
Geranylacetone	Linear ApoC 9,10	37.46	43.00
Pseudoionone	Linear ApoC 9,10	41.01	69.00
3-methylfuran	L	8.75	82.00
1-penten-3-ol		11.17	57.10
1-penten-3-one		11.27	55.10
3-pentanone		11.66	86.00
Pentanal		11.77	44.00
2-ethylfuran		11.88	81.00
(E)-2-pentenal		14.03	83.00
1-pentanol		14.41	70.00
(Z)-3-hexenal		15.74	83.00

Hexanal		15.83	71.90
(E)-2-hexenal		17.98	83.00
Heptanal		19.81	70.05
(E,E)-2,4-hexadienal		20.25	81.00
(E)-2-heptenal		21.97	68.10
1-octen-3-one		22.64	70.05
2-pentylfuran	L	23.20	81.00
(E,Z)-2,4-heptadienal		23.45	110.00
(E,E)-2,4-heptadienal		24.02	81.00
(E)-2-octenal		25.63	97.00
(E,Z)-2,4-decadienal		33.27	81.00
(E,E)-2,4-decadienal		34.02	81.00
Ethanol	N	4.97	45.10
Phenylacetaldehyde		25.45	91.10
2-phenylethanol	Phe	28.05	91.10
Benzyl nitrile		28.64	117.10
1-nitro-2-phenylethane		33.82	104.10
Methanethiol		4.671	47.00
Dimethyl disulfide	S	13.87	94.00
Methional		20.13	48.00
Dimethyl trisulfide		23.11	126.00
p-cymene		24.75	119.00
Limonene		24.95	68.10
(Z)-linalool oxide		26.32	59.00
(E)-linalool oxide	T	26.85	59.00
Linalool		27.00	93.00
Ocimenol		29.15	136.15
alpha-terpineol		30.59	59.10
2-carene-10-al		33.62	107.00

In benzenoid/phenylpropanoid (B)-derived VOCs, methyl salicylate was increased in most edited lines, whether the *Slmax1* lines also displayed higher levels of guaiacol and eugenol. Within the branched-chain amino acids (BCAA) derivatives, 3-methyl butanoic acid was highly reduced in *Slccd7*, *Slccd8* and *Slmax1* (up to a threefold reduction), as was 1-nitro-3-methylbutane, which resulted almost tenfold lower compared to the non-edited control. An additional subclass of VOCs compromised in the CRISPR-KO lines was that arising from the phenylalanine (Phe) pathway, with lower concentrations in *Slccd8* and *Slmax1* lines of 2-phenylethanol and of 1-nitro-2-phenylethane. The level of this last compound was also affected in the *Slccd7* lines.

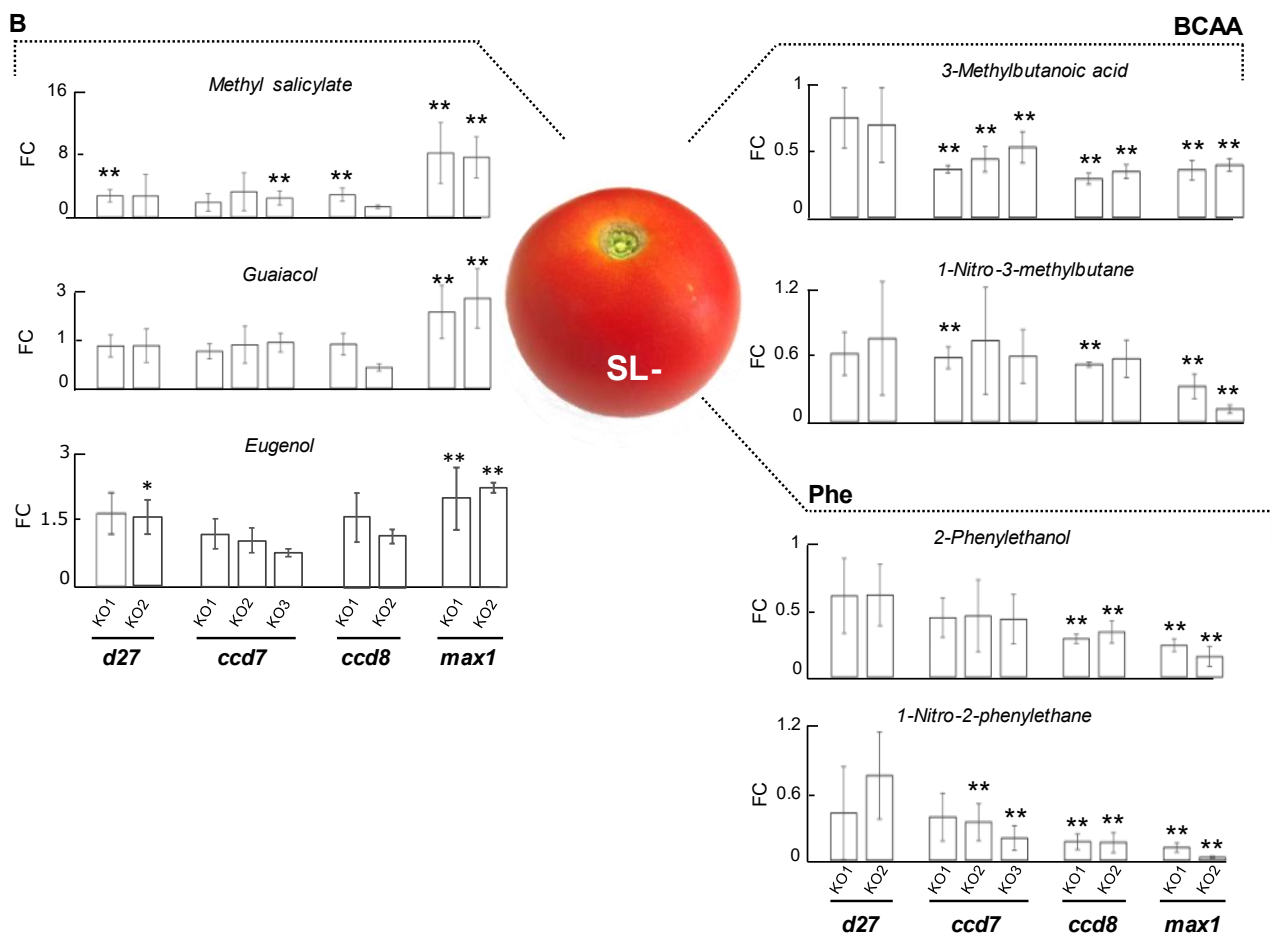


Figure 3. Alterations in key volatile organic compounds (VOCs) in fruits of CRISPR/Cas9 knock-out (KO) lines targeting strigolactone biosynthesis genes. The bar charts display the Avg. fold changes (FC) $\pm$ SD for the four genes relative to non-edited control plants (wild type, WT). VOCs are categorized by metabolic pathways (B: Benzenoid/phenylpropanoid; BCAA: Branched-chain amino acid related; Phe: Phenylalanine derivative). $n = 4$ for each KO line; statistically significant differences are indicated with asterisks: $p \leq 0.05$ (*); $p \leq 0.01$ (**).

The VOC metabolites belonging to the fatty acid (L), apocarotenoid (ApoC) and terpenoid (T) pathways seemed to be not strongly affected by mutations in SL biosynthesis genes. In **Table 5** are reported all the compounds, and the relative lines, in which a significant variation was observed.

Table 5 Volatile organic compounds (VOCs) that have displayed significant variations in the CRISPR/Cas9 knock-out (KO) lines targeting core strigolactone biosynthesis genes (*Sld27*, *Slccd7*, *Slccd8* and *Slmax1*). VOC levels are expressed as fold changes relative to the non-edited control plants (wild type, WT). Each value represents the average of four biological replicates (n = 4). Avg.s statistically different from the WT are marked by asterisks (t-test p-values *: $p \leq 0.05$, **: $p \leq 0.01$). ApoC: apocarotenoid; L: fatty acid derivative; T: Terpenoid.

Compound	Pathway	Genotype	KO1		KO2		KO3	
			Avg.	SD	Avg.	SD	Avg.	SD
Pseudoionone	Linear ApoC 9,10	<i>Sld27</i>	1.57*	0.29	1.98*	0.70	-	-
		<i>Slccd8</i>	1.41*	0.15	1.59*	0.27	-	-
<i>p</i> -cymene	T	<i>Slccd7</i>	1.90*	0.22	1.98*	0.28	1.88*	0.25
		<i>Slccd8</i>	1.86*	0.56	2.42**	0.51	-	-
Linalool		<i>Slccd7</i>	0.79*	0.36	0.71*	0.09	0.80*	0.11
Ocimenol		<i>Slccd7</i>	0.86*	0.50	0.73*	0.16	0.83*	0.18
alpha-terpineol		<i>Slccd7</i>	0.81*	0.42	0.72*	0.10	0.8*	0.10
2-carene-10-al		<i>Slccd7</i>	1.93*	0.41	2.02*	0.44	1.83*	0.05
3-methylfuran		<i>Sld27</i>	1.58*	0.33	1.63**	0.29	-	-
(E)-2-pentenal		<i>Slccd8</i>	0.66*	0.09	0.66**	0.03	-	-
	<i>Slmax1</i>	0.70*	0.11	0.59**	0.09	-	-	
(E)-2-heptenal	L	<i>Slccd8</i>	0.77*	0.08	0.69*	0.09	-	-
1-octen-3-one		<i>Slccd8</i>	0.68*	0.13	0.68*	0.11	-	-
2-pentylfuran		<i>Slccd8</i>	0.77*	0.17	0.86*	0.07	-	-
(E,Z)-2,4-heptadienal		<i>Slccd8</i>	0.89*	0.18	0.76*	0.09	-	-
(E,E)-2,4-heptadienal		<i>Slccd8</i>	0.63*	0.08	0.62*	0.05	-	-
(E)-2-octenal		<i>Slccd8</i>	0.73*	0.12	0.54**	0.09	-	-

To summarize and describe the lines behaviour on the basis of the VOCs content, an LDA was performed, simultaneously ranking the various compounds by their significance in group differentiation (**Figure 4A**). Four main groups have been identified: 1) non-edited control plants; 2) *Sld27* lines; 3) *Slccd7* and *Slccd8* lines; 4) *Slmax1* lines. The canonical variable LD1 accounted for 57.19% of the variability and was positively correlated ($r \geq 0.60$) with two BCAA related compounds (3-methylbutanoic acid, 1-nitro-3-methylbutane), one L compound (E-2-pentenal) and two Phe compounds (2-phenylethanol, 1-nitro-2-phenylethane). Conversely, LD1 exhibited negative correlation ($r \leq -0.60$) with two T compounds (*p*-cymene, 2-carene-10-al). The second canonical variable, LD2, explained 25.69% of the variability and was negatively correlated ($r \leq -0.60$) with two B compounds (guaiacol, methyl salicylate) and positively correlated ($r \geq 0.60$) with one BCAA-related compound (3-methylbutanenitrile) (**Figure 4B**).

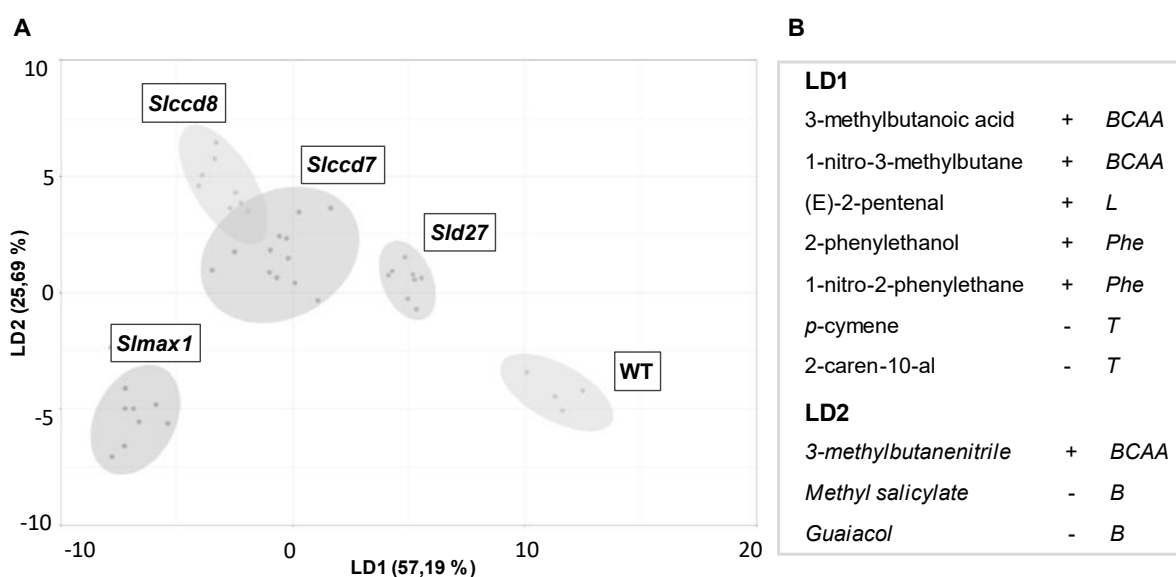


Figure 4. Bidimensional distribution of genotypes (ovals) and lines (dots) after linear discriminant analysis (LDA) of volatile organic compounds (VOCs). **(A)** Bidimensional distribution of scores assigned to CRISPR/Cas9 knock-out (KO) lines targeting strigolactone biosynthesis genes: wild type (WT), *Sld27* (*Sld27* KO1, *Sld27* KO2), *Slccd7* (*Slccd7* KO1, *Slccd7* KO2, *Slccd7* KO3), *Slccd8* (*Slccd8* KO1, *Slccd8* KO2) and *Slmax1* (*Slmax1* KO1, *Slmax1* KO2). Scores for each line were derived using linear discriminant functions LD1 (x-axis) and LD2 (y-axis), which together account for 82.88 % of the observed variability. **(B)** Selected VOCs with strong correlations to LD1 and LD2 are highlighted (+: positive corr., $r \geq 0.6$; -: negative corr., $r \leq -0.6$). Metabolic pathways associated with the VOCs are indicated as follows: BCAA, Branched-chain amino acid related; L, Fatty acid derivative; Phe, Phenylalanine derivative; T, Terpenoid; B, Benzenoid/Phenylpropanoid.

2.4 Discussion

Strigolactones are multifunctional compounds with the peculiarity of functioning as both environmental signals and phytohormones that regulate plant architecture and development. In the last years, the effects of the inactivation of individual genes of the SL biosynthetic pathway have been profiled in various species (Cuccurullo et al., 2022; Seto & Yamaguchi, 2014), and have supported the identification of the main roles of SLs on plant growth and development. Also in tomato the complete or partial inactivation of these genes have been previously carried out using different strategies and in different varieties (Cuccurullo et al., 2022). Recently, CRISPR/Cas9 KO for the core SLs biosynthesis genes (*SID27*, *SICCD7*, *SICCD8* and *SIMax1*) in the tomato cv “Ailsa Craig” have been developed. The homogeneous genetic background allowed the direct comparison among the different genotypes, that was not possible so far Nicolai et al., (2025). In this thesis, flower and fruit traits – including fruit metabolite content – were investigated in this collection of SL-depleted lines in order to evaluate the effects produced by the inactivation of each of these genes.

2.4.1 The alterations severity on flower and fruit traits increases moving downwards the SLs biosynthesis pathway

The effects of disrupting SL biosynthesis on plant development have been extensively studied in several species in *CCD7*- and *CCD8*-deficient lines produced using various techniques. These mutants were widely described for the vegetative traits, and typically exhibited the “bushy-phenotype” associated with SL deficiency (Cuccurullo et al., 2022; Seto & Yamaguchi, 2014). Flower and fruit traits were altered in these lines, in most dicot species (J. Liu et al., 2013; Pasare et al.,

2013; Snowden et al., 2005) including tomato (Karniel et al., 2024; Kohlen et al., 2012; Visentin et al., 2024). Consistent with this evidence, the *Slccd* CRISPR-KOs considered in the present study revealed an overall impairment in flower and fruit development, albeit not always statistically significant. Regarding flowering, a general decrease in the number of flowers on the third inflorescence in both the CRISPR-*Slccd* lines, albeit not significant in the line *Slccd7*, has been reported. Notably, Visentin and colleagues have recently demonstrated how SLs positively regulate flowering in tomato through the microRNA319 (miR319)-Lanceolate (LA)-Single Flower Truss (SFT) module. The leaf-application of the synthetic SL, GR24^{SDS}, in fact, induces the transcription of the *SFT* gene which boosted flowering in these plants. In contrast, *SFT* expression dropped in the RNAi-*CCD7* silenced mutant used as SL-deficient line, which showed an extensive overall alteration of several reproduction-related genes. The percentage of fruit set also appeared reduced in the same lines, although this reduction was statistically significant only in the line *Slccd7* KO3. Reflecting the effects on flower-traits, fruit traits in the same lines were subjected to a general negative impact, with a detectable reduction in fruit weight and size compared to non-edited lines. The average number of days from anthesis to BR did not change in most of the *Slccds*, yet a tendency towards a delayed onset of ripening was observed. The role of the *CCD* genes – and of SLs – on fruit traits and development is still poorly understood. Allegedly, the altered plant architecture and development reported in these edited lines can in turn be the consequence of an altered nutrient (particularly phosphorous) allocation and crosstalk with other phytohormones, as auxins and cytokinins (Kohlen et al., 2012; Mashiguchi et al., 2021; Umehara, 2011). However, high transcript levels described for the *SlCCD7* gene in the MG and turning stages of tomato fruits, opens to the possibility of a more direct implication of the *CCDs* in fruit development (Vogel et al., 2010).

The phenotype CRISPR/Cas9 KOs for the *Max1* gene was in line with those of the *Slccds*, and was more dramatic, with highly significant reduction of all the flower- and fruit-related parameters accounted for in the characterization. The few flowers present in this line exhibited the lowest % of the fruit set, and the developed berries were the smallest in size. Furthermore, fruits were delayed in reaching the onset of ripening (BR stage).

The differences highlighted in these traits in the *Slmax1* CRISPR-lines compared to the other lines could be due to several factors. As for the *Sld27*, *Slccd7* and *Slccd8*, the SLs content in the *Slmax1* CRISPR-lines was under the detection limit at the UPLC-MS/MS analysis of the root exudates (Nicolia et al, 2025). Nevertheless, a minimal rate of germination was observed in both *P. ramosa* and *O. minor* bioassay (Nicolia et al, 2025). This discrepancy can be attributed to the release of residual SLs by the *Slmax1*, which were not recorded due to instrumental detection limits, and is supported by the traces of canonical tomato SLs identified both in root exudates and root extracts detected previously in the *Slmax1* TILLING mutant (Zhang et al., 2018). These SL traces could be generated by yet unidentified routes, although there is no current evidence of alternative *Max1* cytochromes involved in CLA generation in tomato (L. Wang et al., 2023). Additionally, an

accumulation of carlactone (CL) – the product of the *AtCCD8* – could be speculated in the *Slmax1* lines, as described for the transgenic *Atmax1* mutants (Abe et al., 2014). Carlactone itself functions as a weak-SL, restoring the WT tillering in rice and Arabidopsis of SL-deficient mutants (Alder et al., 2012; Scaffidi et al., 2013), and inducing seed germination of the parasitic weed *S. hermonthica* (Alder et al., 2012); though CL was not individuated in root exudates, but only in root extracts of rice and Arabidopsis (Seto et al., 2014). Moreover, contrarily to the immobile substrates of *CCD7* and *CCD8* (Booker et al., 2005), CL is a mobile signal (Al-Babili & Bouwmeester, 2015; Booker et al., 2005; Dun et al., 2023). Nevertheless, despite the SLs residues retained in *Atmax1* and *Slmax1* and the possible high levels of CL, the *Slmax1* showed the most compromised phenotype. This evidence could be related to the loss of function of the *SlMax1* enzyme, suggesting a broad range of functions for this gene and this could explain – together with the strong deficiency in SL content – the extreme phenotype emerged in the edited KO lines. Contrarily to the other SL biosynthesis genes, which are mainly expressed in the root, *Max1* is expressed also in the shoot (Al-Babili & Bouwmeester, 2015; Booker et al., 2005; Scaffidi et al., 2013), suggesting a possible connection between the expression of this gene and reproductive and fruit-traits. Recently, it has been demonstrated that the upstream region of all the five *OsMax1* copies present in rice includes TF motifs related to flowering (Marzec et al., 2020). Also, in strawberry and in the solanaceous tobacco (*Nicotiana tabacum* L.) a high expression of the *Max1* orthologues was detected in young complete flowers (Wang et al., 2023; Wu et al., 2019). These studies and the results of this thesis suggest a strong role of *SlMax1* in tomato flowering and fruit development that should be further investigated, and the edited lines analysed in this thesis represents a valuable resource for these investigations.

In contrast to the marked SL- phenotypes observed in *Slccds* and, even more so, in *Slmax1*, the CRISPR/Cas9 *Sld27* showed normal flower and fruit production, with none of the analysed traits being evidently compromised. These loss-of-function mutants exhibited a control-like phenotype also for vegetative-traits (Nicolia et al., 2025). Moreover, the root exudates from the *Sld27* lines showed undetectable levels of SLs (orobanchol, dihydro-orobanchol, solanacol, phelipanchol and epiphelipanchol) in UHPLC-MS/MS, and accordingly their ability in inducing the seed germination of parasitic weeds *P. ramosa* and *P. aegyptiaca* was reduced up to 90%. This evidence supports the essential role of *SID27* (*Solyc09g065750*) in SL biosynthesis (Nicolia et al., 2025). *D27* was previously described as the iron-containing β -carotene isomerase located upstream on the pathway and required for the SL biosynthesis in rice (Alder et al., 2012; Hao et al., 2009) and Arabidopsis (Waters et al., 2012). Similarly to the *Sld27* line described in this thesis, *Osd27* and *Atd27* KO lines presented a milder SL- phenotype, although they were impaired in both SL content in root exudates and the ability to trigger *Striga hermonthica* L. seed germination (Lin et al., 2009; Yang et al., 2023). The explanation underlying the counterintuitive weak-phenotype in the *d27* SL- lines may rely on the non-enzymatic isomerization of all-*trans*- β -carotene into the 9-*cis* isomer (Waters et al., 2012). However, recent studies have identified additional *D27* β -carotene isomerases (*D27-Like 1* and *D27-*

Like 2) in rice (Liu et al., 2020) and Arabidopsis (Gulyás et al., 2022; Kobuna et al., 2025; Yang et al., 2022), which are active in SLs biosynthesis. These enzymes are supposed to supply small amounts of SLs, possibly localised at shoot level, sufficient to allow normal plant development in *d27* KO lines. In the present thesis, the generation and characterization of CRISPR/Cas9 KO lines have been carried-out for the tomato *D27*-LIKE genes in order to investigate this hypothesis (see **Chapter 3**).

2.4.2 *SlCCDs* and *SlMax1* specifically impact the production of VOCs during fruit ripening

In the present study the profiling of the tomato fruit metabolites was carried-out on the entire collection of the SL core biosynthesis CRISPR/Cas9 KOs. This result represents a novelty, since there are no current evidences on the impact of SL-deficiency on fruit quality.

In all the edited lines analysed, no substantial alterations have been evidenced in the fruits for both polar (sugars and flavonoids) and non-polar metabolites (carotenoids and tocopherols). These results were unexpected for the *Slccds* and *Slmax1*, which showed more pronounced pleiotropic effects for other flower- and fruit-related traits. Nonetheless, in a breakthrough study, the exogenous application of GR24 accelerates the ripening process, increases the accumulation of β -carotene and lutein, and promotes the differentiation of chromoplasts and plastoglobule in the *Yellow Fruited Tomato 3* (*YTF3*) tomato mutant, with an impaired carotenoid-content. Furthermore, a synergistic effect of GR24 and ABA was observed (Naeem et al., 2025).

To provide a comprehensive picture of the effects of the SLs depletion on fruit quality, the metabolic characterization was extended to VOCs. For these compounds, significant alterations were highlighted in the Benzenoid/Phenylpropanoid (B), branched-chain amino acid (BCAA) and phenylalanine pathways, with *Slid27* KOs showing the minor changes and *Slmax1* being the most affected.

Concerning the B pathway, the SL-impaired lines, and in particular *Slmax1*, reported a general accumulation in the fruits of methyl salicylate (MeSA), guaiacol and eugenol. Methyl salicylate derives from the methylation of the phytohormone salicylic acid (SA) and acts as a transduction signal in systemic acquired resistance (SAR). In tomato fruit, MeSA concentration typically peak at 15 days after pollination, then decline and remains constant during ripening. As a volatile compound, it is generally associated with a wintergreen flavour, which is often negatively perceived by consumers (Frick et al., 2023; Kaur et al., 2023; Tieman et al., 2010). Guaiacol is similarly synthesized from SA (Mageroy et al., 2012; F. Zhou et al., 2021). This metabolite is generally not appreciated since it confers a “smoky” or “medicinal” flavour to the berries. It is prominently emitted in tomato fruits at the MG stage in both “smoky” and “non-smoky” varieties, although its levels drop in non-smoky varieties after the BR stage (Tikunov et al., 2013). The tomato variety Ailsa Craig, in which the CRISPR/Cas9 collection was generated, belongs to the “smoky” group, and thus retains higher concentrations of guaiacol in the ripe fruit. Eugenol is related to guaiacol for the odour profile, despite its concentrations in fresh tomato aroma is normally below the odour threshold (Pardo-

García et al., 2013). As for MeSA, eugenol participates to SAR mainly in response to virus infections (Wang & Fan, 2014). In general, the B pathway can be influenced by external causes, such as biotic stresses or plant treatments (Huang et al., 2020). Nevertheless, in Kusajima et al., 2022, the *Arabidopsis* KO mutants for *More Axillary Growth 4 (Max4)*, homolog of the *SlCCD8*, displayed higher free SA levels in leaves compared to the KO mutant of *Max3 (CCD7)*, this latter being assimilable to the WT. This evidence could suggest that the perturbation of SA homeostasis may increase upon disruption of the SL biosynthetic pathway. The further category of VOCs modified by the SL-deficiency are those amino acid-derived, which forms during the ripening process (Kaur et al., 2023). Metabolites positively contributing to the flavor and derived from the downstream metabolism phenylalanine (2-phenylethanol: nutty, fruity flavor; 1-nitro-2-phenylethane: musty, earthy flavor) and leucine (1-nitro-3-methylbutane) were strongly reduced in the SL-KOs. Contrarily, the two aldehydes positively associated to the tomato aroma, 2-phenylacetaldehyde (floral flavor) and 3-methylbutanal (malty, earthy flavor) respectively related to the Phe- and BCAA-related pathway, were not significantly affected in the edited lines.

The results reported in this thesis showed no remarkable changes in VOCs pathways more closely related to SL, namely the ApoC, L, and T pathways. However, statistically significant variations have been detected in some compounds from these pathways (**Figure 4**), although they allegedly do not have a significant impact on the overall metabolite profile. Among the ApoC class, pseudoionone was found to be mildly increased in the *Sld27* and *Slccd8* lines. Pseudoionone is an important component of the tomato flavor, and is a product of the breakdown of carotenoids through the activity of *Carotenoid cleavage dioxygenase 1A* and *1B (CCD1A* and *CCD1B)* (Simkin et al., 2004). It can be speculated that minimal changes in the carotenoid composition (lycopene/ δ -carotene) could have led to such changes. VOCs from the terpenoid-pathway were mainly altered in the *Slccds* KOs, with the only exception of *p*-cymene that resulted increased in both *Slccd7* and *Slccd8*. This metabolite is more typical in flowers and fruits, and it is associated to plant defense against pests (*viz.* whitefly) (Bleeker et al., 2009). Further terpenoid-derived VOCs are specifically affected in the *Slccd7* lines, being generally reduced, as in the case of linalool, ocimenol and alpha-terpineol. These compounds positively contribute to tomato aroma (D'Angelo et. al., 2018). Interestingly, in rice mutants for the *D17 (CCD7)* gene the content of the acyclic monoterpene alcohol linalool, also resulted reduced (S. Li et al., 2023). In rice, the disruption of SL biosynthesis appeared to have, indeed, an broad effect on terpenoids accumulation, as demonstrated by a further study on the *d10 (CCD8)* (Zhou et al., 2020). The same study reported additional modification in the *Osd10* for the lipid metabolism, that is further evidenced herein, primarily in *Slccd8* KOs, in which the lipid-related VOCs tend to be decreased. Most of these compounds are aldehydes with a determinant role in establishing the tomato flavor.

Overall, the analysis of fruit metabolites reflects what observed for the other vegetative (Nicolia et al., 2025) and reproductive (flower and fruit) traits, highlighting the *Slccd8* and *Slmax1* as the most

severely impaired. Concerning fruit quality, the inactivation of the genes involved in SLs biosynthesis did not lead to major changes in the accumulation of primary metabolites, as fructose and glucose, and of secondary metabolites, as flavonoids and carotenoids. VOCs, showed more marked modifications, which are again, minimal in the *Sld27*. It remains to be established whether these modifications in VOCs are sufficient to affect tomato aroma and flavor. However, the fluctuations in VOCs as MeSA and guaiacol, which typically varies during fruit ripening, make it plausible to hypothesize a role for the *CCD* and *Max1* genes in this process. The investigation of SLs roles in the ripening processes of both climacteric and non-climacteric species remains an underexplored but compelling area of research (Ferrero et al., 2018; Li et al., 2023, 2024) and the results of this thesis contribute valuable insights into these dynamics.

2.5 Concluding remarks

In this chapter the characterization of a tomato collection of CRISPR/Cas9 mutants targeting each of the genes involved in SL biosynthesis (Nicolia et al., 2025) was presented. This collection has a great potential, and there is currently no similar repertoire of lines available in the same genetic background (Ailsa Craig). This feature, indeed, allowed the direct comparison and the identification of gene-specific effects. Indeed, the *Sld27* and *SImax1* have been found to exhibit the least and the most affected phenotype, respectively, for the considered flower- and fruit-related traits, and showed a similar trend in fruit quality. This latter aspect represents a novelty original of this work, since there is no current evidence on the fruit metabolites composition in SL deficient lines. In this regard, we have highlighted remarkable rearrangement in VOCs, with a possible impact on the tomato fruit aroma. Nonetheless, since they derive from a diversity of metabolic pathways, VOCs may function as important metabolic checkpoints, suggesting possible direct or indirect effects of SL deficiency on the overall plant metabolism. A further result of this study is the *Sld27* lines, which showed negligible alterations in the parameters here analyzed, as well as in vegetative traits (Nicolia et al., 2025), and therefore represents a valuable material for future investigations (**see Chapter 3**).

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CHAPTER 3 – Functional analysis of *DWARF27*-Like (*D27*-Like 1 and *D27*-Like 2) genes in tomato using loss-of-function mutants revealed novel effects on plant physiology and secondary metabolites composition

Note: Manuscript in preparation.

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Abstract

The *Dwarf 27* (*D27*) gene family encodes the only β -carotene isomerases currently known. The canonical *D27* enzyme catalyzes the 9-*cis*- isomerization of the double bond of the all-*trans* beta carotene, thus initiating the strigolactone (SL) biosynthesis. Furthermore, *D27* is hypothesized to mediate the interaction between SL and abscisic acid (ABA), as it is presumably located at the crossroads of the biosynthetic pathway of both these carotenoid-derived phytohormones. The presence of the two *D27*-Like orthologues 1 and 2 has been recently reported in rice and *Arabidopsis*, although their roles are yet to be fully elucidated. In this chapter, the generation of the CRISPR/Cas9 mutants for both the orthologous genes in tomato is presented. The effects of these mutations on the plant phenotype were evaluated, including the canonical *Sld27* mutants to provide a complete picture of the entire gene family. The vegetative development, flower fertility, fruit productivity and root architecture were assessed. In addition to morphological traits, physiological parameters were also considered, together with the content in secondary polar and non-polar metabolites, including ABA. Herein it is presented the first comprehensive study on the *D27* gene family in tomato. The CRISPR/Cas9 knock-out (KO) lines produced offer unique materials for future investigations directed to elucidate the function of these genes in SLs and ABA biosynthesis. Moreover, the *D27*-Likes were found to provide additional β -carotene isomers as described for *Arabidopsis*, that can be in turn the source of diverse class of apocarotenoids, influencing the content of secondary metabolites in the fruit.

3.1 Introduction

Carotenoids are isoprenoids based on a linear or cyclic C₄₀ polyene backbone (tetraterpenoids) and varying degrees of double bond conjugation (Hirschberg, 2001). This last feature makes carotenoids the second most common pigment in photosynthetic organisms, after chlorophylls. These molecules, in fact, participate to photosynthesis both as accessory light-harvesting pigments associated to chlorophylls, and as protectors of photosystems, dissipating excess energy and preventing damage from photodegradation (Swapnil et al., 2021; Young, 1991). Being powerful scavengers and antioxidants, carotenoids are also essential phytonutrients, with tomato (*Solanum lycopersicum*, L.) being one of the most important sources of these compounds – mainly lycopene and β -carotene – that accumulates in the fruit chromoplasts during the different ripening stages (Martí et al., 2016). From the carotenoid breakdown originates apocarotenoids, molecules that possess multifaceted roles as nutraceuticals (*viz.* provitamin A) and aroma and flavour components (apocarotenoid-derived VOCs). Additionally, specific classes of apocarotenoids have signalling properties. The apocarotenoid phytohormones strigolactones (SLs) and ABA represents the main examples in this regard (Simkin, 2021).

Strigolactones are the most recently identified phytohormones, and were found to be important regulators of plant shoot and root architecture and development; SLs are released into rhizosphere by the root apparatus to establish beneficial interactions with arbuscular mycorrhizal fungi (AMF), but, at the same time, stimulating the seed germination of noxious parasitic weeds (Al-Babili & Bouwmeester, 2015; Wang et al., 2017). ABA similarly affect plant shoot and root development, but it is primarily known for being involved in biotic and abiotic stress response, stomatal aperture/closure regulation mechanisms, and maintenance of seed dormancy (K. Chen et al., 2020; Merilo et al., 2015; Nambara & Marion-Poll, 2005; Shu et al., 2016).

Increasing evidence highlighted also a correlation between SLs and ABA (Cheng et al., 2013; Korek & Marzec, 2023). The study of *cis*-regulatory elements in the promoter of SL biosynthesis genes of *Arabidopsis* and rice has revealed various regions predicted as responsive to hormonal regulation, most of them being identified as responsive to ABA (Marzec & Muszynska, 2015). *In vivo* experiments confirmed the existence of an interaction between eudicots (positive regulation) and monocots (negative regulation) (reviewed in Korek & Marzec, 2023).

Both SLs and ABA biosynthesis starts from β -carotene, which is cleaved by *Carotenoid Cleavage Dioxygenases 7 and 8* (CCD7 and 8) in the case of SLs, and by *9-cis-epoxycarotenoid cleavage dioxygenase* (NCED) in the case of ABA. The resulting cleavage-products are carlactone (CL) and xanthoxin, the two direct precursors of SLs and ABA, respectively (Al-Babili & Bouwmeester, 2015; Nambara & Marion-Poll, 2005; Wang et al., 2017). Nonetheless, CCD7 and NCED enzymes requires a 9-*cis* stereospecific configuration of the double bonds at the cleavage site. Accordingly, CCD7 mediates the cleavage of 9-*cis*- β -carotene (Alder et al., 2012; Jia et al., 2018), and NCED processes both the 9-*cis*-epoxycarotenoids, 9-*cis*-violaxanthin and 9'-*cis* neoxanthin (Nambara & Marion-Poll,

2005; Perreau et al., 2020). In SL biosynthesis, the isomerization of all-*trans* to 9-*cis*- β -carotene is the first step of the pathway and is carried-out by the *Dwarf 27* (*D27*) β -carotene isomerase. *D27* was initially described in rice (Lin et al., 2009), and subsequently in Arabidopsis (Abuauf et al., 2018; Waters et al., 2012). The *Osd27* and *Atd27* SL-deficient lines showed the typical branched shoot-phenotype observed in the mutants of the other genes involved in SL-biosynthesis, although showing a milder intensity (Tolnai et al., 2023). This evidence raised the hypothesis of the existence of additional *D27* β -carotene isomerases, that can complement the activity of the already known one, being possibly active as alternative sources of the 9-*cis* isomer or of diverse β -carotene-*cis* configurations. The presence of these *D27*-Likes genes was effectively confirmed in both rice and Arabidopsis (Gulyás et al., 2022; Kobuna et al., 2025; X. Liu et al., 2020; Yang et al., 2023), along with their role in SL biosynthesis. *In vitro* enzymatic assays revealed also the novel 13-*cis*/15-*cis*/9-*cis* beside the 9-*cis*/all-*trans*- β -carotene isomerization activity for the *AtD27*-Like 1 (Yang et al., 2023). Furthermore, the involvement of *D27*-Likes in ABA biosynthesis was also speculated. The potential involvement of a common mediator that can act as a regulatory-hub among SLs and ABA was already postulated in the work from Haider et al., 2018 for the canonical *D27* in rice. The *Osd27* mutant showed a reduced content in ABA in the shoot, contrarily to the increased concentration of this metabolite observed in the mutant for *d10* (*ccd7*) and *d17* (*ccd8*), thus possibly suggesting a key role in ABA-SL crosstalk. However, the mechanism underlying this crosstalk was not elucidated by the authors. *Osd27*-Like 1 and -2 mutated lines displayed a similar tendency (X. Liu et al., 2020). In Arabidopsis, no variation in the shoot ABA levels were observed in *Atd27*, whereas the *Atd27*-Like 1 and the *Atd27d27*-Like 1 double mutant showed a significant increased accumulation of this metabolite (Yang et al., 2023). Hypothetically, the *D27*/*D27*-Like 1 genes can directly participate to the 9-*cis* isomerization of the all-*trans*-violaxanthin and all-*trans*-neoxanthin ABA biosynthesis-intermediates, or they can rather optimize the *trans/cis*-carotenoid pool in a more indirect way (Tolnai et al., 2023). However, the physiological roles of the *D27* and *D27*-Like gene remained to be elucidated, also considering the contradictory results observed in the recent study on the single, double and triple mutants for all *AtD27*-gene family members, in which the role of these genes was confirmed for SL biosynthesis, but not for ABA biosynthesis (Kobuna et al., 2025).

Recently, CRISPR/Cas9 *d27* knock-out (KO) lines were produced in the tomato cultivar Ailsa Craig (AC) by Nicolai et al., 2025, that prevent the germination of parasitic weed seeds by reducing the SLs in the root exudates.

The plant phenotype of the *Sld27* resulted in an even more mild phenotype than that described in rice and Arabidopsis, with no substantial alterations in vegetative traits, flowering and fruit traits, including quality (**see Chapter 1**).

Here it is described the generation of various KO lines for the entire *D27* gene family via CRISPR/Cas9 genome editing in two different genetic background, cv. Ailsa Craig and MoneyMaker (MM). The AC KO lines were subsequently characterized, assessing seed germination, plant shoot,

root, flower and fruit traits. Furthermore, physiological traits and metabolite composition (ABA, polar and non-polar secondary metabolites) have been characterized in leaves.

This collection provides novel materials of interest to elucidate the functions of the β -carotene isomerases in a crop species; considering the relevant expression level of *SID27*-Like genes, and particularly *SID27*-Like 1, in fruits.

3.2 Materials and methods

3.2.1 Plant materials and growth conditions

The tomato lines produced in this study were obtained in the two cultivars Ailsa Craig (AC) and Moneymaker (MM). The *Sld27* KO1 and *Sld27* KO2 were previously obtained, see **Chapter 2**.

Seeds were surface sterilized using a 70% (v/v) ethanol solution for 1 min, followed by 15% sodium hypochlorite at commercial concentration for 20 min, rinsed 5-6 times with double-distilled water.

For *in vitro* growth, the seeds were germinated in *in vitro* containers with Full MS + 1% sucrose and 0.8% agar, and placed in a dedicated walk-in chamber being maintained at 24°C in the dark. At the appearance of cotyledons, a 16h light/ 8h dark photoperiod was adopted to allow the complete seedling development. The batch of seeds intended for the *in vitro* study of root architecture, were similarly germinated, and positioned in 245 mm × 245 mm squared petri dishes (three seeds/plate) at the emission of the primary root.

For the *in vivo* cultivation, the seeds were pregerminated on sterile filter-paper. Completely formed seedlings were moved in the plant growing facility (25 °C, 16h light/ 8h dark photoperiod) in trays containing a mixture of peat and vermiculite. Thirty-days old plants were finally transplanted in 7L pots with the 80% of peat and 20% perlite and transferred to the greenhouse, with optimal conditions for tomato plants development.

3.2.2 *In silico* phylogenetic identification of the *SID27* likes orthologues and CRISPR/Cas9 single-guide RNA (sgRNA)-design

HMM profile PF13225 (Beta-carotene isomerase *D27*-Like, C-terminal) was retrieved from Pfam. The hmmsearch program (e-value 10e⁻⁵) was used to search against the protein complement of the following species: Brassicaceae: *Arabidopsis thaliana* (TAIR 10), *Brassica Napus* (AST_PRJEB5043_v1), *B. oleracea* (BOL). Solanaceae: *Solanum lycopersicum* cv Heinz 1706 (v 4.1); *S. pimpinellifolium* (v 1.1) *S. lycopersicoides* LA2951 (v 2.0); *S. habrochaites* SH1353; *S. pennellii* (v 2.0); *S. tuberosum* (v 3.0); *S. melongena* (v 4.1); *Capsicum annuum* cv Zunla (v 2.0); *Petunia inflata* (v 1.0.1); *Petunia axillaris* (v1.6.2). Poaceae = *Triticum aestivum* (IWGSC); *Oryza sativa* subsp. *Japonica* (IRGSP-1.0); *Hordeum vulgare* (MorexV3_pseudomolecules_assembly); *Zea mays* (Zm-B73-REFERENCE-NAM-5.0) The blastdbcmd utility from the BLAST+ suite was used to extract the identified protein sequences from the FASTA files retrieved from Ensembl Plants

(<https://plants.ensembl.org>) and Sol Genomics Network (<https://solgenomics.net>). All protein sequences were, then, multiple-aligned using Clustal Omega (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo?type=protein>; Sievers & Higgins, 2018). Mega XI (<https://www.megasoftware.net/>; Kumar et al., 2018) was used to construct a neighbor-joining (NJ) tree with 1000 rapid bootstrap inferences. Tree was visualized with Figtree (<https://tree.bio.ed.ac.uk/software/figtree/>).

The overall plant expression profiles of *SID27*, *SID27-Like1* and *SID27-Like 2* genes was retrieved at the Plant eFP at bar.utoronto.ca/eplant (Sato et al., 2012; Waese et al., 2017).

For the single-guide RNAs (sgRNA) design of the *D27*, *D27-Like 1* and *D27-Like 2* was used the CRISPR-P 2.0 software (H. Liu et al., 2017). One sgRNA for *D27-Like 1*, and two sgRNAs for *D27* and *D27-Like 2* were selected, considering the position along the sequence, and the on-target and off-target scores.

3.2.3 CRISPR/Cas9 vector design and validation in the Hairy Root (HR) system

The vectors for the editing of the *D27-Like 1* and *D27-Like 2* were assembled through the Golden Gate Cloning (Engler et al., 2014). The sgRNA vector for the *D27-Like 1* was constructed in the pEgPubi4_237-2A-GFP vector (courtesy of Prof. Yuriko Osakabe, Tokyo Institute of Technology, Japan) as described in Ueta et al., 2017. For *D27-Like 2*, for which two sgRNA were designed, the pDIRECT_22C vector (purchased from ADDGENE, <https://www.addgene.org/>) was chosen, and the assembly reaction was performed according to (Čermák et al., 2017).

The resulting CRISPR-vectors, named pEgPubi4_237-2A-GFP-*D27-Like 1* and the pDIRECT_22C-*D27-Like 2* were independently transferred into *Agrobacterium rhizogenes* (strain ATCC15834) for being validated by Hairy Roots (HR) assay (Ron et al., 2014). Briefly, ~ 50 cotyledons were collected from *in vitro* cultured tomato seedlings and co-cultured with transformed *A. rhizogenes* harboring either pEgPubi4_237-2A-GFP-*D27-Like 1* and pDIRECT_22C-*D27-Like 2* for 48h at 24°C in dark conditions. Subsequently, they were moved on a cultivation media containing the proper antibiotic selection until the formation of well-developed adventitious roots (*viz.* HRs). Each HR represent an independent editing event. 1-1.5 cm long HR were sampled and the editing site was analyzed by PCR (Extract-N-Amp™ Tissue PCR Kit, Sigma-Aldrich®), in which 5'-carboxyfluorescein labelled primers (*i.e.*, 5'-FAM) was included (**Table 1**). The obtained amplification products were resolved by High Resolution Fragment Analysis (HRFA) run by a SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, US) to individuate insertion/deletion (indel) events. The sgRNAs efficiency was calculated as the ratio of HRs carrying mutations on the total analyze.

Table 1 PCR primers used in the amplification of the *D27*-Like 1 and *D27*-Like 2 regions targeted by the editing.

Target gene	Primer	Primer sequence (5'-3')
<i>D27</i> -Like 1	For	TCATGACTCTATTCGCACGC
	Rev	TTGAATTCTGCTGCCCACTG
<i>D27</i> -Like 2	For	CCGCGAAAGGAAGGAAGATAG
	Rev	AGTTGTTACCGATGCGTCTC

3.2.4 Generation of the edited tomato plants for the *D27* gene family

The validated vectors were separately cloned into *Agrobacterium tumefaciens* strain EHA 105. The *in vitro* regeneration of tomato stable-transformed plants was performed as indicated in the protocol of Qiu et al., 2007 in either AC and MM. Fully expanded cotyledons from ~ 10 days old seedlings were soaked in the liquid bacterial suspension, and then rinsed and placed on the co-cultivation media (dark conditions at 24°C). After 2 days, infected cotyledons were disposed on the selection media containing the required antibiotics, and were grown a 16h light/ 8h dark photoperiod at 24°C. The regenerated seedlings were tested for the presence of the Cas9 transgene and editing at the target site by PCR and HRFA screening, respectively. The positive individuals, referred as the T0 generation, were acclimatized to standard cultivation conditions in soil and subsequently transferred to the greenhouse. In the following self-pollinated T1 generation were identified the Cas9 (*i.e.*, T-DNA) negative progenies carrying the edited alleles in a homozygous state. The Sanger Sequencing was performed at the target site in order to characterize the editing and effectively confirm the presence of a frameshift mutation and the premature insertion of a stop codon, which ultimately leads to the gene KO. Two different KOs were finally chosen for each gene, and advanced to the T2 generation.

3.2.5 Evaluation of phenotypic traits and physiological parameters

A total of nine parameters were comprehensively assessed for the characterization of plant, flower and fruit traits. Six biological replicates were considered for the non-edited Wild-Type (WT) control (cv. Ailsa Craig), and the edited plants – *Sld27* KO1, *Sld27* KO2, *Sld27*-Like1 KO1, *Sld27*-Like1 KO2. Data on *Sld27*-Like 2 for these characters, as well as physiological traits, must be considered preliminary, as they refer to genetically heterogeneous T1 mutants, harboring KO alleles in both homozygous and biallelic conditions. Plant height was recorded at 30- and 40-days post-transplant (DPT); at 30 DPT were further measured the number and length of internodes, and the number of secondary branches present on the main shoot with a length > 5 cm. The flower number *per* inflorescence was calculated on the first, second and third inflorescences, and the total number of inflorescences *per* plant was counted at 48 DPT. The percentage of fruit set on the first inflorescence was similarly established at 48 DPT, and was calculated as the number of formed fruits on the total number of flowers. The fruits were harvested at complete maturation (mature red, MR), and the fruit

weight (g) was established on a minimum of nine fruits *per* plant. The seeds were extracted from three casually picked fruits for each biological replicate ($n = 18$) and counted.

Physiological measurements were performed at 35 DPT. SPAD-502 Plus (Konica Minolta Optics, Tokyo, Japan) was used to calculate the chlorophyll (Chl) content on fully expanded leaves (two leaves *per* plant, $n = 12$). Stomatal conductance (g_s , $\text{mol m}^{-2} \text{s}^{-1}$) and Chl fluorescence (Φ_{PSII}) were determined with a LI-600 porometer/fluorometer (LI-COR, Lincoln, NE, USA) on the third leaf from the apical meristem ($n = 6$).

3.2.6 *In vitro* evaluation of germination rate and root architecture

To calculate the germination rate, 30 seeds *per* each genotype were sown on the MS media. Three plates *per* genotype were considered. After seven days, the number of germinated seeds over the total were counted.

The root architecture was assessed on 9 individuals *per* genotype. After 1 week after the primary root emission, the primary root length (PRL), number lateral roots (LRN) and the root area (RA; cm^2) were assessed using the ImageJ software (Schneider et al., 2012). Lateral root density (LRD) was calculated as the number of lateral roots per unit length of the primary root (No. cm^{-1}).

3.2.7 Extraction and HPLC/HRMS analysis of tomato leaves polar metabolites and ABA

The HPLC/HRMS analyses for polar and non-polar metabolites were performed as described in Sevi et al., 2025 on leaves pools collected from the WT and by each line of the KO mutants; the sampling was performed at 30 DPT. The same considerations reported in paragraph 3.2.5 apply to the *Sld27-Like 2*.

For polar metabolites and ABA, lyophilized leaf tissue was homogeneously ground and split into four replicates of 10 mg each. To the leaf tissue thus prepared were added 0.75 mL of cold 75 % (v/v) methanol, 0.1 % (v/v) formic acid, spiked with 1 $\mu\text{g/ml}$ of daidzein as internal standard. The samples were well mixed by vortexing for few seconds, and were then placed into a Mixer Mill 300 (Qiagen ©, Aarhus, Denmark) 40 min at 20 Hz. Plant debris were separated by centrifuging the tubes at room temperature (RT) 20 min at 20,000 rcf. 500 μl of the clean supernatant were transferred into HPLC tubes, and loaded for LC-ESI-HRMS. A single injection was run *per* sample ($n = 4$). The Liquid chromatography (LC) was carried out on a Phenomenex C18 Luna column ($100 \times 2.1 \text{ mm}$, $2.5 \mu\text{m}$) used as stationary phase, and water-0.1 % formic acid (A) and acetonitrile-0.1 % formic acid (B) as mobile phase. The applied gradient consisted in 95% A:5% B (1min), a linear gradient to 25%A:75%B over 40min, 2min isocratic, before going back to the initial LC conditions in 18min.

The starting material for LC-APCI-HRMS analysis of non-polar metabolites is made-up by 5 mg of leaf tissue initially processed as described above for polar metabolites. The extraction was performed in 1 mL of 25 % (v/v) methanol, 50 % (v/v) chloroform, 25 % (v/v) 50 mM-Tris-HCl, added with 50 $\mu\text{g/ml}$ DL- α -tocopherol acetate as internal standard. The mixture was thoroughly homogenized by vortexing the tubes for 5 sec., and subsequently centrifuged at 4 °C for 10 min at

14,000 rpm. The resulting organic hypophase was collected. The aqueous phase was subjected to a second extraction with the same reagents and volumes described before. The pooled organic extracts were dried in a Speed Vac concentrator, and the residue was resuspended in 200 µl of ethyl acetate. The 4 sample *per* line were singularly injected one time ($n = 4$). LC separation was performed on a C30 reverse-phase column (100 × 3.0 mm; 3µm, YMC Europe); the solvent system used methanol (A); 75 % methanol (v/v), 25 % water (v/v) and 0.2 % ammonium acetate (B); tert-butyl-methyl ether (C). The elution was carried-out as following: 0–6 min 95 % A, 5 % B, and 0 % C; 1 min 80 % A, 5 % B, and 15 % C; 5 min 80 % A, 5 % B, and 15 % C; 20 min 30 % A, 5 % B, and 65 % C; 22 min 30 % A, 5 % B, and 65 % C; 18 min 95 % A, 5 % B, and 0 % C. A volume of 5 µl was injected, and a flow-rate of the mobile phase of 0.25 and 0.8 mL for LC semi-polar and non-polar runs, respectively.

The mass spectrometry (MS) analysis was performed in a quadrupole-Orbitrap Q-exactive system (Thermo Fisher Scientific Inc., Waltham, MA), operating in positive/ negative heated electrospray ionization (HESI) or atmospheric pressure chemical ionization (APCI) coupled to an Ultimate HPLC-DAD system (Thermo Fisher Scientific Inc., Waltham, MA). For polar metabolites, the MS parameters were set as follows: capillary and vaporizer temperatures of 30 and 270°C, discharge current 4.0 KV, probe heater temperature at 370°C, S-lens RF level at 50 V. The mass acquisition scanning ranged from 110/1600 mass-to-charge ratio (m/z), with resolution 70,000, microscan 1, AGC target 1e6, and maximum injection time 50. Full scan MS with data-dependent MS/MS fragmentation was used for the detection of metabolites. For non-polar analysis, the following APCI parameter were applied: nitrogen set to 20 and 10 units was used as sheath and auxiliary gas; the vaporized temperature was optimized at 300°C, and those of capillaries at 250°C. Discharge current was set to 5.5 µA, and S-lens RF level was adjusted at 50. All the solvents were CHROMASOLV® LC-MS high-purity grade, purchased from Sigma-Aldrich®.

Metabolite relative quantification was obtained by normalization on the internal standards (daidzein and DL- α -tocopherol acetate) amount. Targeted metabolite identification was carried-out by comparing chromatographic (retention time, RT) and spectral properties (m/z) with authentic standards (if available) and reference spectra from the in-house database of the ENEA-Green biotechnologies laboratory (Dr. Gianfranco Diretto), PubChem database (<http://pubchem.ncbi.nlm.nih.gov>) for monoisotopic mass identification, or Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator (<http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/>) in the case of adduction detection. The metabolite-related peaks were examined with the Thermo Scientific™ Xcalibur™ and TraceFinder™ Software.

3.2.8 Statistical analysis

The Student's T-test was applied to detect statistically significant differences between the means of the different treatments; the significant threshold was fixed at $p \leq 0.05$.

3.3 Results

3.3.1 Two orthologues for the *D27* β -carotene isomerases were individuated from in silico phylogenetic analysis in tomato, with different expression profiles compared to the canonical *SID27*

The presence of additional *D27* β -carotene isomerases was assessed through *in-silico* phylogenetic analysis, including both dicot and monocot in the screening. The list of *D27* and *D27*-Like genes identified across 16 species belonging to the Solanaceae, Brassicaceae, and Poaceae families is reported in **Table 2**.

Table 2 List of the *D27* and *D27*-Like genes identified across 16 species among monocot (Poaceae) and eudicot (Solanaceae and Brassicaceae). Family, Genus, Species, gene ID, chromosome/scaffold (Chr/Scaff), start and stop positions on the sequence, and the position on the + or – strand were indicated.

Family	Genus	Species	ID	Chr/Scaff	Start	Stop	Strand
Solanaceae	<i>S. lycopersicum</i>		Solyc06g084610.4.1	6	47244152	47246963	-
			Solyc08g008630.4.1	8	3023763	3025448	+
			Solyc09g065750.3.1	9	60124430	60129781	-
	<i>S. pennellii</i>		Sopen06g035980.1	6	60707997	60710947	-
			Sopen08g004470.1	8	3267047	3269641	+
			XP_015087576.1/LOC107030862	9	75349892	75355970	-
	<i>S. lycopersicoides</i>		Solyd06g079950.1	6	90008770	90011339	-
			Solyd08g054020.1	8	4628000	4629132	+
			Solyd09g065330.1	9	98522313	98524258	-
	<i>S. pimpinellifolium</i>		SPI06g05431.1	6	49905785	49909958	-
			SPI08g00561.1	8	3257826	3261277	+
			SPI09g03698.1	9	62328930	62334456	-
	<i>S. habrochaites</i>		scaffold10.000309.1	scaffold10	12584728	12586375	-
			scaffold13.001476.1	scaffold13	11098548	11101380	-
			scaffold42.000242.1	scaffold42	5129627	5135432	-
	<i>S. melongena</i>		SMEL4.1_06g030760.1.01	6	96689396	96692938	-
			SMEL4.1_08g000690.1.01	8	603840	606901	+
			SMEL4.1_09g016040.1.01	9	77201868	77208601	+
	<i>S. tuberosum</i>		Sotub06g035130.1.1	6	58014544	58017555	-
			Sotub08g009070.1.1	8	4632350	4633910	+
			Sotub09g020580.1.1	9	41678220	41683062	-
Capsicum	<i>C. annuum</i>		Capana00g000614	0	276243000	276244763	-

Capsicum		Capana01g004487	1	300974735	300976491	+	
		Capana03g003272	3	201551459	201569018	-	
		Capana06g000026	6	313810	317870	+	
Petunia	P. inflata	Peinf101Scf00571g14015.1	Scf00571	1405364	1410024	-	
		Peinf101Scf02382g01034.1	Scf02382	93429	95181	-	
		Peinf101Scf03382g00048.1	Scf03382	140851	145171	+	
		Peinf101Scf05693g00011.1	Scf05693	76	5121	-	
	P. axillaris	Peaxi162Scf00140g01356.1	Scf00140	1365300	1368933	-	
		Peaxi162Scf00165g00212.1	Scf00165	240085	248004	+	
		Peaxi162Scf01257g00030.1	Scf01257	97807	99514	-	
Arabidopsis	A. thaliana	AT1G03055.1	1	710018	711803	-	
		AT1G64680.1	1	24036021	24037401	+	
		AT4G01995	4	873054	874772	+	
Brassicaceae	Brassica	BnaA03g26410D-1	LK031864	1555748	1560707	-	
		BnaC03g31230D-1	LK031870	101142	102976	-	
		BnaC09g12720D-1	LK031985	140850	141827	+	
		BnaC09g11560D-1	LK032176	338116	339447	+	
		BnaA09g51010D-1	LK032295	232717	234599	-	
		BnaA09g11220D-1	LK032333	215529	216858	+	
		BnaC08g45810D-1	LK032400	125398	127247	+	
		BnaCnng08800D-1	LK032414	127418	129898	-	
	BnaA09g11690D-1	LK032418	293491	294812	-		
	B. oleracea	Bo3g052870	3	20937814	20939500	-	
		Bo8g117140	8	41110952	41112615	+	
		Bo8g117980	8	41551026	41553313	-	
		Bo9g029380	9	10340017	10341020	+	
		Bo9g029390	9	10342601	10343604	+	
		Bo9g035190	9	11748569	11749547	-	
Poaceae		Oryza	O. sativa subsp. Japonica	Os11t0587000	11	22221628	22226596
	Os05t0131100		5	1830765	1833326	-	
	Os05t0131401		5	1840616	1844553	-	
	Os08t0114100		8	751421	753191	+	

Poaceae	Triticum	T. aestivum	TraesCS1A02G073000	1A	57189794	57192224	+
			TraesCS1A02G073000	1A	57189794	57192224	+
			TraesCS1A02G073500	1A	57392464	57394799	-
			TraesCS1B02G092200	1B	94184760	94186873	-
			TraesCS1B02G092300	1B	94200895	94208581	-
			TraesCS1D02G075900	1D	58448976	58451293	+
			TraesCS1D02G075900	1D	58448976	58451293	+
			TraesCS7A02G317900	7A	458710485	458712352	-
			TraesCS7A02G317900	7A	458711134	458712352	-
			TraesCS7A02G418900	7A	610449707	610455225	-
			TraesCS7B02G319100	7B	568679226	568684405	-
			TraesCS7D02G314600	7D	400468878	400470513	-
			TraesCS7D02G411500	7D	530127113	530131818	-
			TraesCSU02G097100	Un	86365233	86366785	-
Zea	Z. mays	Zm00001eb417120	10	86710199	86712996	-	
		Zm00001eb417120	10	86710238	86712984	-	
		Zm00001eb422930	10	120762827	120763678	-	
		Zm00001eb114610	2	233979183	233983258	+	
		Zm00001eb114610	2	233979193	233982905	+	
		Zm00001eb114700	2	234333870	234338031	+	
		Zm00001eb260820	6	9279222	9280771	-	
		Zm00001eb381060	9	42873242	42873850	-	

The phylogenetic tree identified several putative *D27/D27*-Like homologs (**Figure 1**). In tomato, two candidate paralogs of *SID27* (Solyc09g065750) were detected: *SID27*-Like1 (Solyc08g008630) and *SID27*-Like2 (Solyc06g084610).

The expression profile of the *SID27*-Likes was checked using the Tomato eFP Browser 2.0 (https://bar.utoronto.ca/eplant_tomato/; **Figure 2**). Differently from the canonical *SID27*, which is mainly expressed in the roots, the *SID27*-Likes are actively transcribed in shoot organs, mainly leaves, flower buds and fully-opened flowers. The *SID27*-Like 1 appeared overall more highly expressed than *SID27*-Like 2, showing moderate transcript abundance also in fruits at the mature green stage.

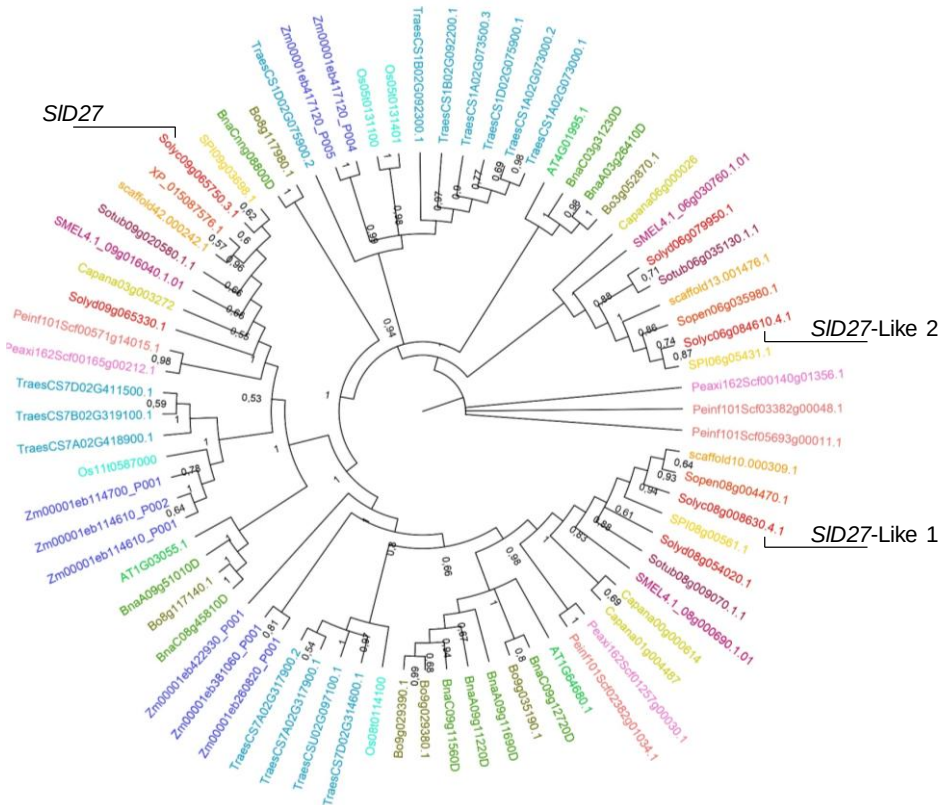


Figure 1 Phylogenetic tree based on the multiple alignment of full-length D27 protein sequences (neighbour-joining method, 1000 bootstrap replicates). The canonical *SID27* and the *SID27*-Like 1 and *SID27*-Like 2 genes are evidenced. *S. lycopersicum* (Solyc); *S. pennellii* (Sopen); *S. pimpinellifolium* (SPI); *S. habrochaites* (scaffold*); *S. lycopersicoides* (Solyd); *S. tuberosum* (Sotub); *S. melongena* (SMEL); *Petunia inflata* (Peinf); *P. axillaris* (Peaxi); *Capsicum annuum* (Capan); *A. thaliana* (AT); *Brassica napus* (Bna); *B. oleracea* (Bo); *Oryza sativa* (Os); *Zea mays* (Zm); *Triticum aestivum* (Traes); XP_015087576.1= Sopen gene.

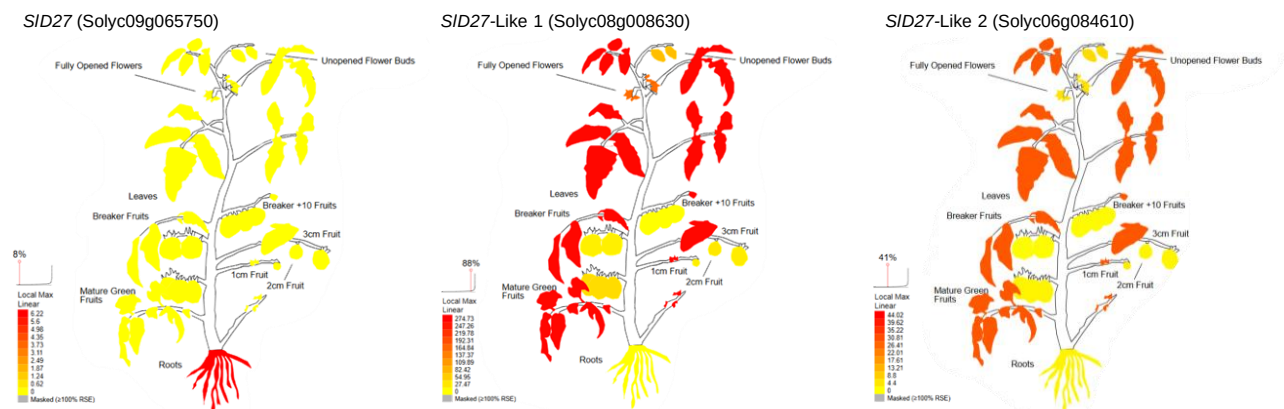


Figure 2 Expression pattern comparison among the canonical *SID27* and the *SID27*-Like genes obtained using the tomato eFP Browser 2.0.

3.3.2 Generation of the CRISPR/Cas9 edited tomato lines for the *D27*-gene family

The sgRNAs for the *SID27*-Likes genes were designed using the CRISPR-P software, and selected based on localization, GC content and on-target and off-target scoring (**Figure 3**). Accordingly, one and two sgRNAs were selected for *SID27*-Like 1 and *SID27*-Like 2, and cloned into the pEgPubi4_237-2A-GFP-D27-Like 1 and the pDIRECT_22C-D27-Like 2 CRISPR/Cas9 vectors, respectively. The *in vivo* efficiency of these vectors was validated in the HR system, and resulted of

55% for the single sgRNA pEgPubi4_237-2A-GFP-*D27-Like 1*, and of 62,5% for the multiple sgRNA pDIRECT_22C-*D27-Like 2*.

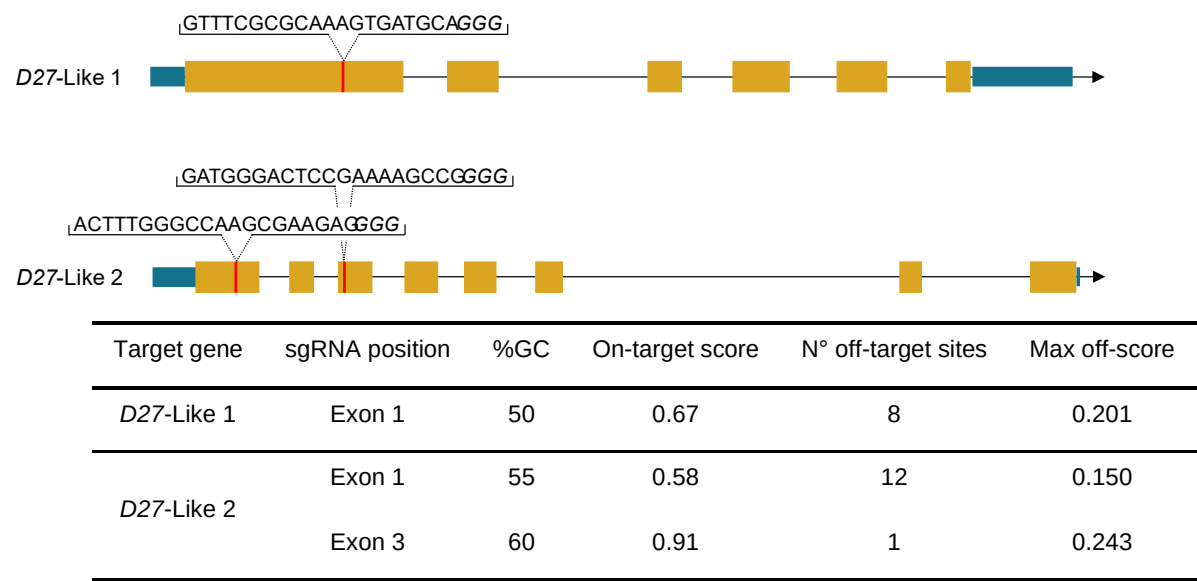


Figure 3 Schematic representation of the sgRNAs selected for the CRISPR/Cas9 editing of the *SID27-Like 1* and the *SID27-Like 2* genes. The gene structure is represented by a black arrow in a 5'-3' orientation, harboring yellow and blue boxes, which refer to the exons and the 5' and 3' untranslated regions (UTRs), respectively. The red lines indicate the location of the sgRNAs alongside the corresponding exons; the 20-nucleotide (nt) sgRNA sequences are shown above, with the PAM region in italics. Further information on the used criteria considered for the sgRNA selection are summarized below.

The stable transformation was carried out in both the tomato variety AC and MM. In the AC background, edited KO alleles were successfully generated for *SID27-Like 1* and *SID27-Like 2*. In MM, the CRISPR/Cas9 mutants were obtained for *D27* and *D27-Like 1*. The entire panel of indel identified through HRFA, is summarized in **Table 3**.

The AC mutants were progressed to the T2 generation, and selected for the following characterization. Independent lines for each of the *D27-Like* genes, segregating for the Cas9 transgene and homozygous for the edited allele, were selected and designated as *Sld27-Like1* KO1 and KO2, and *Sld27-Like2* KO1 and KO2. These mutants were further characterized by Sanger Sequencing (**Figure 4**). Regarding the *Sld27-Like1*, the KO1 mutant showed the insertion of a -T residue and the KO2 a deletion of -7 bp (-AAAGTGA). In both cases, the open reading frame was shifted downstream the mutation site, altering the protein sequence with the formation of a premature stop codon.

Table 3 Summary of the CRISPR/Cas9 knock-out (KO) alleles generated for the *Sld27* gene family in the tomato cultivars Ailsa Craig (AC) and Moneymaker (MM).

Tomato cv.	<i>Sld27</i> -Like 1 KO alleles					<i>Sld27</i> -Like 2 KO alleles				
AC	+1	-2	-3	-5	-7	-2	-3	-4	-6	-11
<i>Sld27</i> KO alleles					<i>Sld27</i> -Like 1 KO alleles					
MM	+1	-1	-3	-5	-7	-2				

In the *Sld27*-Like 2 both the targeted sites were edited. *Sld27*-Like 2 KO1, harbours a 2 bp (-TC) deletion in the site 1, followed by a further deletion of 4 bp (-AAAG) in the site 2. The deletion of 2 bp at site 1 and the consequent shift in the ORF appears to be complemented by the subsequent deletion of 4 bp at site 2 partially restoring the reading frame due to the overall -6 bp deletion. However, correct translation of the *Sld27*-Like2 KO1 protein from an alternative in-frame ATG start is unlikely and disfavored. In *Sld27*-Like 2 KO2 a -3 bp deletion in the site 1, provoked the in-frame deletion of a single amino acid (phenylalanine). Nonetheless, the 8 bp deletion (AAAAGCCG) at site 2, ultimately determines the loss-of-function of the protein.

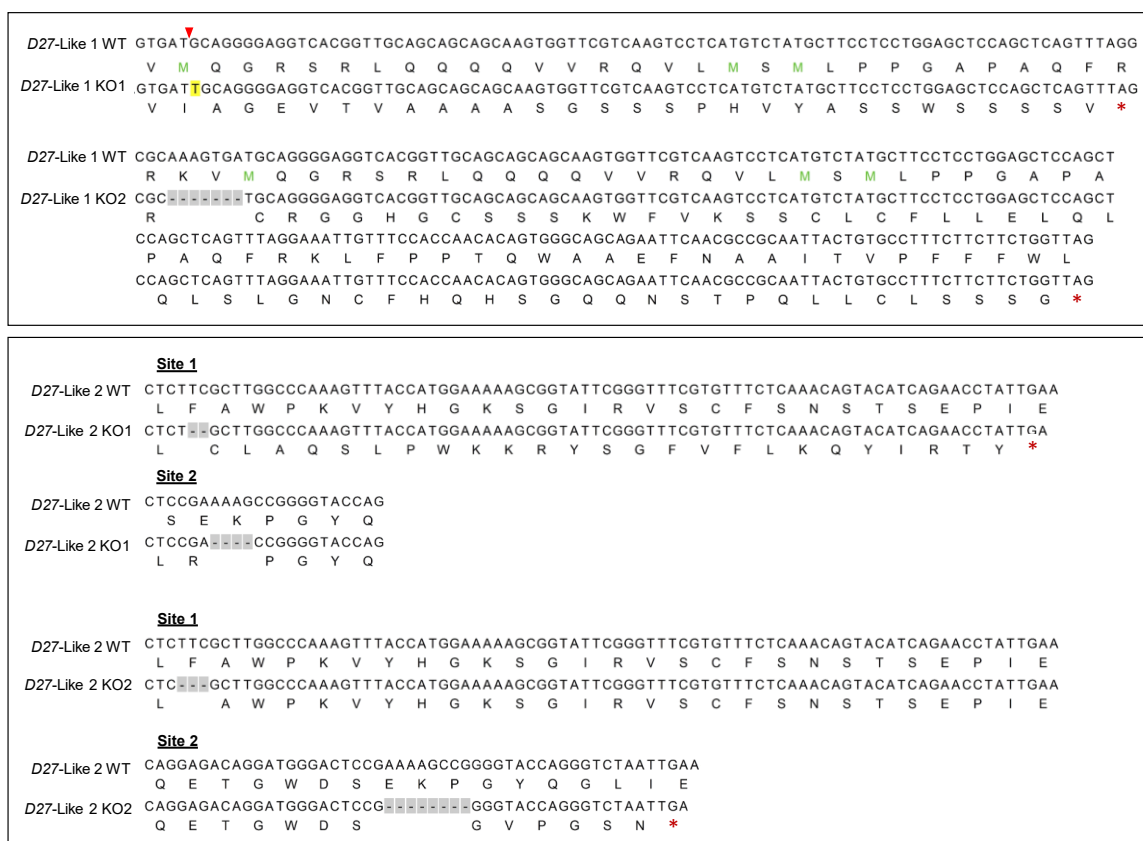


Figure 4 Results of the Sanger sequencing, illustrated as the local alignment between the cDNA and amino acid sequences of Wild Type (WT) and CRISPR/Cas9 edited lines for the *Sld27*-Like 1 and *Sld27*-Like 2. Insertions and deletions are shadowed in yellow and grey, respectively. The red reversed triangle on the WT nucleotide sequence indicates the localization of insertions. Red asterisks define the stop-codon position.

3.3.3 Shoot and root development, flower traits, fruit traits, seed germination, physiological traits

The effects of the inactivation of the CRISPR/Cas9 *Sld27*-Like1 and ACS*Sld27*-Like 2 lines were evaluated in a comparative characterization, with the *Sld27* KO1 and KO2 (**Chapter 2**) to have a complete picture on the *SID27*-gene family. Plant shoot and root traits were assessed, along with physiological traits, fruit traits, and seed germinability.

Being the *D27*-Likes genes potentially involved in the SLs biosynthesis, vegetative traits typically affected by a SL-deficiency were considered. Plant height appeared slightly increased in *Sld27*-Like1 KO1 and KO2 at 30 DPT, although no remarkable differences emerged for this trait at 40 dpt between the different lines. The internode length was substantially reduced only in the *Sld27*-Like 2 lines, whereas this trait did not vary in any of the *Sld27* and *Sld27*-Likes. The number of lateral branches > 5 cm resulted significantly higher in *Sld27* KO1 and *Sld27* KO2 (**Table 4**).

Table 4 Plant vegetative traits significantly different at the comparison between Ailsa Craig (AC) non-edited wild-type (WT) and the CRISPR/Cas9 lines KO for *SID27* and *SID27*-Likes genes. The Student's T-test was applied as statistical test ($n = 6$). Avg, average; SD, standard deviation, p -value, statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

	Plant height (30 DPT)			Lat. branch. > 5 cm			Internode lenght		
	Avg (cm)	SD	p val.	Avg (cm)	SD	p val.	Avg. (cm)	SD	p val.
WT	48.50	2.35	-	5.83	1.33	-	2.95	1.48	-
<i>Sld27</i> KO1	51.17	4.22	0.21	9.50	2.59	0.01	2.96	1.45	0.99
<i>Sld27</i> KO2	50.80	5.54	0.38	6.40	1.34	0.50	3.34	1.78	0.14
<i>Sld27</i> -Like 1 KO1	55.40	4.88	0.01	6.60	1.52	0.39	3.51	2.12	0.06
<i>Sld27</i> -Like 1 KO2	60.40	9.50	0.02	8.00	2.12	0.07	3.38	2.00	0.13
<i>Sld27</i> -Like 2	44.60	4.51	0.10	8.00	1.87	0.05	2.31	1.25	0.004

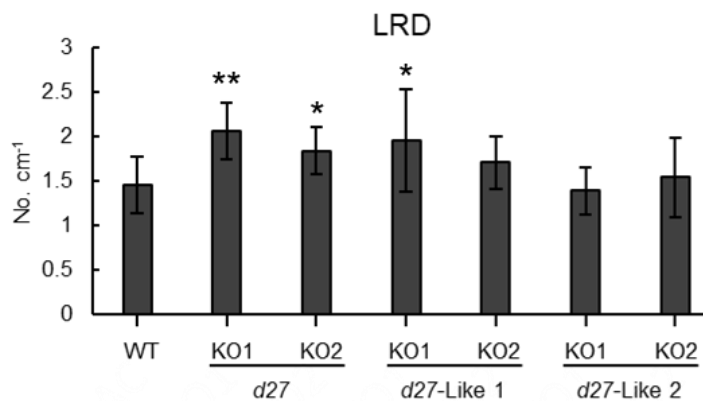
The impact of the mutations on flower production and development was established by evaluating the number of flowers *per* inflorescence (flowers/inflorescence), and the total number of inflorescences *per* plant (45 DPT). While no alterations were observed for this latter index, the *Sld27* KO1 generally produced a lower number of flowers on the first three inflorescences (**Figure 5**). Nonetheless, the estimated fruit-set were not compromised in the *Sld27* and *Sld27*-Like KOs. The average fruit weight (avg. fruit weight) was decreased by ~ 5 g in the *Sld27* KO1; these fruits also contain less seeds *per* fruit (seeds/fruit), whose germination is not impaired.

Figure 5 Plant flower (flowers/inflorescence) and fruit traits (avg. fruit weight, seeds/fruit) that resulted significantly different at the comparison between Ailsa Craig (AC) non-edited wild-type (WT) and the CRISPR/Cas9 lines KO for *SID27* and *SID27*-Likes genes. The Student's T-test was applied as statistical test. Bars represent the standard deviation (SD) of the mean. * $p < 0.05$, ** $p < 0.01$



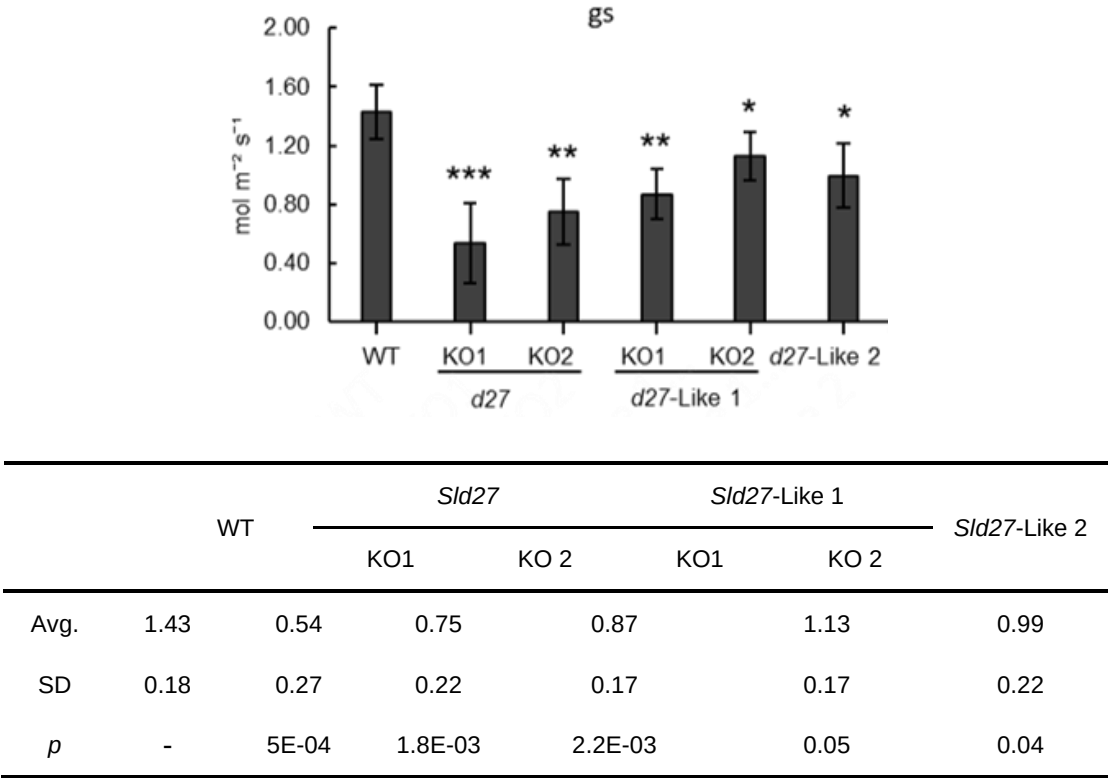
Concerning the architecture and development of root apparatus, we observed main alterations in the LRD of both *Sld27* lines and in the *Sld27*-Like1 KO1 (**Figure 6**). The other measured parameters – PRL, LRN and RA remained unchanged.

Figure 6 Lateral root density (LRD) in Ailsa Craig (AC) non-edited wild-type (WT) and in CRISPR/Cas9 lines KO for *SID27* and *SID27*-Likes genes. The Student's T-test was applied as statistical test ($n > 6$). Bars represent the Standard deviation (SD) of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Physiological parameters were analyzed at 35 DPT. A general reduction in gs appeared in all the edited lines (**Figure 7**). The *Sld27* KOs showed the strongest reduction, with decreases of 62.3% and 47.5% in the *Sld27* KO1 and KO2 respectively. The two *Sld27*-Like, this trend was maintained with a decrease of 39% in *Sld27*-Like 1 KO1, 21% in *Sld27*-Like 1 KO2, and 30.8% in *Sld27*-Like 2. No evident changed emerged regarding the Φ PSII and SPAD index in the mutated lines compared to the WT lines.

Figure 7 Stomatal conductance (gs; mol m⁻² s⁻¹) in Ailsa Craig non-edited wild-type (WT) and in CRISPR/Cas9 lines KO for *SID27* and *SID27*-Likes genes. The Student's T-test was applied as statistical test (*n* = 6). Avg, average; SD, standard deviation, *p*-value, statistical significance. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001.



3.3.4 The depletion of *SID27* and *SID27*-Likes genes impact ABA and polar metabolites in leaves, showing minor effects of carotenoids and non-polar metabolites

Leaf polar and non-polar metabolites analyzed in *Sld27* and *Sld27*-Like lines collection are summarized in **Table 5**.

Table 5 Polar and non-polar compounds analyzed in the present study. *m/z*: mass-to-charge ratio; RT: retention time.

Compound class	Polar compounds	<i>m/z</i>	RT
ABA metabolites	ABA	263.13	10.41
	7'-OH ABA	279.12	10.41
	ABA GE	427.20	6.57
Vitamin C	AsA	177.04	1.33
AA	Pro	116.07	1.24
	Phe	166.09	2.79
	CGA	353.09	6.9
	Rutin	611.16	9.29
Polypohenols	ChNar	271.06	14.25
	FA-hex	355.10	6.89
	QA	193.07	1.3

	4-CQA	355.10	7.06
	Hyp	463.09	9.65
	K-3-G	447.09	10.4
Alcaloids	α -Tomatine	1034.55	10.93
	De-H-tomatine	1032.54	10.66
	OH-tomatine	1050.55	9.5
Compound class	Non-polar compounds	<i>m/z</i>	RT
Carotenoids	PTF	543.49	6.02
	at β C	537.45	6.9
	9c β C	537.44	7.05
	Z	569.43	4.41
	V	601.42	2.85
	9cV	601.42	3.1
	t-neo	601.42	2.42
	at α C	537.45	6.27
	L	551.42	4.1
	LX	601.42	3.53
Chlorophylls	Chl_a	893.54	4.4
	Chl_b	907.52	3.48
	PQH ₂ -9	751.64	5.5
	PQ	749.62	5.92
Ubiquinones	CoQ10	863.69	6.2
Vit K	PK	451.36	3.28
Vit E	α T	431.39	2.5
	α -TQ	429.37	2.01
	α -T3	425.34	3.2
Fatty acids	β SS	397.38	4.2
	Stig	413.38	3.17
Fatty acids	LA	279.23	0.78
	LNA	277.22	0.71

ABA and ABA-related metabolites content in leaves deeply changed in the edited lines (**Figure 8**). ABA content generally increased in all the KO lines but mostly in *Sld27* KO1 and KO2, where the content of this metabolite was 56 and 59% higher than the content detected in the WT, respectively. In the *Sld27*-Like 1 and *Sld27*-Like 2, this trend was still maintained, although no changes were demonstrated in the *Sld27*-Like 1 KO2. 7'-OH ABA showed a decreasing trend in the *Sld27*,

displaying a fold change (FC) almost halved in *Sld27* KO1. Conversely, in the *Sld27*-Like 1 this compound was augmented of up to the 27% in the KO2; in *Sld27*-Like 2 no substantial variations emerged. The content in ABA GE is reduced in all the KO of the *SID27* gene family members, with the strongest reduction observed in the *Sld27* and *Sld27*-Like 2 lines.

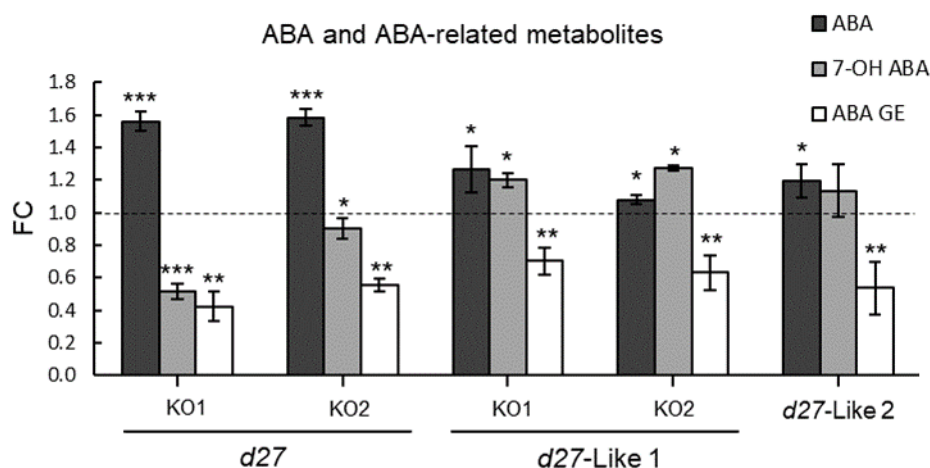


Figure 8 ABA and ABA-related metabolites in the leaves of edited lines for the *SID27* and *SID27*-Like genes. The content for each compound is expressed as fold-change (FC) relative to the non-edited wild-type (WT; dashed line). Statistically significant differences were assessed at the Student's T-test ($n = 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Regarding polar metabolites, five different classes for a total of fifteen compounds were considered (**Table 6**). Most of these metabolites resulted significant at the statistical test, although a more stringent criteria was further applied to evidence fold changes ≤ 0.6 and ≥ 1.5 . Accordingly, Asa resulted more than doubled in the *Sld27* and *Sld27*-Like 1, with a peak up to 2.83-folds in *Sld27* KO2. Among amino acids, Pro increased exclusively in the *Sld27* KO line. The content of polyphenols appeared generally reduced. The most evident changes appeared in ChNar, Hyp and K-3-G accumulation. ChNar levels decreased in the KOs for *Sld27*, whereas Hyp and K-3-G levels decreased also in the *Sld27*-Like 1 KOs. Nonetheless, QA was the only polyphenolic compound that substantially increased in the entire panel of mutants. No remarkable changes emerged in the accumulation of alkaloid-related metabolites.

Table 6 Polar metabolites content in the *Sld27* and *Sld27*-Likes lines. Data are expressed as fold-change (FC) on non-edited wild-type (WT; $n = 4$). Avg: average; SD: standard deviation. Statistical significance was assessed at the Student's T-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Compound	<i>d27</i> KO1		<i>d27</i> KO2		<i>d27</i> -Like 1 KO1		<i>d27</i> -Like 1 KO2		<i>d27</i> -Like 2	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
AsA	2.14 **	0.39	2.83 *	1.19	2.20 *	0.52	2.11	0.83	1.12	0.45
Pro	6.30 ***	0.34	1.54 *	0.25	1.45 *	0.19	1.15	0.26	0.64	0.36
Phe	1.10 *	0.05	0.96	0.02	0.99	0.02	1.04	0.09	1.07	0.09
CGA	0.82 **	0.06	0.70 **	0.02	0.97	0.06	1.09 *	0.05	0.97	0.11
Rutin	0.69 **	0.06	0.46 ***	0.02	0.95	0.14	0.84	0.12	0.82 *	0.12
ChNar	0.52 **	0.10	0.31 ***	0.02	1.10	0.16	1.35	0.24	1.12	0.15
FA-hex	0.83 *	0.08	0.48 ***	0.02	0.76 ***	0.03	0.83 **	0.04	0.81 *	0.11
QA	2.24 ***	0.24	2.41 ***	0.36	1.96 ***	0.11	2.08 ***	0.12	1.84 **	0.34
4-CQA	0.74 **	0.08	0.60 ***	0.03	0.92	0.03	1.06	0.06	0.82 *	0.15
Hyp	0.44 ***	0.03	0.33 ***	0.04	0.60 ***	0.07	0.55 ***	0.04	0.62 ***	0.10
K-3-G	0.59 ***	0.03	0.32 ***	0.02	0.58 ***	0.04	0.45 ***	0.02	0.75 *	0.16
α -Tomatine	0.86 ***	0.01	0.79 ***	0.01	0.97	0.07	0.84 ***	0.04	1.06	0.10
De-H-tomatine	0.82 ***	0.03	0.75 ***	0.02	0.87 *	0.08	1.68 ***	0.10	0.92 *	0.06
OH-tomatine	1.14	0.20	0.62 *	0.01	0.70	0.13	0.70	0.07	1.02	0.16

No appreciable changes emerged within the twenty-three non polar metabolites analyzed (**Table 7**). Only minor variations in the carotenoid composition were detected. The at β C precursor PTF appeared increased in the sole *Sld27* KO2, and at β C itself was only slightly decreased in the KO lines for *Sld27*-Like 1 and -Like 2. Overall, 9c β C amount was not impacted, whereas for Z, a reduction was observed in *Sld27* KO2, *Sld27*-Like1 KO1 and *Sld27*-Like 2. V and its 9-*cis* isomer were mostly unaltered. T-neo, ataC and L showed a decline exclusively in the *Sld27*-Like 1 KO2, while LX level increased in *Sld27*-Like 1 KO1 and *Sld27*-Like 2. The content of Chl_A and Chl_B was compromised in *Sld27*-Like1 KO2 and *Sld27*-Like 2. Contrarily, PQ level, a photosynthesis-related molecule, increased in both the *Sld27* KOs, and the level of the reduced PQ form, PQH₂-9 increased as well in the *Sld27* KO2. CoQ10 (ubiquinone 10) levels was mostly affected in the *Sld27*-Like1 KO2, in which also PK level decreased. Slightly higher levels of α -TQ were displayed in *Sld27*-Like1 KO 2 and *Sld27*-Like 2. Within the fatty acids class, the content in β SS was almost halved in the *Sld27*-Like, and LA and LNA levels decreased in the *Sld27*-Like1 KOs.

Table 7 Non-polar metabolites content in the *Sld27* and *Sld27*-Likes lines. Data are expressed as fold-change (FC) on non-edited wild-type (WT; $n = 4$). Avg: average; SD: standard deviation. Statistical significance was assessed at the Student's T-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Compound	<i>Sld27</i> KO1		<i>Sld27</i> KO2		<i>Sld27</i> -Like 1 KO1		<i>Sld27</i> -Like 1 KO2		<i>Sld27</i> -Like 2	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
PTF	1.07	0.19	1.73 **	0.26	0.94	0.21	0.94	0.41	1.42 *	0.30
at β C	0.93	0.17	0.87	0.14	0.71 *	0.05	0.59 **	0.14	0.67 **	0.09
9c β C	1.00	0.16	1.10	0.20	0.83	0.15	0.85	0.18	0.88	0.17
Z	1.00	0.05	0.69 *	0.12	0.61 *	0.13	0.99	0.21	0.75 *	0.17
V	1.14	0.20	1.14	0.25	0.87	0.12	0.73 *	0.15	1.07	0.09
9cV	1.02	0.17	1.12	0.13	0.93	0.04	0.68	0.16	1.01	0.14
t-neo	0.98	0.15	0.89	0.07	0.81	0.07	0.70 *	0.12	1.06	0.09
at α C	1.07	0.08	0.94	0.05	0.98	0.19	0.61 **	0.16	0.87	0.18
L	0.95	0.07	0.97	0.14	0.96	0.20	0.69 *	0.17	0.88 *	0.07
LX	1.01	0.04	0.90	0.11	1.34 **	0.14	0.97	0.67	1.23 **	0.11
Chl_a	0.92	0.07	0.94	0.18	0.93	0.17	0.65 **	0.14	0.78 ***	0.04
Chl_b	0.98	0.03	0.95	0.17	0.96	0.09	0.65 **	0.16	0.88 *	0.08
PQH ₂ -9	1.07	0.06	1.68 **	0.25	1.15	0.17	0.99	0.24	0.84	0.21
PQ	1.52 ***	0.04	2.43 ***	0.29	1.24	0.30	1.31	0.32	1.04	0.11
CoQ10	0.85	0.08	0.85	0.15	0.90	0.19	0.46 *	0.33	0.77 **	0.07
PK	0.98	0.08	0.97	0.01	1.01	0.13	0.71 **	0.10	0.96	0.06
α T	0.93	0.06	1.15	0.13	1.02	0.29	1.11	0.30	1.22	0.21
α -TQ	0.94	0.08	1.09	0.25	0.95	0.20	1.33 *	0.20	1.25 *	0.15
α -T3	1.05	0.06	1.01	0.06	1.00	0.05	1.07	0.17	1.10	0.08
β SS	0.62	0.19	1.28	0.25	0.87	0.19	1.02	0.39	0.53 **	0.11
Stig	1.03	0.12	1.00	0.15	1.01	0.10	1.04	0.12	1.04	0.12
LA	1.09	0.04	1.08	0.19	1.14	0.10	0.75 **	0.09	0.98	0.19
LNA	1.02	0.10	1.34	0.23	0.74 *	0.08	0.73 *	0.09	0.84	0.12

3.4 Discussion

Carotenoids are ubiquitously present in plants, being fundamental photosynthetic pigments with additional protective antioxidant functions against ROS. From carotenoid-cleavage originates apocarotenoids, heterogeneous compounds involved in several developmental processes, plant-

environment interactions and fruit aroma and quality. In addition, two of the most important phytohormones are apocarotenoids, namely SL and ABA. Both these molecules, in fact, derive from carotenoid precursors, which are specifically required in a *cis*-configured form for subsequent cleavage operated by the downstream enzymes CCDs and NCED.

In this regard, *D27* has long been considered the only β -carotene isomerase, since it converts all-*trans* into 9-*cis*- β -carotene, this latter compound being the substrate for CCD7, and ultimately for SLs biosynthesis. Recently, two *D27*-Like orthologues were described in rice and Arabidopsis. These genes have been demonstrated to be involved in the synthesis of SLs, further influencing ABA levels, and therefore suggesting a possible role of the *SID27*-Likes in coordinating the crosstalk between these two phytohormones.

This work focuses on the functional characterization of the tomato CRISPR/Cas9 loss-of-function lines for *SID27*-Like 1 and *SID27*-Like 2 genes, in order to provide insights on their roles in tomato.

3.4.1 Successfully produced loss-of-function lines for the two *in silico*-predicted *SID27*-Like 1 and 2 genes showed less alterations in plant shoot- and root-phenotype compared to the KO lines for the canonical *SID27*

The hypothesis of additional *D27* β -carotene isomerases initially emerged from phenotypic observations in *Osd27* and *Atd27* mutants, which was later confirmed in tomato (Nicolia et al., 2025). These mutants displayed a milder SL-deficient phenotype than mutants of downstream genes in the SL biosynthetic pathway – *i.e.*, *Osd17/Atmax3 (ccd7)*, *Osd10/Atmax4 (ccd8)* (Lin et al., 2009; Waters et al., 2012) – suggesting an incomplete depletion of SLs. The residual SL levels could result from a spontaneous β -carotene isomerization process, potentially triggered by light exposure. However, Waters et al., 2012 proposed gene redundancy within the *D27* family based on phylogenetic analyses of *D27* homologs in land plants, green algae, and cyanobacteria. Notably, beyond the canonical *AtD27* – which clustered within the same clade as *OsD27* – two additional Arabidopsis proteins with similarity to *AtD27* and *OsD27* were identified, belonging to distinct clades (2 and 3). Consistent with these findings, our phylogenetic tree constructed from the multiple alignment of *D27* protein sequences from monocot and eudicot species revealed a similar pattern for *SID27*. The canonical *SID27* grouped within the same monophyletic clade as *AtD27* and *OsD27*, while two orthologues, *SID27*-Like 1 and *SID27*-Like 2, clustered into separate clades, respectively.

The expression pattern of the *SID27*-Like genes along with the *SID27* was explored using the *in-silico* tool Tomato eFP Browser 2.0. The *SID27* is a low-expressed gene, being mostly transcribed in the roots; contrarily, the *SID27*-Like transcripts are present in the shoot-part of the plant, including flowers and MG fruits for *SID27*-Like 1. These results are well aligned with those recorded in Arabidopsis for *AtD27*-Like 1. In this species, the mRNA of *AtD27*-Like 1 was detected in phloem sap and in the roots from WT scions grafted onto *Atd27*-Like 1 rootstocks, demonstrating a shoot-to-root transport for this transcript. Moreover, different studies establishing the spatial and temporal

localization of the *AtD27*-Like expression by GUS assay, have confirmed the active transcription of these genes in the shoot organs; importantly, a remarkable GUS staining was observed in flower stigma and style, and in pods for *AtD27*-Like 1 (Gulyás et al., 2022; Yang et al., 2023). Whereas, *AtD27*-Like 2 is more highly expressed in seeds, with low level of transcription retained in the pods. This latter evidence suggests a role for *D27*-Like1 in flowering and fruit development.

In order to explore the potential phenotypic effects of inactivating the *SID27*-Like genes, we analyzed shoot and root traits and seed germination. For the sake of completeness, *Sld27* KOs (*Sld27* KO1 and KO2), which are impaired in SL production, were included in this study (Nicolia et al., 2025). Vegetative traits were mostly unaltered in *Sld27* KOs, confirming previous results (Nicolia et al., 2025), and *Sld27*-Like lines, although in this case an augmented lateral branching was detected in the *Sld27* KO1 and *Sld27*-Like 2. In general, the tomato *Sld27* mutants appeared less affected than those for the orthologues genes in other species (*viz.* *Arabidopsis* and rice; Lin et al., 2009; Waters et al., 2012). The “bushy” phenotype associated to the SL- condition was completely absent in the *Sld27*-Likes, consistently to those showed in the orthologue genes *d27*-Like 1 and 2 mutants in both *Arabidopsis* and rice (Kobuna et al., 2025; X. Liu et al., 2020; Yang et al., 2023). Flower and fruit traits exhibited the same trend: a reduced number of flowers *per* inflorescence, fruit weight and seeds *per* fruit were only reported in the *Sld27* KO1, although with high variability. *Sld27*-Like 1 and 2 displayed a normal phenotype. Nonetheless, both these genes were found to be expressed in flower, fruit and seeds in *Arabidopsis* (Gulyás et al., 2022; Kobuna et al., 2025; Yang et al., 2023). A more accurate investigation of the *SID27-like* genes in tomato would provide important insights to elucidate the *Sld27* KOs counterintuitive phenotype.

Strigolactones have long been established as essential players in the regulation of root apparatus remodeling in response to phosphorus (Pi) deficiency conditions (Kapulnik et al., 2011; Ruyter-Spira et al., 2011; Santoro et al., 2020, 2021). In this work, the development of root apparatus was assessed in an *in vitro* assay in order to evaluate changes in macroscopic root features, as root morphology and architecture, and under normal nutrient conditions. Our data did not highlight variations in the PRL and LRN individually, however, the LRD index was increased in both the *Sld27* KOs and in the *Sld27*-Like 1 KO1. A similar increase in this parameter was reported in the SL-depleted *Arabidopsis* mutants *Atmax3/ccd7* and *Atmax4/ccd8* (Kapulnik et al., 2011), but not in the *Osd27* and *Osd10/ccd8* (Sun et al., 2014), when grown without nutrient starvation. Tomato RNAi-CCD7 root apparatus was mostly unaffected in presence of full-nutrients, with main alterations appearing in low Pi and complete Pi deficiency (Santoro et al., 2020). An *in vivo* evaluation will be essential to definitively ascertain the effects of *SID27* and *SID27*-Likes inactivation on root apparatus architecture and development.

3.4.1 The *Sld27* and *Sld27*-Likes showed altered stomatal conductance and ABA and ABA-related metabolites

In our study we have assessed key physiological parameters in *Sld27* and *Sld27*-Likes plants. Although no alterations in Chl content and fluorescence were evidenced, important changes were highlighted in the stomatal conductance (*gs*). Particularly, we have observed a general reduction in this parameter, which is more strongly impaired – up to 63.2% – in the *Sld27* KOs. A reduced *gs* generally suggest a lower stomatal aperture. In eudicots, the impairment in SL content is associated to increased *gs*, which is restored by the exogenous application of the synthetic SL analogous GR24, in the *ccd7* and *ccd8* mutants. Higher *gs* is also associated to higher susceptibility to abiotic stresses, such as drought (Ha et al., 2014; J. Liu et al., 2013, 2015; Visentin et al., 2016). Overall, our results on *d27* and *d27-like* genes do not agree with these lines of evidence but, to our knowledge, there are no other comparable studies in tomato.

In order to assess whether the decreased *gs* is associated to variations in ABA levels, we have analyzed its content in leaves. We further checked its degradation and storage metabolites, respectively 7'-OH ABA and ABA GE. Noteworthy, we have reported substantial changes in all these compounds. In recent years, evidences are accumulating that highlighted the presence of a SL-ABA crosstalk, and the possibility that such interaction is mediated by *D27* and/or by *D27*-Likes has been postulated in rice and Arabidopsis (Haider et al., 2018; X. Liu et al., 2020; Yang et al., 2023). Both SLs and ABA, in fact, require the respective carotenoid precursors in a 9-*cis* configuration, and *D27* has long been established as an all-*trans*/9-*cis*- β -carotene isomerase (Abuauf et al., 2018; Alder et al., 2012; Bruno & Al-Babili, 2016). This activity was also exhibited by *AtD27*-Like 1, that also shows a 13-*cis*/15-*cis*/9-*cis* interconversion (Yang et al., 2023), and accepts the ABA-precursors zeaxanthin and violaxanthin as substrates in addition to all-*trans*- β -carotene. Concerning *Sld27* lines, ABA content increased of at least 56%, therefore supporting the observed *gs* reduction. This increase in ABA was associated to low levels of both 7'-OH ABA and ABA GE, potentially indicating a perturbation in the ABA degradation and storage. From the data of *gs* and ABA/ABA-related metabolites, it could be hypothesized that *Sld27* lines behaved as under a moderate constitutive stress. Previously, the endogenous ABA levels were investigated in *Atd27* shoots, but no deviations from the WT levels were detected. Conversely, *Atd27*-Like 1 mutants displayed higher ABA content, consistent with our findings regarding *Sld27*-Like 1 (Yang et al., 2023). Furthermore, in contrast to *Sld27*, *Sld27*-Like 1 KOs exhibited elevated levels of 7'-OH ABA, an ABA catabolite with weak ABA-like activity. However, the specific functions of 7'-OH ABA remain unclear (Nambara & Marion-Poll, 2005; Zhou et al., 2004). *Sld27*-Like 2 mirrors *Sld27*-Like 1 in terms of the composition of these metabolites.

3.4.3 The depletion of genes of *SID27* family affects leaf polar and non-polar secondary metabolites

To gain a comprehensive picture of the impact of *SID27* gene family member depletion, and to elucidate their functions on overall plant status, we added a further piece to the puzzle by analyzing polar and nonpolar metabolites in leaves from the mutant collection.

For polar metabolites, two groups with opposite behavior can be distinguished. The first group includes AsA and amino acids (Pro, Trp, Phe), which appeared slightly increased in the edited lines. Contrarily, the polyphenolic compounds and alkaloids levels decreased. All these compounds are related to the capacity of plants to cope with various types of stresses, and different studies reported the positive role of exogenous application of SLs (*i.e.*, GR24) in promoting their accumulation particularly under stress condition (X. Chen et al., 2022; H. Liu et al., 2022; Luqman et al., 2023; Richmond et al., 2022; Trasoletti et al., 2022). The knowledge about the specific effects of the depletion of *D27*, and even more of the *D27*-Likes genes is relatively scarce, and the current evidences on the effects of these mutations on AsA and amino acids are scarce. The accumulation of proline, which is usually associated with stress, increased in the *Sld27* KOs. However, proline measurements in RNAi-*SICCD7* leaves did not previously reveal such an increase (Agliassa et al., 2025). With regard to polyphenols, a positive correlation among the polyphenol-compounds catechin and the *D27* expression was proposed by Shen et al., 2025 in *Camelia sinensis*, that is in accordance with our results. Furthermore, the same study found that reduced damage in the *D27* over-expressing lines was associated with reduced oxidative membrane damage due to lower levels of MDA.

Within non-polar secondary metabolites, only minor changes have emerged among the different classes of compounds that we have analyzed. We were particularly interested in carotenoids, since they represent the common precursors of both SLs and ABA. In literature, the effects of the inactivation/over expression of the *D27* and *D27*-Likes genes were available for rice and Arabidopsis (reviewed in Tolnai et al., 2023). We have detected a decreasing trend in all-*trans*- β -carotene in all the investigated lines, but which was only statistically significant in the *Sld27*-Likes. In Arabidopsis, no particular changes in the levels of this metabolite were reported in *Atd27* and *Atd27*-Like 1 KOs (Yang et al., 2023); however, slightly lower levels of this metabolite were observed in rice (Haider et al., 2018). No substantial variations were recorded in any of the other carotenoids and xanthophylls analyzed. In Arabidopsis a strong increase in 9-*cis*-violaxanthin was evidenced (Yang et al., 2023), that was not visible in our case. Among other non-polar metabolites, Chl_a and Chl_b were not subjected to major variations, however these pigments displayed a lower concentrations in *Sld27*-Like 1 KO2 and *Sld27*-Like 2. This phenotype was consistent to those described in the *SI-ORT1* line, flawed in the SLs content due to the mutation in the *SICCD7* gene. Mayzlish-Gati et al. (2010) reported a reduced content in chlorophylls and a reduced expression of light- responsive genes. It has to be underlined that our mutants did not showed variations in the SPAD index compared to the

WT, therefore this aspect requires further investigations. PQ levels appeared significantly higher in *Sld27* KO1 and KO2. There is no direct evidence in literature about direct effects of the inactivation of SL biosynthesis related genes on this specific compound; however, both SLs and PQ are plastid isoprenoids deriving from the methylerythritol phosphate (MEP) pathway (Barja et al., 2021; Matúšová & Bouwmeester, 2005), therefore, wide range genetic perturbations in this context cannot be ruled out. Fatty acids were minimally affected, with the most important changes which consists in the contraction of β SS content in *Sld27*-Like 2, and in a slight decrease in LNA exhibited in the *Sld27*-Like 1 KOs. In fact, a down-regulation of the fatty-acid metabolism was described by Richmond et al., 2022 in the roots of the Arabidopsis SL- line *Atmax4/ccd8*.

3.5 Concluding remarks

In this chapter was described the phylogenetic identification, generation and characterization of the CRISPR/Cas9 mutants for two additional members of the *D27* β -carotene gene family, *Sld27*-Like 1 and Like 2, respectively. The existence of these genes was postulated considering the mild phenotype displayed by the mutants for the “canonical” *D27* gene, the first committed gene in SL biosynthesis (Alder et al., 2012; Nicolai et al., 2025; Waters et al., 2012). *D27*-Likes genes were, in fact, demonstrated to contribute to SLs biosynthesis, possibly maintaining local amount of these phytohormones in specific conditions (Korek & Marzec, 2023; X. Liu et al., 2020; Yang et al., 2023). Moreover, recent evidence in rice and Arabidopsis raised the intriguing question of whether the *D27* and/or the *D27*-Likes can mediate the SL-ABA crosstalk at biosynthetic level, with conflicting results obtained so far (Kobuna et al., 2025; Korek & Marzec, 2023; X. Liu et al., 2020; Yang et al., 2023). Therefore, extending the knowledge in this regard is fundamental. The *Sld27*-Like 1 and Like 2 CRISPR/Cas9 tomato lines produced during this experiment were fundamental at this purpose, further representing the first collection of mutants for these genes available in a model crop species. The results from the characterization of these lines are partially in accordance to those observed in the literature, and additional investigations will be required to provide solid information about the effective role of these genes, and on their impact on plant physiology and metabolic functions. Finally, an effect of the *D27*-Likes genes on the fruit quality cannot be excluded. In fact, in tomato, these genes appeared expressed in the above-ground part of the plant including fruits. Interestingly, *SID27*-Like 1 produces novel 13-*cis* and 15-*cis*- β -carotene isoforms (Gulyás et al., 2022; Yang et al., 2023), that could have affects the apocarotenoid composition in fruits.

Acronyms for metabolites

ABA: abscisic acid

7-OH ABA: 7-hydroxy ABA

ABA GE: ABA-glucose ester

AsA: ascorbic acid

Pro: proline

Phe: phenylalanine

De-H-tomatine: dehydrotomatine

OH-tomatine: hydroxytomatine

CGA: chlorogenic acid

ChNar: naringenin chalcone

FA-hex: ferulic acid hexose

QA: quinic acid

4-CQA: 4-caffeoylquinic acid

Hyp: hyperoside (quercetin-3-galactoside)

K-3-G: kaempferol-3-O-glucoside

PTF: phytofluene

at β C: all-*trans*- β -carotene

9c β C: 9-*cis*- β -carotene

Z: zeaxanthin

V: violaxanthin

9cV: 9-*cis*-violaxanthin

tNeo: all-*trans*-neoxanthin

at α C: all-*trans*- α -carotene

L: lutein

LX: luteoxanthin

Chl_A: chlorophyll A

Chl_B: chlorophyll B

PQH₂-9: plastoquinol-9

PQ: plastoquinone

CoQ10: ubiquinone-10

PK: phylloquinone (vitamin K)

At: α -tocopherol

α -TQ: α -tocopherol quinone (vitamin E)

α -T3: α -tocotrienol

β SS: β -sitosterol

Stig: stigmasterol

LA: linoleic acid

LNA linolenic acid

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CHAPTER 4 – The inactivation of two *Ascorbate Oxidases* lead to increased ascorbic acid accumulation in tomato fruits

Note: Manuscript in preparation.

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Abstract

The ascorbic acid (AsA) content is among the factors that most affect the tomato fruit nutritional quality, due to the importance of this molecule as antioxidant and metabolically active compound. Tomato, being one of the most diffused and consumed vegetables in the worldwide, represent an important source of this essential nutrient, however, decades of breeding-for-yield had generally reduced the AsA pool of tomato, especially in the commercial variety. Recently, agricultural research has devolved relevant efforts in exploring different strategies aimed at generating high-accumulating AsA genotypes.

Beyond through the biosynthesis pathway, maintenance of adequate AsA levels in plants is ensured by a AsA recycling pathway in which AsA in its active form – which is the reduced AsA – is oxidized into monodehydroascorbate (MDHA) and dehydroascorbate (DHA) by the activity of enzymes known as ascorbate peroxidase (APX) and ascorbate oxidase (AO).

Previously, a repertoire of tomato CRISPR/Cas9 knockout (KO) lines for the *SlAO* gene, and for a further ascorbate oxidase candidate – *SlLAC* – were generated by a research group at the Department of Agricultural Science, University of Naples Federico II, in the tomato cultivar “Red Setter”. In this chapter is described the advancement in T2 generation of *Slao* and *Sl/ac* KOs, their genetic characterization and the production of genetically stable edited lines. The evaluation of morphological-, flower-, and fruit-traits was also performed and did not highlight relevant changes between the mutants and the non-edited wild-type (WT). A higher content of reduced AsA was detected in leaves, roots and fruits of the *Slao* and *Sl/ac* KO lines. Overall, we confirmed the *SlAO* gene as a good target to increase AsA levels in fruit and confirmed that the *SlLAC* gene code for as an additional ascorbate oxidase potentially involved in AsA metabolism.

4.1 Introduction

The importance of vegetables as an indispensable component of a balanced diet has long been established, since they represent a unique source of beneficial nutrients. Indeed, vegetables provide several of those compounds known as “essential organic compounds”, necessary for the proper functioning of the metabolism and ultimately for human-health, but which the human body cannot produce on its own. Therefore, their uptake exclusively occurs with the diet. Among vegetables, tomato is the most diffused, being ubiquitous in the Mediterranean regions, but equally widespread on world markets for fresh consumption or in the form of processed products. Therefore, this vegetable has a huge impact on nutrients intake of macro- and micro-nutrients (Vats et al., 2022). With regard to the latter, tomato fruits are rich in micronutrients referred as phytonutrients, a variegated class of compounds with recognized benefits for human-health, as well as for plant-health (Ali et al., 2020; Davies et al., 1981). These macromolecules, are characterized by a specific structure which confers them antioxidant properties. The most abundant phytonutrients and antioxidants present in tomato are carotenoids, as lycopene, β -carotene, lutein and their derivatives (Inbaraj & Chen, 2008). These are lipid-soluble long-chain isoprenoids with polyunsaturated double bonds, which confers them the Reactive Oxygen Species (ROS)-scavenging capacity (Martí et al., 2016). Contrarily, ascorbic acid (AsA), commonly referred as vitamin C (vit. C), is the most relevant water-soluble antioxidant present in this vegetable (Davies et al., 1981; Mellidou et al., 2021). Ascorbic acid is a low molecular weight six-carbon cyclic-ester, that includes in its structure an enediol-group, whose reversible oxidation allows the ROS quenching. pH strongly influences the form in which AsA can be found, being this molecule highly sensible to this and other physiological and environmental conditions, as temperature and light (Paciolla et al., 2019). In fact, AsA plays plenty roles in metabolism, most of which are shared between plants and humans, especially those related to its antioxidant activity, which are ultimately essential in protecting cells from ROS-induced damages, maintain the redox-status and regenerate further antioxidants as α -tocopherol (Alberts et al., 2025; Gallie, 2013; Gęgotek & Skrzydlewska, 2022). In plants, these key-functions intervenes in the response against biotic and abiotic stresses (Xiao et al., 2021), whereas, in humans they are fundamental in preserving immune-system and musculoskeletal health by promoting collagen synthesis for strong connective tissues, and in preventing several types of cancer (Alberts et al., 2025; Mellidou et al., 2021). As previously mentioned, AsA is an essential phytonutrient since humans lost their ability of autonomously biosynthesizing this compound due to a spontaneous mutation in a biosynthesis gene (Fenech et al., 2019). Therefore, its intake exclusively occurs with the diet. For this reason increasing AsA content is one of the priorities of tomato genetic improvement, with the dual aim of improving the nutritional quality of the fruit and increase the plant's tolerance to biotic and abiotic stresses. In last decades, various strategies have been used to increase AsA content in tomato, with remarkable results obtained by conventional breeding (Mellidou et al., 2021; Rigano et al., 2014), or using more straight-forward genetic engineering techniques

(Yazdani et al., 2019; Koukounaras et al., 2022). This latter approach was primarily focused on the direct or indirect manipulation of the AsA biosynthesis pathway, that starts from sugars and in plant occurs from the main and fully characterized d-mannose/l-galactose (d-Man/l-Gal) route – also referred as Smirnoff–Wheeler (SW) route. Three alternative pathways – the d-galacturonate, L-gulose, and myo-inositol pathways – are hypothesized to participate in the generation of this metabolite (Quiñones et al., 2024). Moreover, beyond the *de novo* biosynthesis, the AsA metabolism additionally encompasses a recycling route – the Foyer-Halliwell-Asada cycle – in which AsA is initially oxidised by ROS, and subsequently regenerated into its bioactive reduced form (Asada, 1999; Foyer & Halliwell, 1976; Smirnoff & Wheeler, 2024). The oxidation process of AsA is driven by the enzymes ascorbate peroxidase (APX; Davey et al., 2000) and ascorbate oxidase (AO; Hoegger et al., 2006).

Ascorbate peroxidases are heme-containing enzymes, whose isoforms have been found associated to various intracellular compartments as mitochondria and chloroplasts. These enzymes are, in fact, involved in the detoxification of hydrogen peroxide (H_2O_2), which are generated at high rate by respiration and photosynthesis processes. Particularly, APX converts H_2O_2 via AsA, by oxidising this latter into monodehydroascorbate (MDHA), which rapidly disproportionate into dehydroascorbate (DHA) and AsA. Subsequently, DHA is reconstituted by glutathione (GSH)-dependent dehydroascorbate reductase (DHAR; Caverzan et al., 2012). Instead, the AO enzymes are transmembrane multicopper oxidases, mainly located in the apoplast, and whose activity relies in the reduction of molecular oxygen (O_2) by forming DHA and H_2O (Mellidou & Kanellis, 2024). Nonetheless, the activity of the AO enzyme, which has only recently been elucidated, seems to be not only restricted to the ROS-scavenging. In fact, it has been proposed that AO has more complex roles in preserving the proper redox homeostasis finely tuning the AsA/DHA ratio and the ROS concentration in the apoplast, with important consequences in key physiological processes as the regulation of cell-wall expansion. Moreover, AO activity is linked to further physiological processes as gas exchange and stomatal conductance, flowering time, and senescence (Mellidou & Kanellis, 2024; Stevens et al., 2017).

Previously, the accumulation of AsA in tomato has been increased by suppressing the enzymes involved in its oxidation (Chen et al., 2003). For instance, RNA interference (RNAi)-mediated silencing of a mitochondrial APX gene improved AsA accumulation in tomato fruits (Zhang et al., 2011). Consistently, the CRISPR/Cas9 knockout (KO) of a fruit-specific APX, the *APX4*, similarly displayed a higher content of AsA (Do et al., 2022). In tomato, the partial inactivation of the AO gene was similarly obtained, with RNAi-AO down-regulated lines showing augmented levels of AsA in the fruit (Abdelgawad et al., 2019; Zhang et al., 2011). These lines also displayed an improved tolerance to drought stress (Zhang et al., 2011).

In this chapter, a collection of CRISPR/Cas9 mutants for the *SIAO* (*Solyc04g054690*) gene produced in the tomato cultivar “Red Setter” were characterised for various morphological and fruit related

traits. A CRISPR/Cas9 mutant for the *Laccase 22/L-ascorbate oxidase* (*SILAC*; *Solyc07g052230*) gene was also characterized. This gene was firstly described in the tomato wild species *Solanum pennellii*, as a potential gene controlling AsA content in tomato fruits (Calafiore et al., 2016). Subsequently, it was further identified in tomato by Sacco et al., 2019, as an additional ascorbate oxidase involved in the AsA recycling pathway by oxidising AsA to MDHA/DHA.

4.2 Materials and methods

4.2.1 Plant genetic materials and growth conditions

Seeds from the first generation of CRISPR/Cas9 transformants (T1) for the *SIAO* (*Solyc04g054690*) and *SILAC* (*Solyc07g052230*) genes used in this work were previously obtained by the research group led by Prof. M.M. Rigano (Department of Agricultural Science, University of Naples Federico II) in the tomato cv. Red Setter using the pDirect_22C CRIPR-vector harbouring two sgRNAs for each gene (**Figure 1**).

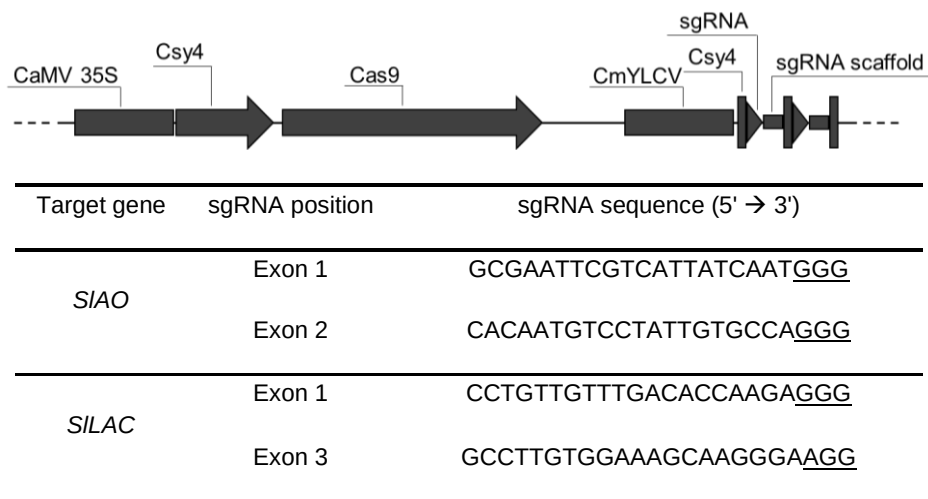


Figure 1 Schematic representation of the CRISPR/Cas9 cassette in harbored by the pDirect_22C plasmid. CaMV 35S: Cauliflower mosaic virus 35S promoter; Csy4: Csy4 recognition site; CmYLCV: Cestrum yellow leaf curling virus promoter; sgRNA: single-guide RNA. sgRNA sequences are reported below, with the PAM sequence being underlined.

Surface sterilization of the seeds was carried out by repeated washes in 70% (v/v) ethanol for 1 min, followed by 20 min in 15% commercial sodium hypochlorite. Seeds were then rinsed in autoclaved distilled water 5-6 times for 5 min.

For the *in vivo* cultivation, seeds were placed on petri dishes lined with moistened filter paper, until germination. Young seedlings were subsequently transferred in trays containing a mixture of peat and vermiculite, and grown into a walk-in chamber with controlled temperature and light (25 °C, 16h light/ 8h dark photoperiod). After 25-30 days (fourth-/fifth-leaf stage) six plants *per* genotype ($n = 6$) were transplanted into 20 x 20 pots containing 80% peat and 20% agriperlite, and randomly distributed in the greenhouse, where optimal conditions for tomato growth were adopted.

Plants intended for the analysis of AsA in the root ($n = 10$) were *in vitro* cultivated using proper boxes with a full MS + 1% sucrose and 0.8% agar substrate.

4.2.2 Selection of non-transgenic CRISPR/Cas9 homozygous genotypes

The pool of T1 seeds edited for the *SIAO* and *SILAC* genes was subjected to molecular screening to select individuals that were Cas9-transgene free and homozygous at the editing site. The genomic DNA (gDNA) extraction from cotyledon or leaf tissue were performed using the Extract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich®). The PCR reactions for the amplification of the Cas9 transgene and of the editing target-region were carried-out with specific primers (**Table 1**).

Table 1 Primer used for the amplification of the editing target regions on the *SIAO* and *SILAC* genes.

Target gene	sgRNA	Primer	Primer sequence (5'→3')
<i>SIAO</i>	1	For	TGCCATATTTGATTGACCAAGCC
		Rev	AATTTACCTGTCTAATTCCATGCC
	2	For	GGCATGGAATTAGACAGGTAAATT
		Rev	TACCTGAGGTTCTCCAACCC
<i>SILAC</i>	1	For	GCAAATAGTAAAGTGGGGAGCG
		Rev	TGGAACAGAGTAAAACAGAGAGACA
	2	For	GATGCCAATTCAGGAACGGTG
		Rev	TGGAAGGGGAGAACTCACAC

Primers for the amplification of PCR products intended for the High-Resolution Fragment Analysis (HRFA) were fluorescently labelled at the 5'. This analysis was run in a SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, US). Homozygous mutations thus individuated were characterized by Sanger sequencing (Eurofins Scientific ©, www.eurofins.com), and the electropherograms and the effects of the potential mutations were assessed using the CLC Main Workbench software (QIAGEN, Aarhus, Denmark). Plants carrying deleterious and homozygous alleles at the editing sites were chosen as parental lines for the T2 generation, that includes the stably edited non-transgenic genotypes.

The plant expression profiles for both genes were retrieved using the Plant eFP (bar.utoronto.ca/eplant; Sato et al., 2012; Waese et al., 2017).

4.2.3 Detection of morphology-, flower-, and fruit-traits

T2 homozygous edited plants were characterized to detect possible effects of the mutations on the plant phenotype.

Vegetative traits were recorded from 30 days post-transplant (DPT). Plant height (cm) and number of leaves were assessed at 30 DPT; plant height was further measured at 40 DPT and at the start of fruit harvest (90 DPT). The date of the first flower opening and of the first developed fruit was annotated, and expressed in DPT. The number of flowers *per* inflorescence were counted on the

first, second, and third inflorescence, and considered both separately *per* each inflorescence and as a total. The onset of fruit-set (DPT) was also estimated as the number of days required for the first fruit to be detectable. Fruit weight (g) was calculated on the total of harvested fruits ($n \geq 54$). For fruit number all the fruits produced during the productive cycle were considered. Seeds were extracted from each fruit at complete ripening stage (mature red, MR), and counted ($n \geq 31$).

On fruits at the complete ripening stage (mature red, MR), the soluble solids content (SSC) and firmness were assessed. The SSC (degrees Brix, °Bx) was assessed on few drops of tomato juice obtained by mixing at least two fruits from the same plant using a Refracto™ 30PX Portable Refractometer (METTLER TOLEDO™). A minimum of six independent measurements ($n = 6$) were performed for each genotype. The fruit firmness was determined on at least 47 fruits with a Bareiss HPE II BARCOL Shore digital durometer mounted on a Bareiss BS 61 II support (Bareiss North America Inc., Stouffville, L4A 0Y2, Canada).

4.2.4 Spectrophotometric determination of ascorbic acid in leaf, roots and roots

The AsA content was assessed in leaves, fruits and roots.

The third fully-opened leaf from the growing tip were collected for each plant. Root were obtained by creating a single pool *per* each genotype from the roots collected by ten *in vitro* grown plantlets ($n = 10$). Leaves and roots were snap-frozen in liquid nitrogen or dry ice, and stored at -80°C.

Fruits were harvested at mature green (MG), turning (T) and MR stages. For each plant ($n = 6$), pools of at least two fruits *per* ripening stage were formed. The fruit pericarp was obtained upon removing the seeds, locular gel and placenta, and it was immediately put in liquid nitrogen or dry-ice, and then stored at -80°C until processing.

The content of reduced AsA was evaluated in a colorimetric assay, as described by Francesca et al., 2022. Briefly whole leaves and roots, and the fruit pericarp were finely ground in liquid nitrogen. To 500 mg of the obtained powder were added 600 µl of ice-cold 6% w/v trichloroacetic acid (TCA). The obtained mixture was incubated up to 15 min on ice to avoid direct light, and subsequently centrifuged at 14,000 rpm for 20 min. A volume of 20 µl of the supernatant, representing the sample extract was collected separately from the solid debris, and was added to 20 µL of 0.4 M phosphate buffer (pH 7.4), 10 µL of double distilled (dd) H₂O and 80 µL of color reagent solution prepared by mixing 5.5 mL of solution A (31% w/v H₃PO₄, 4.6% w/v TCA and 0.6% w/v FeCl₃) with 2 mL of solution B (4% w/v 2,20-Dipyridyl). The resulting mixture was incubated at 37°C for 40 min, after which and 100 µL for each sample were transferred to a microplate and the absorbance at 525 nm was read using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific™, Waltham, Massachusetts). The final concentration of reduced AsA was expressed as mg 100 g⁻¹ of fresh weight (FW). At least three technical replicates *per* each biological repetition were considered.

4.2.5 Statistical analysis

Data were analyzed using the Student's T-test. Statistically significant differences between the means of the different treatments were considered significant with a $p \leq 0.05$.

4.3 Results

4.3.1 Evaluation of the *in-silico* expression profiles of *SIAO* and *SILAC* genes and generation of the T2 Cas9-free tomato knockout plants

The expression for the *SIAO* and *SILAC* genes was evaluated *in silico* using the Plant eFP (bar.utoronto.ca/eplant; Sato et al., 2012; Waese et al., 2017; **Figure 2**). Overall, the two genes showed a similar expression intensity and localization – being mainly transcribed in roots and fruits. Nonetheless, the expression profiles were slightly different, with *SIAO* having high and moderate expression levels respectively in roots and MG fruits and *SILAC* being strongly transcribed in one and two cm developing fruits, and moderately in roots and three cm fruits.

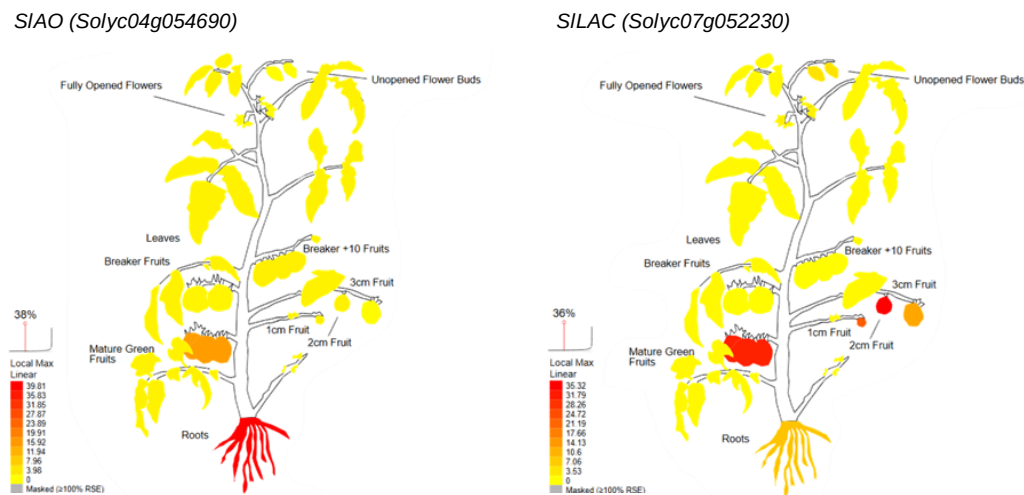


Figure 2 Expression profiles of the genes *SIAO* and *SILAC* according to the Plant eFP.

A genetic selection was carried out on CRISPR/Cas9 KO *Siao* and *Sllac* T1 lines previously generated which were subjected to a first round of PCR screening aimed at detecting the individuals in which the Cas9 transgene was eliminated by segregation. A PCR at the editing sites was then performed on the Cas9-negative plants. A subsequent HRFA analyses allowed the identification of insertion/deletion (indel) events at single base-pair (bp) resolution, and discriminated the homozygous mutant alleles.

Three independent mutants for *SIAO* gene – *Siao* KO1, KO2 and KO3 respectively and one for *SILAC* - *Sllac* – were ultimately isolated and characterized by Sanger sequencing (**Figure 3**).

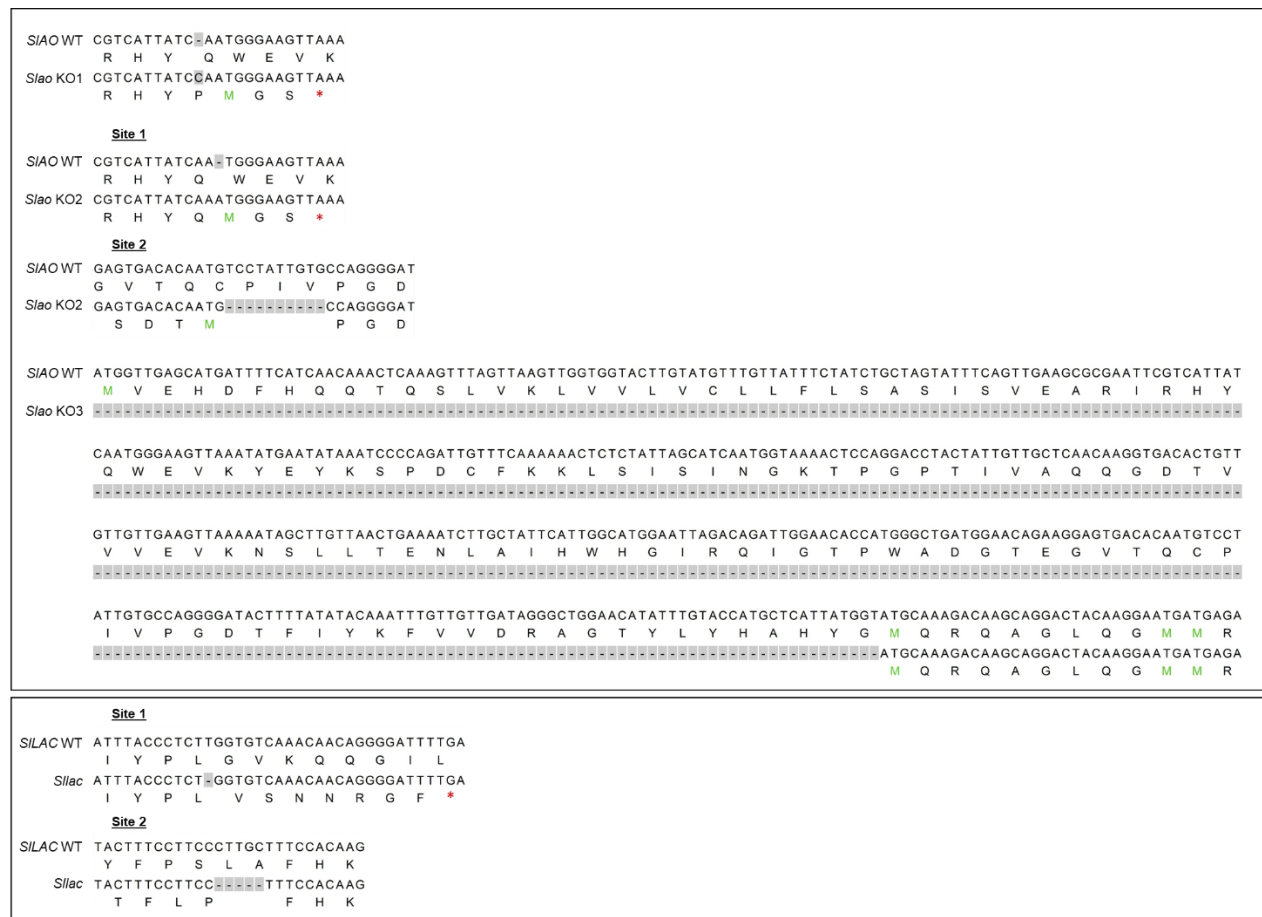


Figure 3 Results of the Sanger sequencing illustrated as the local alignment between the cDNA and amino acid sequences of Wild Type (WT) and CRISPR/Cas9 lines edited for the *SIAO* and the *SILAC* genes. Deleted nucleotides are shadowed in grey. The stop-codon position is indicated by the red asterisks.

Siao KO1 displayed the insertion of a cytosine residue in the exon 1. Exon 2 found to be a WT allele by HRFA was confirmed as not altered. Overall, the +C in the exon 1 induced a frameshift in the ORF, which lead to the formation of an early stop codon. For *Siao* KO2, the HRFA anticipated a single bp insertion on the exon 1 and a 10 bp deletion on the exon 2. Indeed, a +A and a -TCCTATTGTG were highlighted in the first and second exon, respectively, by sequencing. As for *Siao* KO1, the single bp insertion in the exon 1 provoked the abrupt formation of a UAA stop. However, the CDS appeared restored downstream the ten bp deletion, with a possible ORF predicted potentially initiated by an alternative ATG start. Nonetheless, a *de novo* transcription from alternative starts is not probable, and, would even so generate a truncated protein. Concerning the *Siao* KO3, this mutant exhibited a largest deletion of -437 bp, which caused the depletion of most of the exon 1 – including the ATG start, and of part of the intron 1. At protein level, this condition lead to the loss of the first 140 amino acids. However, also in this case, the downstream DNA and protein sequence remained in frame, and could be potentially transcribed and generate a truncated protein from an alternative ATG codon. *Sllac* edited gene was characterized by two deletions of one and five bp occurring at both the editing sites. These deletions consist in a -T in the exon 1 and a -CTTGC in the exon 3. The single bp deletion in the exon 1 caused a frameshift of the canonical ORF, which completely compromised the sequence introducing a precocious translational TGA stop.

The transcription could restart using an alternative ORF; however, its hypothetical translation would produce a mutated protein, with the first 159 amino acids being mis-sense, and overall lacking 75 bp from the original protein.

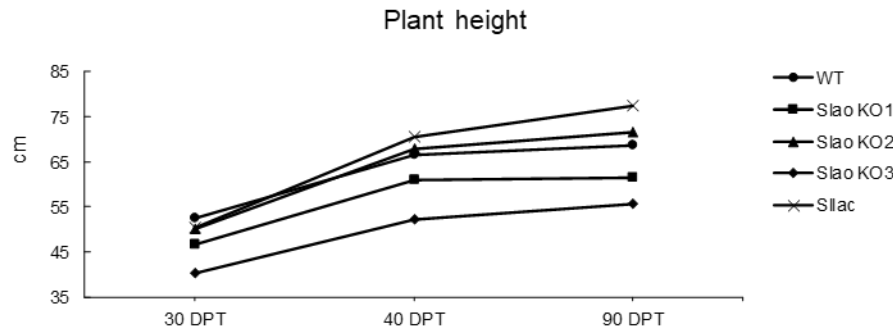
4.3.2 The depletion of *SIAO* and *SILAC* genes resulted in minor changes in plant morphology-, flower- and fruit-related traits

The *Slao* KOs and the *Sllac* T2 mutants were grown in the greenhouse together with the WT (**Figure 4**), and a comprehensive characterization, which included morphological-, flower- and fruit-related traits, was carried-out in order to evidence possible effects of the gene inactivation on the plant phenotype.



Figure 4 Image of the *SIAO* and *SILAC* T2 plants compared to the wild-type (WT) tomato, cultivar “Red Setter”. Scale bar, 10 cm.

Overall, eight traits were considered. Plant height was recorded at three different time points: 30 DPT, 40 DPT and 90 DPT (first fruits harvest). This parameter was not found altered in any of the mutants with the exception of the *Slao* KO3, which showed a reduced size throughout the entire cultivation period (**Figure 5**). The number of leaves, assessed at 30 DPT, remained unchanged compared to the non-edited control.



	30 DPT			40 DPT			90 DPT		
	Av.	SD	<i>p</i> -value	Av.	SD	<i>p</i> -value	Av.	SD	<i>p</i> -value
WT	52.50	6.22	-	66.50	8.02	-	68.75	12.07	-
<i>Slao</i> KO1	46.67	2.34	0.06	60.83	4.67	0.17	61.42	10.31	0.28
<i>Slao</i> KO2	50.17	3.54	0.44	67.83	4.17	0.73	71.50	5.43	0.62
<i>Slao</i> KO3	40.33	3.93	2.3E-03	52.33	6.06	0.01	55.58	7.98	0.05
<i>Silac</i>	50.33	6.62	0.57	70.50	8.02	0.41	77.50	11.36	0.23

Figure 5 Plant height trend at the three measurements times, 30 days post-transplant (DPT), 40 DPT, 90 DPT in the edited plants for *SIAO* and *SILAC* genes compared to the wild-type (WT). No differences emerged for *Slao* KO1 and 2 and *Silac*, while *Slao* KO3 displayed a reduced height over the entire cultivation period. The Student's T-test was applied as statistical test ($n = 6$). Avg, average; SD, standard deviation; *p*-value, statistical significance. Statistically significant *p*-value (threshold ≤ 0.05) are in italics and bold.

The flowering time was assessed at the first flower opening, and appeared delayed by 5 days in the *Silac*, whereas no variations were reported in the *Slao* genotypes (**Figure 6a**). The number of flowers was evaluated both separately *per* inflorescence (first, second and third inflorescence; **Table 2**) and as the total number of flowers in the three inflorescences (**Figure 6b**). When considered separately, no substantial differences emerged for the number of flowers in the first inflorescence, whereas, in the second inflorescence, the *Slao* KO3 had an average of 2.5 less flowers than the WT. On the third inflorescence, the *Silac* had an average of 1.67 more flowers compared to the WT. Nonetheless, the total number of flowers for the three inflorescences evidenced a substantial decrease in the flowers produced by the *Slao* KO3, with an average of 14.40 flowers compared to the 19.60 flowers of the WT.

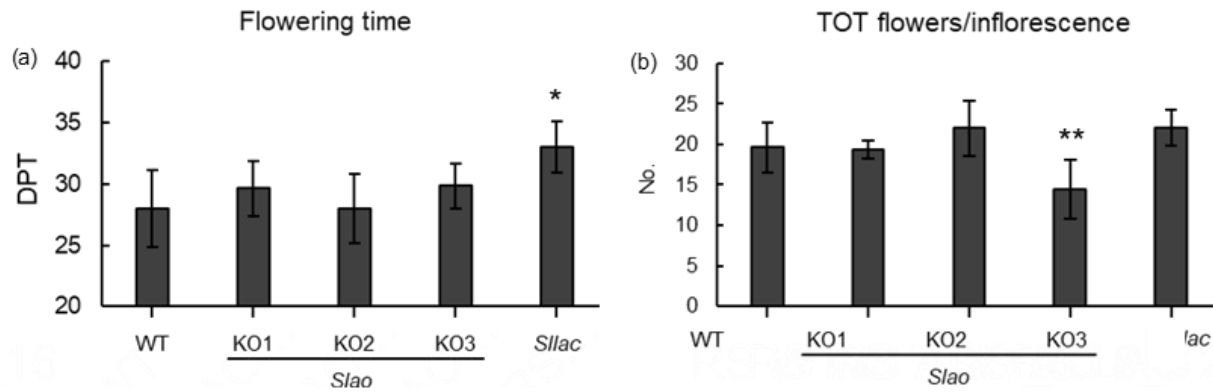


Figure 6 Flowering time (days post-transplant, DPT; a) and total number of flowers *per* inflorescence (b) in the edited plants for *SIAO* and *SILAC* genes compared to the wild-type (WT). *Sllac* showed a significative delay in the first flower opening, whereas *Slao* KO3 exhibited a reduction in total number of flowers on the three inflorescences. The Student's T-test was applied as statistical test (n = 6) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Table2 Number of flowers on the first, second and third inflorescence in the edited plants for *SIAO* and *SILAC* genes compared to the wild-type (WT). *Slao* KO3 showed reduced number of flowers on the second inflorescence, while *Sllac* displayed a slight, but significant, increase in the number of flowers on the third inflorescence. The Student's T-test was applied as statistical test (n = 6). Avg, average; SD, standard deviation; p -value, statistical significance. Statistically significant p -value (threshold ≤ 0.05) are in *italics* and **bold**.

	1 st inflorescence			2 nd inflorescence			3 rd inflorescence		
	Avg.	SD	p	Avg.	SD	p	Avg.	SD	p
WT	7.33	1.75	-	7.00	1.67	-	5.83	1.47	-
<i>Slao</i> KO1	6.33	1.21	0.28	6.83	1.47	0.86	6.33	0.82	0.48
<i>Slao</i> KO2	7.33	1.21	1.0	8.00	3.46	0.54	6.33	0.82	0.48
<i>Slao</i> KO3	5.50	1.64	0.09	4.50	1.05	0.01	4.60	1.67	0.23
<i>Sllac</i>	7.83	1.72	0.63	6.83	1.47	0.86	7.50	0.84	0.04

Slao KO3 showed 4.66 days delayed in the onset of fruit set, and produced less fruits *per* plant, which were further reduced in fruit weight (**Figure 7a, b, c**). With regard to the number of seed *per* fruit, this parameter resulted increased in the *Slao* KO1 and KO2, and non-affected in the other mutants (**Figure 7d**).

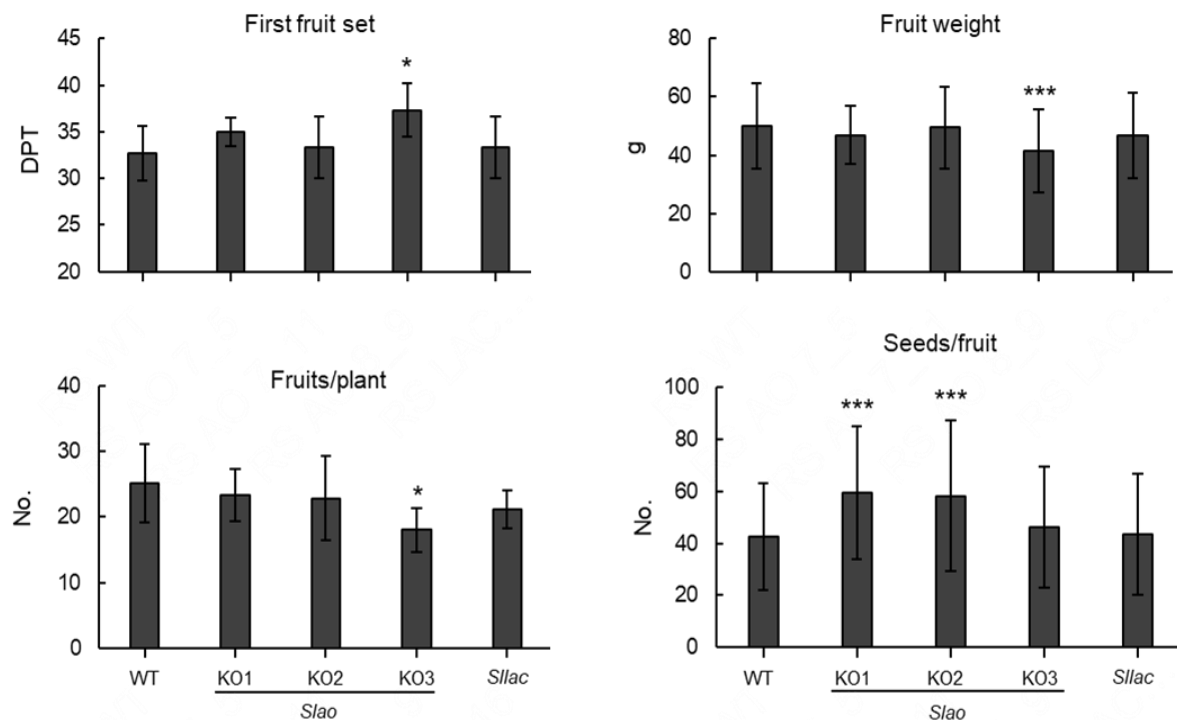


Figure 7 Fruit-related traits assessed in the edited plants for the *SIAO* and *SILAC* genes compared to the wild-type (WT). the traits include: fruit set (DPT; a), fruit weight (g; b), number of fruits *per* plant and number of seeds *per* fruit. *Slao* KO3 displayed a delay in the onset of fruit set and a reduced fruit weight and fruit number *per* plant, whereas *Slao* KO1 and KO2 showed and increased number of seed *per* fruit.

4.3.3 The *Slao* and *Silac* displayed reduced firmness and increased soluble solids in fruits and augmented reduced ascorbic acid in leaf, roots and fruits

Fruits from *Slao* and *Silac* were analysed for technological parameters as fruit firmness and SSC, that showed slight – although significant – variations. In all the mutant lines, the fruit firmness showed a minimal decrease (**Figure 8a**), whereas the SSC (°Brix) appeared substantially increased in *Slao* KO1 and KO2 and in *Silac* (**Figure 8b**).

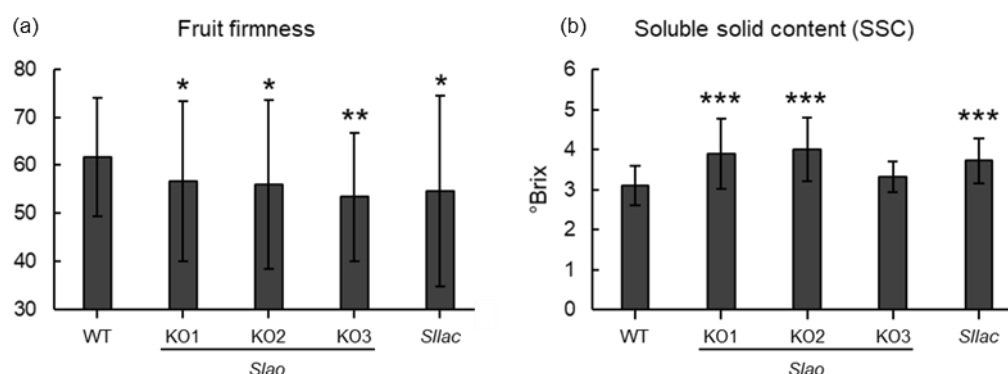


Figure 8 Fruit firmness (a) and soluble solid content (SSC; °Brix; b) in the edited plants for the *SIAO* and *SILAC* genes compared to the wild-type (WT). Fruit firmness overall decreased in all the mutant lines, whereas (°Brix) increased in *Slao* KO1 and KO2 and in *Silac*.

The most significant impact of the mutations in the genes *SIAO* and *SILAC* was on the content of reduced AsA in leaf, roots and fruits.

In leaves of the *Sllac*, the content of reduced AsA was augmented of 35.1 % with respect to the WT, while no statistically significant differences emerged in the *Slao* KOs (**Figure 9a**). In roots, the opposite trend was observed: the *Slao* mutants, in fact, accumulated up to the 80.3% more reduced AsA compared to the non-edited WT, whilst, in *Sllac* no appreciable changes were detectable (**Figure 9b**).

Fruits were analyzed at three different stages, MG, T and MR (**Figure 7c**). At the MG stage, *Sllac* CRISPR line was the sole in which the accumulation of reduced AsA increased (+26.2%). None of the edited genotypes showed variations at the T stage. In MR fruits all the KO lines accumulated more reduced AsA. Particularly, *Slao* KO3 and *Sllac* exhibited respectively 60.5% and 27.6% more reduced AsA compared to the WT.

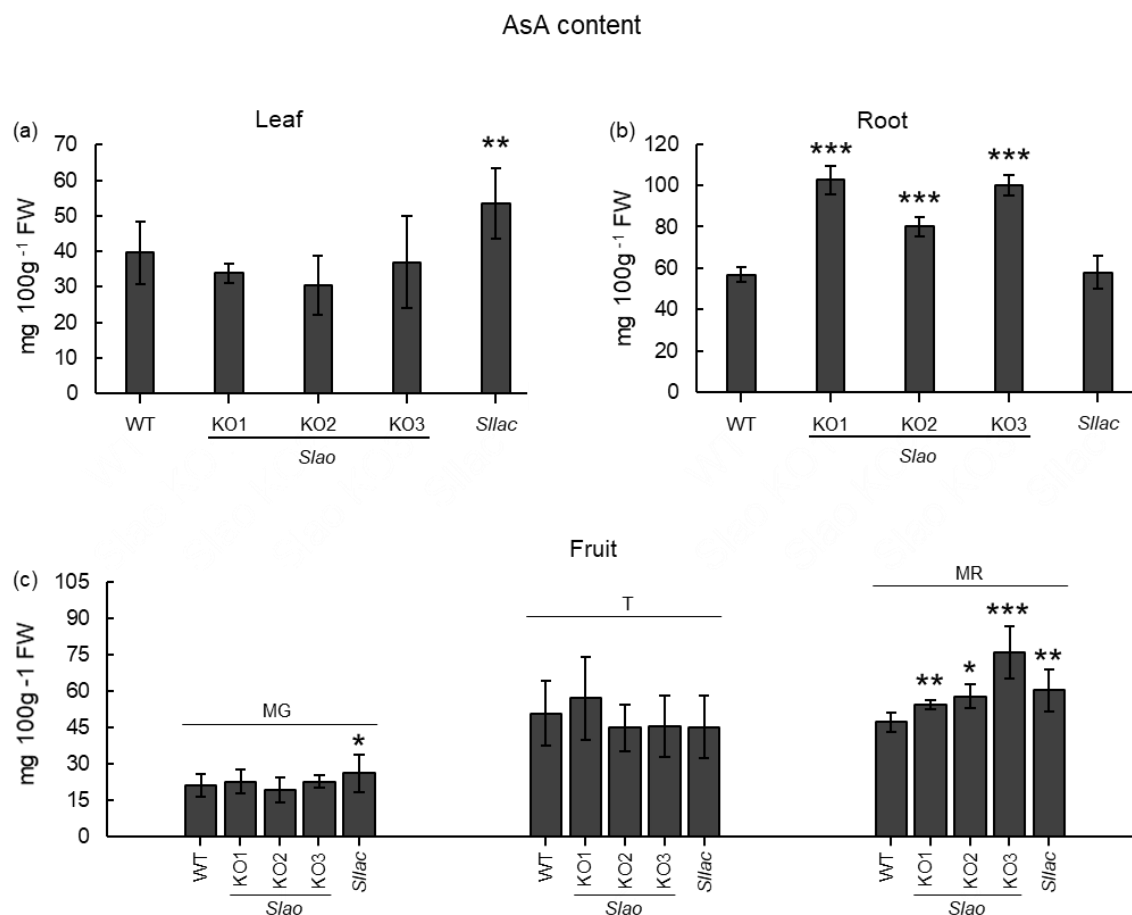


Figure 9 Reduced AsA content in leaf (mg 100g⁻¹ FW; a), root (b) and fruit (c) in the edited plants for the *SIAO* and *SILAC* genes compared to the wild-type (WT). Reduced AsA significantly increased in *Sllac* leaves with no variations in *Slao*. The opposite trend appeared in roots, that showed higher levels of reduced AsA in *Slao*, with no substantial changes in *Sllac*. In fruits, a slight increase in reduced AsA is appreciable in *Sllac* at the mature green (MG) stage. At the turning (T) stage, no significant variations were detectable. In the mature red (MR) fruits, reduced AsA is augmented in all the KO lines.

4.4 Discussion

Ascorbic acid is among the most potent antioxidants and a key metabolite involved in several physiological processes both in plants and animals. Being the most consumed vegetable worldwide, tomato represent an important source of AsA, although decades of breeding-for-yield has decreased its content particularly in the commercial varieties. Accordingly, exploring novel approaches to increase the tomato fruit content in this metabolite has become a priority in agricultural research. In this chapter it was described the production and the characterization of a panel of CRISPR/Cas9 inactivated lines for the *SIAO* and *SILAC* genes, respectively an established and a putative ascorbate oxidase active in the AsA recycling pathway, with relevant results concerning the AsA accumulation in leaves, roots and fruits.

4.4.1 The characterization of plant morphology-, flower- and fruit-related traits in the T2 genetically stable *Slao* and *Sllac* mutants evidenced mild alterations in the overall plant phenotype

T2 *SIAO* and *SILAC* KO lines Cas9-depleted were initially selected. These lines represent the first collection of edited lines for these genes in tomato. In total, three and one KO mutants were obtained for the genes *SIAO* and *SILAC* respectively, that originated from independent editing events and harbours different indels. These indels were characterized by sanger sequencing and their effects on the sequences were assessed through the CLC software that confirmed their deleterious impact on the CDS.

Potential effects on the plant phenotype induced by the depletion of these genes were systematically evaluated considering traits relevant in defining the overall plant development, fertility and fruit-productivity.

AO activity regulates ROS levels in the apoplast and intervene in the cleavage of cell-wall polysaccharides, thus modulating its extensibility during cell expansion. Furthermore, AsA stimulates cell division showing an effect on the G1 to S phases of mitosis. Therefore, both AsA and AO have important roles in finely tuning the overall plant growth, and serious alterations have been described in plants impaired in AsA biosynthesis and/or recycling (Dowdle et al., 2007; Truffault et al., 2016). However, here we haven't found relevant changes in plant height of most of the *Slao* and in the *Sllac*, in accordance to results previously obtained in the RNAi-AO tomato lines (Abdelgawad et al., 2019). The only exception was the *Slao* KO3, that showed a reduced size for the entire growth period. Impaired growth was similarly observed in some of the RNAi-AO lines generated in melon (Chatzopoulou et al., 2020). The number of leaves *per* plant was equally unaltered in all the KO lines, as also found by Abdelgawad et al., 2019 in tomato plants downregulated for the same *SIAO*. The progression in flowering was also assessed, monitoring the flowering time and the number of flowers *per* inflorescence. A slight delay (5 days) in flowering time resulted exclusively in the *Sllac*, as observed in tobacco and Arabidopsis plant expressing sense and antisense AO gene and in

Arabidopsis plants treated with exogenous AsA (Barth et al., 2006; Yamamoto et al., 2005). Regarding flower number *per* inflorescence, this parameter was not found to change in tomato plants in which *S/AO* was down-regulated and grown in standard conditions (Abdelgawad et al., 2019), and our data were largely consistent with this evidence. Nonetheless, the *Slao* KO3 had an average of 2.5 less flowers on the total number of flowers *per* inflorescence, while a small – but significant – increase in this trait emerged in the *S//ac*. This latter result was previously obtained in *Slapx*-edited lines (Do et al., 2022). The impact of the gene mutations on the fruits production was also evaluated. Fruit weight and number was comparable to those of the non-edited control in *Slao* KO1 and KO2 and *S//ac* lines, similarly to what was previously reported in RNAi-AO tomato lines (Abdelgawad et al., 2019; Garchery et al., 2013). The sole line affected for both these parameters was *Slao* KO3. It is possible that such deleterious phenotype could be mutation-specific. Indeed, in the same line alterations were demonstrated also for vegetative and flower-traits, and the in the onset of fruit set. It has also to be mentioned that melon fruit from genotypes in which the *AO* gene was down-regulated displayed important alterations in fruit development, with substantially smaller fruits. The yield of seeds was increased in the *Slao* KO1 and KO2 and in the *S//ac*. In Arabidopsis and tomato, the under expression of ascorbate oxidase led to an increase in seed yield under stress conditions (Garchery et al., 2013; Yamamoto et al., 2005).

4.4.2 *Slao* and *S//ac* CRISPR/Cas9 knockout induced variations in soluble solid content and firmness in mature red fruits

The manipulation of AsA in tomato fruits is often associated to variation in SSC and firmness, two important parameters for the technological quality of tomato fruits (Di Matteo et al., 2010; Osorio et al., 2013).

We have assessed both firmness and SSC in the mutants. *Slao* KO1 and KO2 and *S//ac* fruits were characterised by a general, although small, increase in SSC (°Brix), which is consistent with the literature and with results observed by Abdelgawad et al., 2019 in *S/AO*-down regulated lines. Firmness resulted also marginally altered, although with very small variations, being overall lower in *Slao* and *S//ac* edited lines. This result appeared counterintuitive since a decrease in *AO* activity usually should confer an increased firmness in tomato (Abdelgawad et al., 2019; Stevens et al., 2017). Nevertheless, there are exceptions, as in the case of *AO*-impaired melon lines, in which fruits showed a lower firmness at 40 days after pollination due to an earlier increase of ethylene production (Chatzopoulou et al., 2020). Furthermore, the *Slao* KO3 fruits, in which the reduction in firmness was more pronounced, also showed a reduced fruit weight and size and this could negatively affect the measurement itself.

4.4.3 Edited lines for the *SIAO* and *SILAC* genes accumulate reduced ascorbic acid in leaves, roots and fruits, suggesting *SILAC* as a novel ascorbate oxidase

To evaluate the effects of the inactivation of the *SLAO* and *SILAC* genes, reduced AsA was quantified in leaves, roots and fruits.

In leaves, reduced AsA substantially increased in *Sllac* plants, whereas no variations appeared in any of the *Slao* T2 mutants. It has to be noted that Plant eFP transcription profiles did not evidence particular levels of expressions for *SIAO* and *SILAC* in leaves; this latter gene, in fact, is mainly expressed in fruits, as described in D'Angelo et al., 2022 and Sacco et al., 2019.

In contrast, in roots, only *Slao* KO genotypes showed a higher content of reduced AsA, while in *Sllac* no changes were reported. The results obtained for *Slao* are consistent with the known profile of expression of this gene in tomato, that is known to be predominantly transcribed in roots (**Figure 2**; Garchery et al., 2013; Ioannidi et al., 2009; Stevens et al., 2017). Previously tomato lines impaired for *Slao* demonstrated also an improved tolerance to abiotic stresses – particularly drought and salinity stress (Abdelgawad et al., 2019; Garchery et al., 2013), an aspect that will have to be further analysed for the edited lines produced in this study.

In fruits, reduced AsA was quantified at three ripening stages, MG, T and MR. *Sllac* MG fruit had a slightly higher content in reduced AsA, that is consistent with the expression profile of the *Solyc07g052230* gene, that appeared strongly expressed at the early fruit stages up to the MG stage, as seen using Plant eFP and as described by D'Angelo et al., 2022. In contrast, MG fruits of the *Slao* lines did not showed variations in AsA accumulation compared to the WT. Also, no changes in the accumulation of this metabolite appeared at the T stage in the *Sllac*. Similar results were reported by Garchery et al., 2013, that demonstrated that AsA levels did not change in fruits at these ripening stages. The most relevant impact on the content of reduced AsA was reported in tomato at the MR stage, in which we observed a general increase in this metabolite in all the edited genotypes. Consistently, Abdelgawad et al., 2019 further showed an increase in AsA content in MR fruit in RNAi-AO downregulated lines.

4.5 Concluding remarks

Increasing AsA levels in tomato was extensively explored, and several studies based on transgenesis exists in which exogenous genes from AsA biosynthesis pathway were cloned and induced the accumulation of this metabolite. In this chapter the approach of the gene-KO mediated by the CRISPR/Cas9 technology was investigated as a possible strategy to increase AsA in tomato fruits by inactivating an established ascorbate oxidase – *SIAO* (*Solyc04g054690*) – and a putative ascorbate oxidase – *SILAC* (*Solyc07g052230*). These gene are, in fact, involved in the oxidation of AsA within the recycling pathway, and their depletion is likely to cause an increase in AsA concentration. In tomato, the RNAi-mediated silencing approach was similarly used to down-regulate the expression of the *SIAO* gene itself (Abdelgawad et al., 2019; Y. Zhang et al., 2011), and of other genes from the same pathway – viz. *SIAPX* (Do et al., 2022; Y. Y. Zhang et al., 2011).

Nonetheless, the genotypes herein generated represent the first available genetic materials produced by genome editing. Various morphological-, flower and fruit-related traits were assessed in the *Slao* and *Sllac* plants in order to evaluate the overall development. Among the different edited lines, only few phenotypic variations have been evidenced, which are mostly restricted to the sole *Slao* KO3 genotype. Differently from the *Slao* KO1 and KO2, this genotype was characterised by a larger deletion, that could differently impact the plant phenotype. Nonetheless, the impact of such alterations has a general low impact on relevant aspects (*i.e.*, fruit number, fruit weight, firmness in *Slao* lines). Notably, the content of reduced AsA increased in leaves, roots and fruits of the *Slao* and *Sllac* lines. The inactivation of the *SLAO* gene was confirmed to be a good strategy to improve AsA content in tomato fruits. A remarkable increase of reduced AsA in MR fruit was further obtained in the *Sllac* line, that exhibited slightly higher levels for this metabolite in MG fruits. This evidence therefore supports the hypothesis of *SILAC* being an ascorbate oxidase active in the AsA recycling pathway. Moreover, the accumulation pattern observed for *Slao* and *Sllac* appeared to be complementary, potentially suggesting a differential localization of the two ascorbate oxidases in the plant.

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General conclusions

The process of genetic improvement of tomato (*Solanum lycopersicum*, L.) started even before it reached the Old World, but it was there that it became systematic. The need of having a plant suitable for cultivation, together with the rapid growth of the population, have pushed the selective pressure towards increasing productivity. Nowadays, tomato is a widespread crop, being consumed worldwide. However, the huge success achieved from high-yield varieties had the main drawback of reducing the genetic variability for other traits that contribute to tomato fruit quality. In recent decades to increase tomato fruit nutritional quality notable results have been reached by breeding and, subsequently, by using systems based on the direct manipulation of DNA. One of the latest advancements in this latter technology is represented by genome editing mediated by the CRISPR/Cas9 system, an approach that allow to precisely intervene in the genome.

In this thesis various CRISPR/Cas9 mutants for different genes of the strigolactones (SLs) and ascorbic acid (ASA) pathways have been exploited in order to evaluate the effects of the mutation on tomato fruit nutritional quality and plant phenotype.

Strigolactones are phytohormones with roles in regulating plant development and interaction with the environment, being also the main chemical cue that stimulates the germination of the noxious parasitic weeds. Their biosynthesis occurs from the all-*trans*- β -carotene through the activity of four main genes, *SID27*, *SICCD7*, *SICCD8* and *SIMax1*. Diverse mutants for these genes exist in monocot and eudicot species, and are generally impaired in the vegetative development. In the **Chapter 2** is described the characterization of a panel of mutants previously obtained in the same tomato genetic background through the CRISPR/Cas9 technology for each of the abovementioned genes. All these lines were previously assessed as SL-deficient and parasitic-weed resistant. Several changes related to plant fertility and productivity were highlighted in the edited lines. Moreover, important gene-specific effects were evidenced. Indeed, less changes were seen in *Sld27* while a higher number of changes were demonstrated in the *SImax1* lines. Moreover, since SLs derives from carotenoids, and during their biosynthesis pathway generate apocarotenoids and volatile compounds (VOCs), these categories of metabolite were analysed in fruits. Surprisingly, the carotenoid composition did not show particular changes. Contrarily, VOCs, mainly from the phenylpropanoids (B), branched-chain amino acids (BCAA) and phenylalanine-derivatives (Phe) pathways, appeared to be altered. Also in this case the *Sld27* displayed minor changes, while *SImax1* was more largely affected. Although the impact on the global fruit aroma cannot be predicted from these data, the depletion of *SICCD7*, *SICCD8* and *SIMax1* resulted to overall decrease the accumulation of positive compounds relevant for tomato fruit quality. Nonetheless, *Sld27* was confirmed as a line with negligible variations, also for metabolites composition. The **Chapter 3**, thus, focused on the thorough investigation of the *SID27* gene, which was the only known β -carotene existing in plants, and of two additional *D27*-Likes genes (*D27*-Like 1 and -Like 2) recently described in rice and Arabidopsis. The *D27*-Likes gene have been previously postulated to act in redundancy

with the canonical *D27*, supplying localized amount of SLs in specific shoot areas. Furthermore, these genes were supposed to be active in modulating the SLs interaction with ABA. However, the knowledge in this sense is still scarce and contradictory. From the *in-silico* analysis of the *D27* family members in tomato, the *SID27*-Like 1 and *SID27*-Like 2 genes were identified. For these genes CRISPR/Cas9 inactivated-lines were generated and subjected to a functional characterization. Plant morphology, fertility and productivity were only marginally affected in the *Sld27* KO lines while no differences were highlighted in the *Sld27*-Likes KO lines. The stomatal conductance appeared reduced in the *Sld27* and *Sld27*-Likes lines, and this feature is consistent with the increased levels of ABA found in the leaves of the same lines. Also, ABA-related metabolites, 7-hydroxy-ABA (7-OH ABA) and ABA-glucose ester (ABA GE), showed to be altered in the mutated lines. The impact of these mutations on the secondary metabolite profile was analyzed in leaf. As for the *Slccds* and *Slmax1* mutants (**Chapter 2**), no substantial differences emerged for the non-polar compounds. Conversely, the profile of polar compounds displayed relevant changes, and polyphenols accumulation decreased significantly in the *Sld27* lines. Hence, it could be concluded that the *SID27* and *SID27*-Likes genes have a potential role in the SL-ABA crosstalk, and in the composition of leaf polar metabolites. Overall, these lines represent unique materials to further explore the role of the *SID27* and *SID27*-Likes in SL biosynthesis and SL-ABA interaction in tomato. In the **Chapter 4** the study of the AsA pathway was carried out, through the characterization of a set of CRISPR/Cas9 mutants inactivated for two ascorbate oxidases, the already characterised *Ascorbate Oxidase* (*SIAO*) and the putative *Laccase 22/L-ascorbate oxidase*. *Ascorbate Oxidases*, together with the *Ascorbate Peroxidases* (*APX*), are part of the recycling pathway of AsA. Particularly, they catalyse the oxidation of reduced AsA – being the active AsA form – into monodehydroascorbate (MDHA) and dehydroascorbate (DHA). Accordingly, the depletion of the AO genes should hamper AsA oxidation, consequently leading to the accumulation of reduced AsA. The CRISPR/Cas9 *Slao* and *Sllac* stable T2 mutants were here analysed. Currently, no CRISPR/Cas9 mutants for these genes were described so far, and therefore represent unique genetic materials. Consistently with literature, these plants only showed minimal alteration of the plant phenotype. Notably, mature red (MR) fruits from *Slao* and *Sllac* lines respectively accumulates up to 60.5% and 27.6% more AsA compared to the non-edited lines, hence confirming that the inactivation of ascorbate oxidases is as a good strategy to increase the content of this metabolite in tomato fruit. Reduced AsA have been found to further accumulate in leaves of *Sllac* and in roots of *Slao*. These profiles confirmed the *SIAO* as a root highly-expressed gene, and suggested for *SILAC* a leaf-specific expression.