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CRISPR/Cas9 for the generation of new tomato ideotypes with improved nutritional quality: a multi-omics characterization

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ABSTRACT

Il pomodoro (*Solanum lycopersicum*) è una delle colture orticole più importanti dal punto di vista economico e uno dei principali prodotti dell'industria agroalimentare. Nonostante il pomodoro sia una fonte di molti composti nutrizionali come carotenoidi, flavonoidi e vitamine, è anche una fonte di alcuni composti anti nutrizionali, tra cui glicoalcaloidi, tannini, folati e allergeni. In particolare, i glicoalcaloidi sono un gruppo di composti che forniscono protezione contro l'attacco di agenti patogeni, ma mostrano anche effetti tossici nell'uomo causando disturbi gastrointestinali e neurologici. Un altro aspetto molto importante del consumo di pomodori è la loro allergenicità che riguarda circa il 16% della popolazione italiana.

Lo scopo di questa tesi di dottorato è stato quello di eliminare o ridurre alcune di queste molecole anti nutrizionali e allergeniche utilizzando la nuova tecnologia di breeding "genome editing" e in particolare il sistema CRISPR/Cas9. Nel nostro approccio, sono stati disegnati due gRNA specifici per i geni di interesse, *GAME 4* e *Sola l 4*. Abbiamo scelto il gene *GAME 4* in quanto si trova in un punto cruciale della biosintesi dei glicoalcaloidi (SGAs); più in dettaglio, si trova in un punto di ramificazione della via che porta, da un lato, alla produzione di SGAs e, dall'altro, alla sintesi di una classe di molecole ad alto valore nutraceutico, le saponine. Quindi, intervenendo a livello di questo enzima, è possibile diminuire la concentrazione di sostanze anti nutrizionali, con un contemporaneo aumento di quelle pro-nutrizionali. Per quanto riguarda la scelta degli allergeni, abbiamo selezionato *Sola l 4* perché è uno dei più caratterizzati nel pomodoro, oltre ad avere una maggiore allergenicità nei soggetti con allergie alimentari. La prima parte di questo lavoro si è concentrata sulla caratterizzazione molecolare delle piante editate e sull'applicazione di un approccio multi-omico (proteomica, metabolomica e volatilomica) (**Capitolo 2**) che ci ha permesso di comprendere gli effetti derivati dal "knock-out" dei due geni in studio sulla pianta. Analizzando gli estratti proteici di pomodoro attraverso l'analisi Western blot, non si è osservato alcun segnale in entrambe le linee modificate, confermando l'assenza della proteina nella sua conformazione nativa. A livello metabolomico, è stata effettuata un'analisi semi-polare e non-polare osservando una fortissima riduzione degli SGAs totali, superiore al 99% nelle foglie e nei frutti. Parallelamente è stato riscontrato un marcato aumento di alcune saponine, come ad esempio la tigonina. Inoltre, è stato osservato un aumento significativo del contenuto di zuccheri nelle linee modificate. È interessante notare che, analizzando i dati proteomici nelle foglie, tra le proteine up-regolate ci sono numerose proteine coinvolte nei meccanismi di difesa contro gli stress biotici, come diverse perossidasi, chitinasi e proteine PR. L'analisi dei dati proteomici nei frutti di pomodoro ha rivelato diverse proteine coinvolte nel metabolismo dei carboidrati, in accordo con i

dati metabolomici. Nelle linee editate è stato osservato un aumento del gruppo degli aminoacidi a catena ramificata (BCAA VOCs). In questo gruppo, il composto che si accumula nel frutto in maniera maggiore è stato il 3-metilbutanale sia nello stadio maturo verde che rosso. I nostri risultati suggeriscono che, come conseguenza dell'editing genetico dei due target, è stata ottenuta una rimodulazione del metabolismo secondario nelle piante di pomodoro modificate.

Un secondo obiettivo di questo studio è stato quello di confrontare le comunità batteriche nella rizosfera di pomodori non trasformati e editati per i geni *GAME 4* e *Sola l 4* per valutare se le alterazioni del genotipo potessero riflettersi in una diversa distribuzione della comunità microbica. A tal fine, nel **Capitolo 3** è stata studiata la comunità batterica del suolo mediante metabarcoding del gene batterico 16S rRNA. Inoltre, è stata effettuata un'analisi metabolica delle radici e, come previsto, gli effetti dell'editing genico sul metabolismo secondario osservati in frutto e foglia, sono stati confermati anche nelle radici, con una riduzione del contenuto totale di SGA superiore al 98,0% nelle due linee analizzate. In aggiunta è stato ottenuto un notevole accumulo di saponine nelle radici. L'analisi delle coordinate principali, PCoA, associata ai risultati del PERMANOVA test, ha mostrato una marcata separazione tra le linee wild-type (WT) e quelle editate. Di conseguenza, i nostri risultati confermano il ruolo dei SGAs e delle saponine nella modulazione della comunità batterica del suolo.

Considerando che è possibile applicare la tecnologia CRISPR/Cas 9 non solo per eliminare i composti anti nutrizionali, ma anche per aumentare i composti nutrizionali e, di conseguenza, la qualità del frutto, nel **Capitolo 4** di questa tesi di dottorato è stata effettuata la caratterizzazione metabolica di mutanti "*greenflesh"* di pomodoro in collaborazione con l'Istituto di Biologia Molecolare e Cellulare delle Piante (IBMCP, laboratorio del Professor Antonio Granell). Nello specifico è stato osservato un accumulo di preziosi composti secondari che promuovono la salute, in particolare un aumento dei livelli di carotenoidi e un accumulo significativo di vitamina E.

Poiché la tecnica del "genome editing" è in grado di generare mutazioni precise all'interno del genoma, paragonabili a quelle che si verificano naturalmente o a quelle ottenute con la mutagenesi classica, le nuove varietà vegetali del futuro potrebbero essere prodotte con questa metodica.

ABSTRACT

Tomato (*Solanum lycopersicum*) is one of the most economically important vegetable crops and one of the major products of the agri-food industry. Despite tomato is a font of many nutritional compounds like carotenoids, flavonoids and vitamins, it is also a source of some antinutritional compounds including glycoalkaloids, tannins, folates and allergens. In particular glycoalkaloids are a group of compounds that provide protection against pathogens attack but also show toxic effects in humans causing gastrointestinal and neurological disorders. Another very important aspect regarding the consumption of tomatoes is their allergenicity which affects about 16% of the Italian population.

The purpose of this phD thesis has been to eliminate or decrease, some of these antinutritional and allergenic molecules using the new breeding technology "genome editing" and in particular using the CRISPR/Cas9 system. In our approach, two specific gRNAs for genes of interest, *GAME 4* and *Sola l 4* were designed. We chose *GAME 4* gene, as it is located at a crucial point in the biosynthesis of glycoalkaloids (SGAs); more in detail, it is placed at a branching point in the pathway leading, on one hand, to the production of SGAs and, on the other, to the synthesis of a class of molecules with high nutraceutical value named saponins. Thus, by intervening precisely at this enzymatic step, it is potentially possible to decrease the concentration of anti-nutritional substances, with a simultaneous increase in pro-nutritional ones. Concerning the choice of allergens, we selected *Sola l 4* because it is one of the most characterized in tomatoes, as well as having a greater allergenicity in people with food allergies. The first part of this work focused on the molecular characterization of edited plant and applying a multi -omics approach (proteomics metabolomics, and volatilomics) (**Chapter 2**) that allow us to understand the effects derived by the knock-out of the two genes under study on plant metabolism. In fact, as a result of knock-out of *Sola l 4* gene, analyzing tomato extracts through western blot analysis, no signal was observed in both edited lines, confirming the absence of the protein in its native conformation. At metabolomic level, a semi-polar and non-polar targeted characterization was carried out and as expected, a very strong reduction of total SGAs was achieved, greater than 99% in leaves and fruits. At the same time, a marked increase of some saponins, such as tigonin, was found. Moreover, a significant increase of the sugar content in edited lines was observed. Interestingly, analyzing proteomic data in leaves, we observed that among the up-regulated proteins there are numerous proteins involved in defense mechanisms against biotic stresses, such as several peroxidases, chitinases and PR-proteins. Furthermore, analysis of proteomic data in tomato fruits revealed several proteins involved in carbohydrate metabolism, according with the metabolomic

data. In addition, in edited lines, an increase in branched-chain amino acids group (BCAA VOCs) was observed. In this group the most up-regulated compound was 3-methylbutanal for both MG and RR stage.

Our findings suggest that as a result of genome editing engineering in glycoalkaloids and allergens content, a remodulation of secondary metabolism was observed in tomato edited plants.

A second aim of this study was to compare the bacterial communities in the rhizosphere of WT and GAME 4/Sola l 4 knock-out tomatoes in order to assess if the alterations on the genotype and therefore in the content of certain molecules could be reflected in a different distribution at microbial composition and biodiversity. In the **Chapter 3**, the soil bacterial community has been investigated by metabarcoding of the 16S rRNA bacterial gene both in WT and in edited plants to evaluate the potential impact on microbial diversity and structure. Moreover, a metabolic analysis of roots was performed and, as expected, the same effects of gene editing observed on the secondary metabolism in fruits and leaves were also confirmed in roots, in which a reduction in total SGAs content over 98.0% was achieved in the two analyzed lines. In addition, a great accumulation of saponins in the roots was obtained. A principal coordinate analysis, PCoA, associated with PERMANOVA test, reveals a marked separation between WT and edited lines that clustered together. Consequently, our findings confirm the role of SGAs and saponins in changing the soil bacterial community.

Considering that we can apply CRISPR/Casa9 technology not only to eliminate antinutritional compounds but also to increase nutritional compounds and as a consequence fruit quality, the final research of this thesis was focused on metabolic characterization of *greenflesh* tomato mutants (**Chapter 4**) generate in collaboration with the "Istituto de Biologia Molecular y Celular de Plantas" (IBMCP, laboratory of Professor Antonio Granell). Specifically, an accumulation of valuable health-promoting secondary compounds was observed, in particular an increase in carotenoid levels and a significant accumulation of vitamin E.

Since genome editing technique is able to generate precise mutations within the genome, comparable to those that occur naturally or to those obtained with classical mutagenesis, the novel varieties could be efficiently generated in the future by genome-edited crops.

Chapter 1. General introduction

1.1 Tomato fruits quality

Tomato (*Solanum lycopersicum)* is one of the most economically important vegetable crops. Moreover, it has interesting physiological and botanical features, such as fleshy fruits, compound leaves and sympodial shoot branching. The cultivated tomato is also a diploid species, self-pollinating, easy to maintain, with efficient sexual hybridization, a short generation time and efficient genetic transformation protocols (Kimura & Sinha, 2008). For all these reasons, tomato is considered a model species for both basic and applied plant research, plant breeding and genetics studies.

In particular, tomato is a climacteric fruit, so when it begins to ripen, there is an increase in respiration and ethylene production (S. Li et al., 2019). The ripening process begins approximately two days after the mature green (MG) stage, when a metabolic reorganization, characterized by the boost of ABA synthesis, occurs (Zhang et al., 2009). The accumulation of carotenoids, mostly lycopene and β-carotene, and the simultaneous degradation of chlorophylls, which is accompanied by the conversion of chloroplasts into chromoplasts, leads to change of fruit color from green to yellow-orange, typical of breaker stage (Br) (Carrari, 2006). Subsequently, an increase of rate respiration and peak production of ethylene occurs, with a consequent acceleration of all ripening processes such as cell wall degradation promoting tissue softening, accumulation of simple sugars, acids and emission of volatile compounds (Matas et al., 2009). The final red ripe (RR) stage is characterized by a mature fruit that shows reduced transcriptional activity and ethylene production. Depending on the different cultivars, it occurs between the seventh and tenth day after the breaker. The fruit has completed the accumulation of primary and secondary metabolites: glucose and fructose are the two main sugars in a ripe fruit; instead, malic and citric acids are the most abundant organic acids (Klee & Giovannoni, 2011).

Over the years, improving the quality and nutritional characteristics of tomato fruits has become an important focus in plant breeding. During domestication, ancient and modern breeders have always dealt with traits such as efficiency, productivity and biotic and abiotic stresses; more recently and at the request of consumers, classic and advanced breeding have been started to focus also on other traits related to berry ripening and quality such as, for the latter, the improvement of nutraceutical compounds, color (see below), flavor and aroma (Gascuel et al., 2017). Some of these, e.g., color, flavor, aroma and texture change during fruit ripening process, thus promoting their consumption.

The color of fruit depends on its content of carotenoid and chlorophyll pigments: it can be green, red, orange, yellow, brown or pink. In green berries, chlorophylls a and b prevail, while those that

accumulate carotenoids, predominantly lycopene and β-carotene, are characterized by red and orange color, respectively. In tomato, several natural and induced mutants affected in color are available. Several mutations occur in genes involved in color formation, as in the case of the carotenoid and anthocyanin biosynthetic pathway. For example, it is shown that a truncated non-functional carotene isomerase (*CrtISO*), found in tomato *tangerine* mutants, leads to the production of berries with orange flesh due to accumulation of prolycopene (7,9,7′,9′tetra-cis lycopene) instead of all-trans lycopene which, is predominantly accumulated in red fruits (Isaacson et al., 2002; Yoo et al., 2017). On the other hand, another orange colored fruit, *Beta* mutants, was obtained in the case of an up-regulation in the chromoplast-specific lycopene β-cyclase (*SlCYC-B*) gene, an enzyme that catalyzes the conversion of lycopene to β-carotene (Pecker et al., 1996; Ronen et al., 2000). Lycopene can also be converted to δ-carotene by the enzyme lycopene e cyclase (*LCY-E*). The transcriptional up-regulation of this gene was observed in the so called *Delta* mutant, in which was found a sequence change in the upstream promoter region, leading to the accumulation of δ-carotene and lutein (Ronen et al., 1999). Interestingly, it has been shown that the prolycopene accumulated in *tangerine* tomatoes showed a higher bioavailability (~8.5 times) in humans than the all-trans lycopene (Cooperstone et al., 2015), while an important role of lutein as macular pigment has been assessed (Evans et al., 2013).

Looking at other types of mutations outside the carotenoid pathways, pink tomatoes differ from red ones only in the color of the epicarp: in fact, inside the fruit the color remains unchanged; it has been shown that this difference is due to a mutation in a gene involved in the biosynthesis of flavonoids, which accumulate preferentially in berry skin (Ballester et al., 2009). An interesting case is the one of the purple tomatoes, reviewed in (Gonzali & Perata, 2020), which accumulate high amounts in anthocyanins, mainly due to the presence, in the homozygous status, of the Anthocyanin fruit (*Aft*) and atroviolacea (*atv*) alleles, coding for MYB transcription factors triggering the synthesis of these valuable compounds, usually not detectable in tomato fruits and, for which, a series of positive effects on human health have been associated (Gonzali & Perata, 2020).

Finally, in the case of *gf* tomato mutants, ripe fruits developed a characteristic brown color due to the simultaneous presence of chloroplasts and chromoplasts/plastoglobules, with the concomitant retainment of chlorophylls as consequence of the depletion in a Mg-dechelatase stay-green protein, which plays a fundamental role in chlorophyll degradation (Barry et al., 2008). Of interest, it has been reported that chlorophylls may exert several beneficial properties for animals, including photoprotection, red blood cells boosting, as antioxidant and inflammatory agent and, more

recently, it has also been proposed a positive effect on Sars-Cov 2 infection by a chlorophyll derivative product (Clark & Taylor-Robinson, 2020).

1.2 Nutritional values of tomato fruits

Tomato is one of the most widely consumed vegetables worldwide because of its high nutritional value and delicious taste and flavor. However, these sensorial traits of tomato berries are gradually diminishing in time, mainly due to domestication and agronomical processes albeit they are highly requested by consumers. The molecules mainly involved in these properties are sugars, organic acids, amino acids and volatile compounds (Cheng et al., 2020), which are important target of classic and advanced breeding efforts.

About the nutritional values, the composition and ratios of the tomato's macro- and micronutrients are strongly influenced by different kind of factors such as cultivar type, genotype, agronomic practices, climatic conditions and soil characteristics (García-Valverde et al., 2013; Verheul et al., 2015).

Considering only the macronutrients, tomato would not have a high nutritional value. In fact, for 100 grams of fresh fruit, there is an average of 94% water, 3% simple sugars (glucose and fructose), 2% fibers, 1% protein and 0.2% fat. Nevertheless, the attention of many researchers, engaged in studies of the correlation between food and human health, has for many years focused on the minor constituents that lack nutritional value, but have beneficial effects on our health (Singh et al., 2016) as antioxidants (Table 1). Moreover, many consumers are increasingly interested in functional foods because of their beneficial and protective properties for human health. In this context, it would seem that daily consumption of tomatoes may reduce the occurrence of numerous diseases such as certain types of cancer, cardiovascular diseases, diabetes and obesity (Petruk et al., 2016; Singh et al., 2018). Indeed, tomato fruits are an important source of numerous healthy compounds, mainly secondary metabolites, with therapeutic roles including vitamins, minerals and antioxidants (Chaudhary et al., 2018). These biologically active molecules are both lipophilic (carotenoids, vitamin E and K) and hydrophilic (ascorbic acid, polyphenols and folates), which give the fruit a high antioxidant activity (Martí et al., 2016). Among the lipophilic components, there are carotenoids, tetraterpenes involved in the photosynthetic system, that can be divided into provitamin A carotenoids (α-carotene, β-carotene and β-cryptoxanthin) and non-provitamin A carotenoids (lycopene and lutein) (Raiola et al., 2014). Lycopene, a polyunsaturated compound which confers the characteristic red color to tomato berry, is synthesized during fruit ripening and it becomes the main carotenoid present in red ripe fruit. It can exist both in *trans*- and

Table 1: Nutrient, vitamin and carotenoid content of 100 mg of raw tomato fruit. Data source: USDA (https://www.usda.gov/).

Nutrient	Value/100g	Vitamin	Value/100g	Carotenoid	Value/100g
Energy	18 kcal	Vitamin C	13.7 mg	α-carotene	101 µg
Protein	0.88 g	Thiamin	0.037 mg	β-carotene	449 µg
Total lipid	0.2 g	Riboflavin	0.019 mg	Lycopene	2,573 mg
Carbohydrate	3.89 g	Niacin	0.594 mg	Lutein + zeaxanthin	123 µg
Sugars	2.63 g	Vitamin B-6	0.08 mg		
Fiber	1.2 g	Folate	15 µg		
		Vitamin A	42 µg		
		Vitamin E	0.54 mg		
		Vitamin K	2.7 µg		

cis- configurations. In the raw fresh fruit, the *trans*- form is predominant, which is less bioactive and poorly absorbed by the human body.

During thermal treatments or in presence of light, acids, oxygen, the isomerization of lycopene is induced and the molecule is converted into the more bioactive *cis*-forms (Boileau et al., 2002). Due to the high number of conjugated double bonds, lycopene has a strong antioxidative role. Thus, its consumption would look to reduce the risk of prostate cancer and cardiovascular diseases (Saini et al., 2015). Other carotenoids present in ripe tomatoes include β-carotene and lutein, both involved in the prevention of eye health and, depending on the cultivar, of small amounts of phytoene, phytofluene, δ-carotene, ζ-carotene and neurosporene (Martí et al., 2016).

Another class of liposoluble antioxidants are the vitamin E family. In particular, eight molecules belong to this group and, based on the nature of the isoprenoid chain, we distinguish tocopherols (with a phytyl chain), usually most abundant, from tocotrienol (with a geranylgeranyl chain). Even for vitamin E, it has been observed that an increase in the intake of this phytochemical would decrease the risk of prostate cancer (Kirsh et al., 2006).

The main form of vitamin K found in food is vitamin K1 or phylloquinone. Since it is exclusively produced by plants, algae and cyanobacteria, humans are unable to synthesize it. So, they must achieve vitamin K in a rich plant-based diet as an essential micronutrient. (Oostende et al., 2008). However, tomato berries are typically low in phylloquinone (2.7 µg/100 g), whereas it increases in processed tomatoes reaching the highest value (9.9 µg phylloquinone/100 g) in tomato paste (Damon et al., 2005). Biologically vitamin K1, a conjugated isoprenoid composed of a C-20 phytyl side chain bonded with a naphthoquinone ring, plays a role as a coenzyme of several proteins

involved in blood coagulation, cardiovascular health, and osteoporosis prevention (Vermeer et al., 2004).

Moving on to hydrophilic compounds, we find vitamin C, an essential molecule for humans who cannot synthesize it. In fact, its deficiency can cause scurvy, a disease characterized by dry skin, fatigue and depression (Naidu, 2003). Humans must therefore supplement vitamin C through the diet, in the form of L-ascorbic acid and dehydroascorbic acid. This molecule is easily assimilated by the body, but is not stored. Notably, vitamin C present in tomatoes is very bioavailable, but its concentration in the fruit decreases during ripening. Due to its high electron-donating capacity, ascorbic acid is a powerful antioxidant: it exhibits, indeed, protective activity for cells against DNA damage induced by oxidant species, and it seems especially to have a synergistic effect with lycopene (Riso et al., 2004).

In the tomato, we also find polyphenols, a large family characterized by the presence of phenol units, which play the role of free radical scavengers and are associated with the prevention of inflammatory diseases. Their content in the tomato berry, mainly located in the skin and with naringenin chalcone and rutin as main representatives, is influenced by the spectral quality of solar UV radiation (Luthria et al., 2006). Among these, the most abundant are the flavonoids that give the berry its color. About phenolic acids, which confers astringent taste to the fruit, they are divided into hydroxybenzoic and hydroxycinnamic acids and the most abundant compounds are caffeic acid, ferulic acid and chlorogenic acid (Perea-Domínguez et al., 2018).

Tomatoes are also a source of small amounts of folates (vitamin B), which are essential in fetal growth.

1.3 Antinutrients content in tomato fruit

In addition to beneficial molecules, plant-based foods can also contain anti-nutritional substances, some of which may limit nutrient absorption, while others may cause health problems. Overall, in tomato, two groups of antinutrients can be identified and classified according their contents and/or significance: a major one, in which glycoalkaloids and allergens (from this point they will be referred to as SGAs and ALs, respectively) are founds; and a minor one, composed by oxalates, phytates, tannins and lectins. While SGAs and ALs will be examined in more detail in the next sections, minor antinutritional components will be briefly discussed as follows.

Oxalate, for instance, is an antinutritional compound that can form insoluble salts with minerals and can be found in several plant-based foods as soybean, spinaches and, more in general, leafy greens and potato (Noonan & Savage, 1999). In plants, we distinguish between two types of oxalates: those that are water-soluble (oxalic acid, sodium, potassium and ammonium oxalates)

and those that are insoluble (mainly calcium oxalates) (Petroski & Minich, 2020). They are defined as anti-nutritional because of their chelating action against certain minerals, thus reducing their absorption, as well as can contribute to the formation of kidney stones (Holmes et al., 2001). In tomato, a content between 5 and 35 mg/100g FW was observed (Noonan & Savage, 1999).

Phytic acid, or phytate, is another compound usually listed as an anti-nutrient in the plant field. In tomatoes, it is present albeit in low concentrations, around 3.71 (mmol/kg) (Wei & Gregory, 1998). At structural level, phytic acid is composed of an inositol ring to which are bound six phosphate groups that act as strong chelators by binding to numerous mineral cations such as Cu^{2+}, Ca^{2+}, Zn^{2+} and Fe^{3+} (Castro-Alba et al., 2019). These complexes are insoluble at neutral pH values and cannot be digested by human enzymes, reducing their bioavailability (Mihalache et al., 2020).

In addition to oxalates and phytates, tannins, belonging to the class of polyphenols and found in many plant-based foods, have also been included in the list of antinutrients. However, differently from the others, it has to be mentioned that tannins can also have a role as beneficial compounds, either in terms of sensorial properties (e.g. they confer an astringent taste, and in tomato fruit they are involved in determining the color and flavor) as well as pro-nutritional molecules since some members have strong antioxidant activities (Ojo, 2022). They accumulate during ripening of tomato berries and reach their highest concentration in the outer wall of the pericarp in the red fruit (about 0.025 nmol/g DW) (Bialczyk & Lechowski, 2000). Similarly to the previous compounds, they also act as chelators of minerals such as iron, zinc and copper, thus inhibiting their absorption (Karamać, 2009). In fact, some studies show that they may be responsible for iron deficiency anemia, especially in developing countries (Petry et al., 2010).

A last class of minor antinutritional compounds is lectins. Indeed, they are also known as hemagglutinins, and act as carbohydrate-binding proteins with the ability to reversibly bind to specific sugar moieties. Tomato lectin is a glycoprotein composed of equal amounts of carbohydrate (mainly arabinose and galactose) and protein, and its amino acid composition is similar to the potato lectin with hydroxyproline, cysteine, serine and glycine as most abundant amino acids (Oguri et al., 2008); interestingly, it accumulates mainly in the locular fluid and have a higher concentration in the tomato red fruit (~ 2 mg/kg) (Peumans et al., 2003), but doesn't belong to the toxic one, although it may also affect the immune system. On the contrary, potato lectin has been reported to potentially increase the severity of the clinical symptoms of non-allergic food hypersensitivity in atopic subjects (Preedy & Watson, 2008).

1.3.1 Glycoalkaloids

Steroidal alkaloids (SAs) are biologically active secondary metabolites, found in various plant,

containing a nitrogen atom. Their structure (aglycone) can be modified by the addition of glycosidic molecules to C-3β to form SGAs. SGAs are produced mainly by plants belonging to the *Solanaceae* family, in particular, the genus *Solanum*, and sporadically in *Liliaceae* (Cárdenas et al., 2016). Solanum SGAs share a common steroid skeleton with nitrogen incorporated into the F ring and are accumulated in leaves, roots, tubers, shoots and fruits of plants (Milner et al., 2011). More detailed, tomato glycoalkaloids possess the spirosolanes aglycone structure and their content is influenced by some environmental factors such as temperature, light exposure and by cultivar type and time of harvest (Koh et al., 2013; Zhao et al., 2021).

To date, more than 100 SGAs have been identified in tomatoes. Among these, α -tomatine is the one most commonly found in green leaves and fruits. The high tomatine content is associated with the bitter taste of the green tomato, making it unappetizing. In green fruit, the α-tomatine content is high (500 mg/kg) compared to ripe red fruit (5 mg/kg) (Friedman, 2013). This is because during ripening, α-tomatine is converted into less bitter compounds such as esculeosides A and B through hydroxylation, acetylation and glycosylation reactions (Cárdenas et al., 2019; Itkin et al., 2011, 2013). This event is not occasional, since the "low-SGA" trait has been largely investigated by the breeders to be achieved in cultivated tomato; indeed, varieties grown and commercialized nowadays, and obtained over the years through the process of domestication, are characterized by a low glycoalkaloid content, and less bitter and more palatable fruits. However, a variety of high tomatine bitter-tasting cherry tomato (*S. lycopersicum* var. *cerasiforme*) landraces has been found in Peru, in which they have shown that the bitter trait is monogenic and recessive (Rick et al., 1994). It has recently been discovered that, in these varieties, there is a large deletion in chromosome 3 that falls within the gene coding for a nitrate/peptide family transporter (NPF), called GORKY (Kazachkova et al., 2021). These transporter usually exports α-tomatine and its derivatives from its storage site, the vacuole, to the cytosol where it becomes a substrate for the enzymatic activities listed above, facilitating its conversion into non-bitter forms (Kazachkova et al., 2021).

Although SGAs play a key role in plant's resistance against fungi, bacteria, viruses and insects (Friedman & Levin, 2016; Rudolf & Rudolf, 2016), some of them are considered anti-nutritional compounds for humans due to their toxic effect (Friedman et al., 2009; Milner et al., 2011). The most abundant are α-tomatine and dehydrotomatine and their negative properties are mainly due to their action against bovine and human acetylcholinesterase (AChE), although they are less effective than the glycoalkaloids found in potato (Sucha & Tomsik, 2016). Furthermore, it has been shown that these compounds can bind to cholesterol and other hydroxysteroids, such as cholestanol and lanosterol, damaging the integrity of the cell membrane (Silva-Beltrán et al., 2015)

to the point of causing gastrointestinal and neurological disorders (da Silva et al., 2017; Iablokov et al., 2010). Similarly, it has also been reported that α-tomatine would lead to increased permeability of small intestinal mucosa cells by inhibiting active nutrient transport, which may then be associated with some forms of food allergy. In this context, SGAs may therefore be a factor in food hypersensitivity (Gee et al., 1996). For all these reasons, high levels of SGAs can be harmful to humans and, therefore, undesirable in food products.

Understanding the biosynthetic pathway of SGAs in tomatoes is very important in order to understand the genes involved in the synthesis of α-tomatine, and thus to be able to possibly alter their expression through genetic engineering mechanisms, in order to obtain a more nutritionally valuable tomato fruit. Their biosynthesis is derived from the mevalonic acid (MVA) pathway, an anabolic pathway that, starting from acetyl-coenzyme A (Figure 1), leads to the formation of numerous essential organic compounds, such as such as phytosterols and terpenoids (Sonawane et al., 2017). The starting point of local SGAs metabolism has been identified in cholesterol, a compound that, albeit in trace amounts, has been found in all species, but only in *Solanaceae* is used as a substrate for the significant production of SGAs through hydroxylation, oxidation transamination, and glycosylation reactions. In tomato, numerous enzymes have been identified, encoded by a family of genes called *'GLYCOALKALOID METABOLISM'* (*GAME*) (Itkin et al., 2011, 2013; Sonawane et al., 2018). Similar with other specialized plant metabolites, SGAs are also produced by clustered genes: in fact, most of the *GAME* genes in tomato are clustered in chromosomes 7 and 12. Specifically, seven genes are co-expressed on chromosome 7 (*GAME 6*, *GAME 8*, *GAME 11*, *GAME 1*, *GAME 17*, *GAME 18*, *GAME 2*), while only two are co-expressed on chromosome 12 (*GAME 4* and *GAME 12*). More in detail, cholesterol initially undergoes three successive hydroxylation reactions by *GAME 7*, *8* and *11*, which respectively add an -OH group at position C22, C26 and C16, yielding 16,22,26-trihydroxycholesterol (Itkin et al., 2013; Nakayasu et al., 2017). Subsequently, two oxidation reactions take place operated by the *GAME 6* and *GAME 4* genes, both belonging to the cytochrome P450 family. The former catalyzes a reoxidation of C22 and, together with a probable not yet characterized cyclase, leads to the formation of the furostanol-type aglycone (Itkin et al., 2013; Nakayasu et al., 2017). A very important step in tomato SGAs biosynthesis is the reaction catalyzed by *GAME 4*. This enzyme, indeed, catalyzes the production of furostanol-26-aldehyde, which is the first specific SGA of tomato plant. Furthermore, it is located in a branch point, leading to the formation of SGAs against non-nitrogenous steroidal saponins. Indeed, it has been observed that the silencing of *GAME 4* by RNA interference (RNAi) technique leads to an alteration in the metabolic profile of tomato, which is evidenced, in particular, by a reduction of SGAs and also, albeit to a lesser degree, of certain

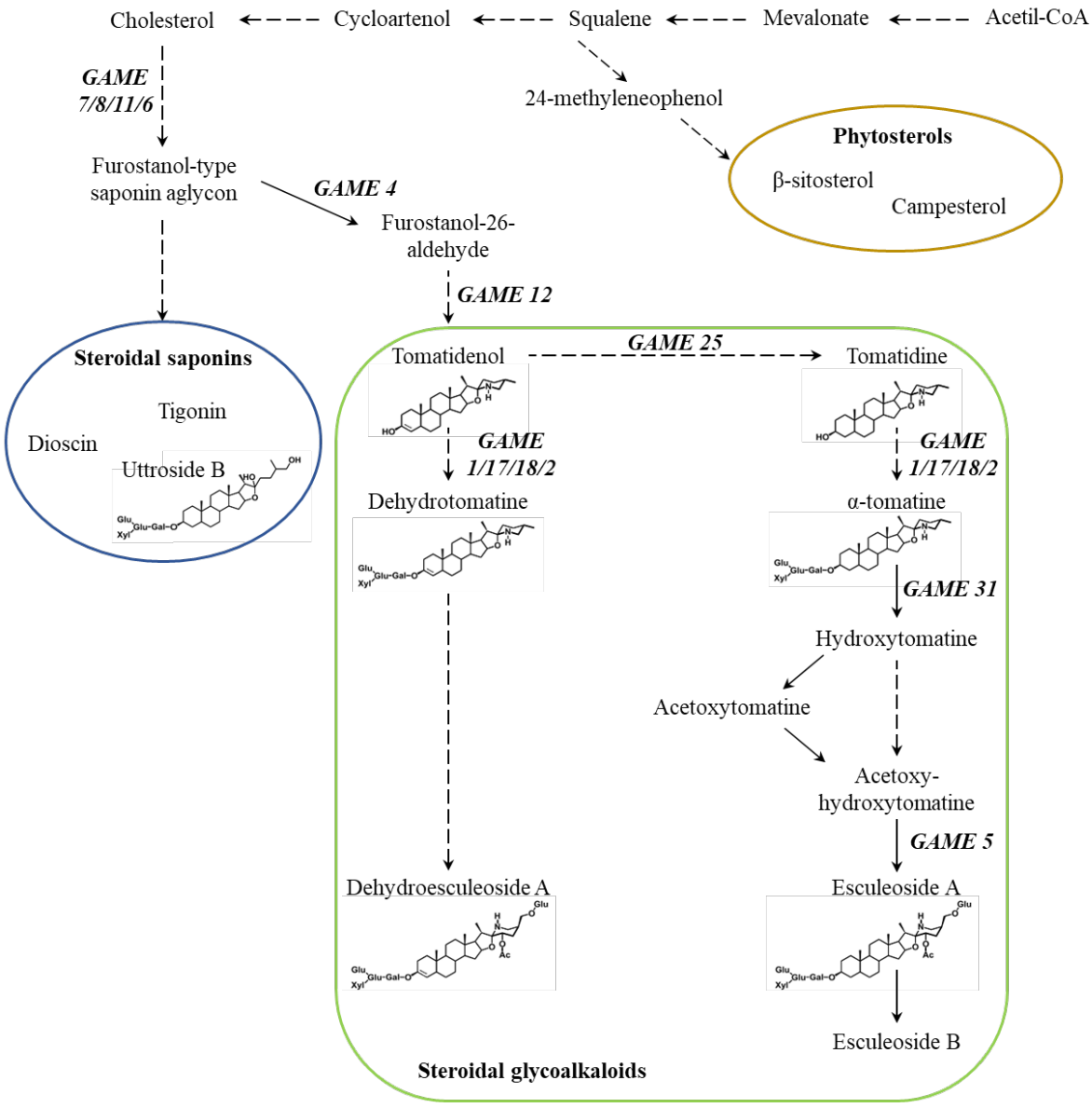

Figure 1. Biosynthetic pathway of SGAs. The figure shows the local metabolism of SGAs, marked by the green outline, the precursors and related pathways (phytosterols and steroidal saponins, marked in dark yellow and dark blue, respectively). Only genes belonging to the GLYCOALKALOID METABOLISM family, which have been characterized so far, are shown in bold italics. The arrows represent reactions catalyzed by single enzymatic step. Dashed arrows represent reactions catalyzed by several enzymatic activities.

phytosterols such as β-sitosterol (Itkin et al., 2013). Studies in potato also show that gene silencing causes a decrease in plant resistance to the fungus *Verticillium dahliae*, and an alteration in plant response to the Colorado potato beetle; however, tolerance to drought and other abiotic stresses remains unchanged (Paudel et al., 2017). The next transamination reaction is crucial for the introduction of nitrogen into the cholesterol skeleton, and is carried out by *GAME 12*, which converts furostanol-26-aldehyde to 26-amino-furostanol (Itkin et al., 2013; Sonawane et al., 2020). Subsequently, the gene that leads to F-loop closure and thus to the formation of dehydrotomatidine (or tomatidenol) has not yet been characterized. At this point, the other SGAs to be formed will

differ in the presence or absence of a double bond at the C-5,6 position. The transition from unsaturated glycoalkaloid (dehydrotomatidine) to saturated one (tomatidine) seems to involve four reactions (oxidation of C3, isomerization, reduction of C5α and reduction of C3). Recently, the *GAME 25* gene, a member of the short-chain dehydrogenases/reductases (SDRs), has been identified, which appears to catalyze all the reactions listed above except the reduction of C5 (Sonawane et al., 2018). Finally, glycosylation reactions are catalyzed by various uridine-5'-diphosphate (UDP)-glycosyltransferases (UGTs) that add several sugar moieties at the C3 hydroxyl group to the steroid alkaloid skeleton (Itkin et al., 2011, 2013). In particular *GAME 1*, a galactosyltransferase that adds a galactose molecule to the C-3β aglycone tomatidine, producing tomatidine galactoside, was the first gene identified in the biosynthesis of SGAs in tomato. As general concept, plants use glycosylation of secondary metabolites as fundamental mechanism to make them more soluble and to detoxify potential negative activities (Gachon et al., 2005). And, indeed, it has been seen that *GAME 1* silencing, using RNAi technology, resulted in an accumulation of tomatidine with a consequent delay in plant growth (Itkin et al., 2011), likely due to the presence of high levels of tomatidine, which has also been shown to exert a toxic effect on plant cells by disrupting the hydro-electrolyte balance (Hoagland, 2009). In addition, other sugars are added by the enzymes *GAME 17* and *GAME 18*, two glucosyltransferases that lead to the formation of γ-tomatine and β1-toamine, respectively. Finally, *GAME 2*, a xylosyltransferase, allows for the formation of α-tomatine (Itkin et al., 2011, 2013).

During fruit ripening, the levels of α-tomatine and dehydrotomatine decrease considerably, favoring the accumulation of esculeosides and dehydroesculeosides, respectively. Two other members of the family *GAME* genes have recently been identified, responsible for these conversion from bitter and toxic compounds (Zhao et al., 2021). The first gene is *GAME 31*, a 2-oxoglutarate-dependent dioxygenase, which adds an -OH group to the C23 of α-tomatine leading to the formation of hydroxytomatine (Cárdenas et al., 2019; Nakayasu et al., 2020). Subsequently two other genes, not yet identified, are responsible for the acetylation of the C23 hydroxyl group and the hydroxylation of the C27, leading to the production of acetoxy-hydroxytomatine. Finally *GAME 5*, a member of the uridine glycosyltransferase family diphosphate (UDP), adds a glucose molecule to the C27 hydroxyl group producing esculeoside A (Alseekh et al., 2015; Szymański et al., 2020).

Although there is great interest in understanding the SGAs pathway, there are few studies in the literature concerning the transcriptional regulation of this biosynthesis. The discovery of *GAME 9*, a transcription factor of the AP2/ERF family, showed how important in glycoalkaloid biosynthesis is not only the control of those genes involved in the 'core' pathway, from cholesterol

to α-tomatine, but also those involved in cholesterol biosynthesis (Cárdenas et al., 2016). For example, *GAME 9* binds also the promoter of the gene 'sterol side chain reductase 2' (SSR2), a reductase of the double bond at position C24-25 of cycloartenol, that catalyzes its reduction to cycloartanol (Sawai et al., 2014).

1.3.2 Allergens

Food allergy is a significant global public health problem for which there is no reliable treatment. Any food containing protein presents a potential risk, indeed, for triggering immune reactions. The majority of reported and verified food allergies are classified as immunoglobulin E (IgE) mediated because they trigger the body's immune system to produce IgE antibodies. Symptoms can range from mild and transient to severe, including death without appropriate and prompt treatments (Remington et al., 2020). The mechanisms of food allergy can differ between countries around the world, due to different dietary habits, as well as to different sensitization of populations to a particular food allergen; therefore, to the complex interplay of genetic, epigenetic, and environmental factors (Tham & Leung, 2018). Notably, the occurrence of food allergy continues to increase every year, and it spreads especially in industrialized states, affecting 8% of children in the Western countries, but it seems to be rising also in the developing countries (Peters et al., 2021).

Although tomatoes are recommended for daily diet, due to the numerous beneficial molecules, they are also common sources of ALs, so their consumption should be avoided by allergic patients (Asero, 2013). As mentioned before, also tomato allergy is IgE-mediated and it seems to be partly originate from pollen cross-reactivity (Włodarczyk et al., 2022). The most common allergic reactions in sensitive patients are cutaneous symptoms (pruritus, erythema) or even hives and oral allergy syndrome. However, gastrointestinal and cardiovascular reactions have also been reported occasionally (Asero et al., 2009; Zacharisen et al., 2002). Cases of tomato allergy are present throughout Europe, with an average of about 4.9%. However, most of the allergic population is present in Southern Europe. For example, in Italy up to 16% of adults are sensitive (Burney et al., 2014; Ortolani et al., 1989).

To date, 27 tomato proteins have been reported as putative ALs, however only preliminary data on their allergenicity are available. For this reason, up to date, seven ALs (Table 2) have been confirmed and are on the list of the International Union of Immunological Societies (IUIS).

Table 2: Tomato allergens, with their biochemical name and molecular weight, officially confirmed by the International Union of Immunological Societies.

Allergen	Biochemical name	Molecular weight (SDS-PAGE)
Sola l 1	Profilin	14-17 kDa
Sola l 2	Beta-fructofuranoside	51-60 kDa
Sola l 3	Non-specific lipid transfer protein	9 kDa
Sola l 4	Pathogenesis-related protein (PR-10)	17.5-18 kDa
Sola l 5	Cyclophillin	19kDa
Sola l 6	Non-specific lipid transfer protein	10 kDa
Sola l 7	Non-specific lipid transfer protein	12 kDa

1.3.2.1 Sola l 1 – Profilin

Profilins, small globular cytoplasmic proteins (approximately 14-17 kDa), are ubiquitous proteins present in all eukaryotic cells. They promote the polymerization of actin filaments and monomers, playing a central role in cytoskeleton regulation and signal transduction (Carlsson et al., 1977) and have, thus, a strong involvement in the processes of cell proliferation, growth and motility. Their participation in all these crucial function explains why they are expressed so widely and are highly conserved (Rodríguez del Río et al., 2018). Most of the profilins, with an amino acid sequence between 100 and 130 aa, have a secondary structure with seven beta sheets and four helices (Włodarczyk et al., 2022). Plant profilins have been described as panallergens; in fact, they are responsible for cross-sensitization between plant pollen and vegetable food extracts (Wensing et al., 2002).

To date, three Sola l 1 isoforms have been described in tomato plants. The first one identified is accumulated mainly in the pollen (Yu et al., 1998). Other two isoforms have been found in various tissues, including fruits (Westphal et al., 2004; Willerroider et al., 2003). Comparing the amino acid sequence of the profilin expressed in the pollen with those present in the fruit, only 76 % and 78% identity was observed, respectively. The two isoforms expressed in the fruit, on the other hand, have an identity percentage of 89% (Yu & Parthasarathy, 2014). The first evidence that tomato profilin is an AL was advanced by (Foetisch et al., 2001), however it was officially recognized as an AL only a few years later (Willerroider et al., 2003). It has recently been shown that Sola l 1 resists to heat and enzymatic digestion. Therefore, sensitive patients may experience allergic reactions even when consuming processed tomatoes (Kiyota et al., 2017).

In order to reduce the allergenicity of tomato fruits, RNAi technique was used by (L. Le et al., 2006) to target the *Sola l 1* gene. The resulting transgenic plants showed a decrease in profilin levels and skin prick tests confirmed a reduction of the allergenic response from 63% to 100% in

patients with an exclusive IgE-Sola l 1 reaction and between 16-25% in patients with additional allergies to other tomato components. On the other hands, unfortunately, tomato plants were also characterized by a dwarf phenotype associated also with a decrease in fruit number and size. Furthermore, the flowering time was delayed by several weeks compared to WT plants (L. Le et al., 2006). All these effects are likely due to fundamental cellular functions played by profilins on cell cytoarchitecture and plant morphogenesis, as aforementioned.

1.3.2.2 Sola l 2 – β-fructofuranosidase

β-fructofuranosidase catalyzes the reaction of sucrose hydrolysis, yielding glucose and fructose. This well-known enzyme, also known as acid invertase, is present in a variety of plants (Daimon et al., 2008). It has also been identified in tomato, called Sola l 2, where it is involved in the process of hexose accumulation at the stage of fruit ripening. There are two Sola l 2 isoforms: the most expressed has a molecular mass of 60 kDa, the other has an insertion of 86 bp, and a premature stop codon is created generating a protein of 51 kDa (Ohyama et al., 1992; Westphal et al., 2003). Petersen proposed, for the first time, that tomato β-fructofuranosidase was a potential AL (Petersen et al., 1996) and its hypothesis was confirmed few years later (Foetisch et al., 2001). However, it was shown that in a group of people sensitive to glycoproteins, only 46% recognized the Sola l 2 allergen in immunoblot assays, highlighting its low allergenic power (Westphal et al., 2003). In these perspectives, more investigations are required to fully comprehend the allergic mechanisms connected to Sola l 2.

1.3.2.3 Sola l 3, Sola l 6 and Sola l 7 – Lipid transfer proteins

Non-specific lipid transfer protein (ns-LTPs) are small proteins (6.5-10 kDa) only found in terrestrial plants, with a basic isoelectric point, between 8.8-12. They have the capacity to bind several hydrophobic molecules, such as phospholipids, fatty acids, fatty acyl-coenzyme A and cutin monomers (Liu et al., 2015). They contain eight conserved cysteine residues, forming four disulphide bridges that hold four α-helices together resulting in a compact cylinder-like structure (Salminen et al., 2016). Although their mode of action is not entirely understood, it has been observed that ns-LTPs play a defense role against infection caused by bacteria, fungi and viral pathogen; in fact, they belong to the pathogen-related (PR) protein family 14 (Van Loon & Van Strien, 1999). Being involved in defense mechanisms, they usually localize in the surface layers of certain fruits; however, in others, seeds seem to contain larger concentration of ns-LTPs (Asero, 2011). Originally grouped into two types, with the discovery of new ns-LTPs, a new classification

method was developed based on sequence similarity and distance of cysteine residues in the molecules (Boutrot et al., 2005).

ns-LTPs have been identified as major ALs in many foods and are responsible of several allergic reactions, including anaphylaxis especially in sensitive adults living in the Mediterranean area (Asero et al., 2018; Volpicella et al., 2015). Moreover, for their stable and compact secondary structure, they resist to gastrointestinal proteolysis, pH changes and heat treatment (Breiteneder & Mills, 2005).

Three of the seven tomato ALs registered in the list of IUIS belong to this class. Sola l 3, a 9 kDa protein, was identified in flesh and epicarp of tomato fruits (L. Q. Le et al., 2006; Volpicella et al., 2015), while Sola l 6 (10 kDa) and Sola l 7 (12 kDa) were detected in seeds (Martín-Pedraza et al., 2016). In the same research, it was found that both the two ns-LTPs isolated in tomato seeds had a very low percentage amino acid identity (only 22% and 38% respectively) compared to Sola l 3. In the tomato genome, 64 additional possible nsLTP coding sequences were recently identified, suggesting how little is still known about this class of molecules (D'Agostino et al., 2019).

As for profilin, also for Sola l 3 silencing strategy was adopted in order to reduce the allergic content of tomato fruits. Using double-stranded RNA interference, to target both isoforms, a decrease in nsLTPs accumulation was obtained in transgenic tomato plants. Evaluation of the allergenic potential of these tomatoes showed a 10- to 100-fold decrease in histamine release by human basophils (L. Q. Le et al., 2006). Subsequently, to analyze the reduction in the allergenicity of the fruit, a skin prick test (in vivo) was also performed. Notably, a reduction in allergenicity was confirmed and the heritability of silencing genes on the next generation of plants was also evaluated (Lorenz et al., 2006).

1.3.2.4 Sola l 4 – Pathogenesis-related protein (PR10)

Sola l 4 belongs to the PR-10 family, known for their low molecular weight (around 18 kDa), their acidic character, and the fact that they are resistant to proteases (Sinha et al., 2014). Their secondary structure has three α-helices and seven antiparallel β-sheets. These structural elements enclose a large hydrophobic cavity, most likely indicating their potential ability to bind non-polar molecules (Fernandes et al., 2013). People allergic to this class of food allergens develop symptoms after consumption of fresh fruit; in contrast, they can tolerate processed products (Dölle et al., 2011). Previous studies have revealed that PR-10 are involved in biotic stresses showing antifungal (Chen et al., 2007; Xie et al., 2010), antibacterial (Flores et al., 2002; Xie et al., 2010), antiviral (Park et al., 2004) and antinematode (Andrade et al., 2010) activities.

Interestingly, an Italian study showed that tomato-allergic patients are sensitized to the Bet v 1 allergen (Asero, 2013), and these results provide indirect evidence of the presence of an AL homologous to Bet v 1 in tomato fruits. Indeed, two isoforms named Sola l 4.01 and Sola l 4.02, homologous to the Bet v 1 protein present in *Betula verrucosa*, have recently been identified in tomato. The two isoforms differ in both molecular weight and isoelectric point: Sola l 4.01 was 18 kDa with a pI equal to 5.35; whereas for Sola l 4.02, those parameters are 17.5 kDa for mass and pI 5.44 (Wangorsch et al., 2015). Among the two isoforms, Sola l 4.02 showed stronger immunological activities and has been characterized as a major allergen in tomato fruits. Furthermore, this isoform appears to be the most highly expressed in tomato. They also pointed out the problem of cross-reactivity between tomato and pollen: in fact, they found that most of the patients sensitized to Bet v1 also reacted to Sola l 4. Of note, Bet v 1, the main AL in birch pollen, belongs to the same class of PRs (PR-10), and it is known that one of the most prevalent cross-allergies occurs between tomato and birch pollen. Although proteins belonging to this class share only ~ 50% of the amino acid sequence identity, the main reason for cross-reactivity among members of the PR-10 family is the high three-dimensional structure similarity (Sinha et al., 2014). In a recent paper, a deeper characterization of the Sola l 4.02 was performed, and it was observed that the purified recombinant protein has a molecular weight of 18 kDa. A polyclonal antibody against Sola l 4 was also used in an indirect competitive ELISA assay to investigate the correlation between the allergen level and features such as genotype and climate condition. The results seem to indicate that the content of Sola l 4 decreased under climatic conditions of low humidity and high temperatures; it also varied significantly within different cultivars (Kurze et al., 2018).

1.3.2.5 Sola l 5 – Cyclophilins

Cyclophilins are ubiquitous proteins and are present in a wide range of organisms, including plants, with peptidyl-prolyl cis-trans isomerase activity (PPIase), involved in the folding of proteins (Fanghänel & Fischer, 2004). Although cyclophilins have little sequence homology with FK506-binding proteins (FKBPs) and parvulins, due to their PPIase activity, all they belong to the immunophilin family (Thapar, 2015).

Recent developments in genome and transcriptome sequencing have shown that plants have higher numbers of cyclophilins than other eukaryotes. Although cyclophilins are mostly found in the cytosol of plants, they are also localized in the nucleus, mitochondria and chloroplasts, suggesting their involvement in different kind of cellular processes. For example, they participate in control transcription, hormone signaling and in response to both biotic and abiotic stresses (Olejnik et al., 2021; Romano et al., 2004).

Cyclophilin was first identified in tomatoes by (Gasser et al., 1990). Subsequently, it was revealed that the *dgt* mutation, which generates plants characterized by agravitropic growth, lack of lateral root formation and aberrant xylem structure, is located in the Cyclophilin1 (*SlCyp1*) gene. In fact, it was observed that aberrant phenotypes were restored complementing some *dgt* mutants with *SlCyp1* (Oh et al., 2006).

However, little interest has been shown, up to now, in this molecule regarding to its allergenic activity. To date, Sola l 5 is recognized as an allergen by the IUIS; in fact, its up-regulation has been observed in infected tomato lines with the Pepino mosaic virus (PepMV) plant pathogen (Welter et al., 2013).

1.4 Genome editing

Innovation in plant breeding is continuously needed to address the challenges of global changes such as population growth and climate change. These factors are contributing to reduced food availability and lead to the assumption that food production will need to be doubled between 2000 and 2050 (http://faostat.org). Furthermore, plants are continuously exposed to various pathogens including bacteria, fungi and viruses, resulting in a 20-40% yield loss globally (Borrelli et al., 2018; Savary et al., 2012). Agriculture has been able to face these challenges since ancient times until now, trying, through empirical actions first and scientific research later, to pursue methods capable of supplying food in sufficient quantity and of better quality, always with a view to the environment. In particular, genetic improvement continues to provide the main tools for obtaining ever more nutritious crops suited to a rapidly changing world, reducing the environmental impact of agriculture. However, further efforts are needed, so breeders are constantly searching for new genetic improvement techniques. In this context, some of the technologies developed in the last two decades based on genetic engineering have allowed for a massive boost in terms of genetic attempt accuracy and efficiency, and have been grouped together and named New Plant Breeding Techniques (NPBT).

Among these, genome editing technique is a tool that can precisely modify a specific DNA sequence within the genome without moving it from its natural position (van de Wiel et al., 2017). In nuclease-mediated genome editing, a molecular machinery consisting of a DNA-binding domain and a double strand break (DSB) domain is required, using a site-specific endonuclease (Porteus, 2016). The principle is similar to that of mutagenesis: DNA damage, a DSB, occurs and cellular repair mechanisms, in an attempt to correct the damage, can generate errors, thus introducing mutations. The difference with random mutagenesis lies in the mutagenic agents: in this case, indeed, they are no longer represented by chemical or physical agents, but are specific

nucleases, carefully designed in the laboratory, that read the entire genome, recognize a specific DNA sequence and cut at the desired point (van de Wiel et al., 2017). The creation of the DSB triggers DNA repair mechanisms, mainly Non-Homologous End-Joining (NHEJ) and homologous recombination (HR) (Porteus, 2011). Depending on the repair mechanism used, the presence or absence of a templated DNA molecule (exogenous or endogenous), we can distinguish different types of site-directed nucleases (SDNs). If no exogenous DNA is used, we talk about SDN-1 with the simple creation of one or more DSBs that generate random mutations (substitutions, insertions or deletions) repaired by NHEJ (Roth et al., 2012). In general, it could cause gene silencing, gene knock-out or a change in the activity of a gene. With the SDN-2, it is possible to produce specific point mutations, generating two DSB and using the HR system. It is necessary to use a donor DNA template homologous to the targeted area, containing targeted mutations by single-base substitution or short indels (Schaart et al., 2016). Similarly, also SDN-3 method induces two DSBs and require a donor DNA but, in this case, it is an entire gene. It can be inserted either by NHEJ or HR into a desired site in the genome in order to create new phenotypes (EFSA Panel on Genetically Modified Organisms (EFSA GMO Panel) et al., 2020).

1.4.1 The state of art on genome editing legislation

Modern biotechnologies are a step forward compared to traditional GMOs and the international scientific community believes that plants generated with genome editing are substantially different from traditional GMOs and that they must be regulated differently from them. This idea is also confirmed by EFSA which, in an opinion requested by the European Commission, declared that some methods of genome editing are not comparable to the techniques that generate GMOs but to conventional techniques (EFSA Panel on Genetically Modified Organisms (GMO), 2012). However, the pronouncement of the Court of Justice of the European Union (CJEU) (judgment in case C-528/16 of 25 July 2018) established that all mutagenesis techniques give rise to GMOs and that only organisms obtained with conventional mutagenesis techniques and with a of use are excluded from the application of EU Directive 2001/18 (the directive that regulates the diffusion of GMOs) as they are expressly indicated in Annex I B of the directive. The possible negative impact of this ruling on commercial and technological development in Europe in the field of genetic innovation, and the technical impossibility (European Network of GMO Laboratories (ENGL), 2019) of detecting and identifying, distinguishing them from those obtained by random mutation, the products obtained using the new technologies, as required by the Directive, prompted the European Council to ask the Commission for a study to evaluate the classification of the new genomic techniques (NGT, genome editing and cisgenesis) within the European legislation and to

propose, if appropriate, a subsequent intervention plan. This study, based on extensive consultation of Member States, expert commissions (EFSA, JRC and Competent National Authorities) and of all the main stakeholders and published on 29 April 2021, confirmed the significant interest both in the EU and globally in the application of NGT. Although the issues of safe use of these technologies, labelling, freedom of choice for the consumer and their possible environmental impact are still the subject of very contrasting positions, it has also been recognized that NGT could make a substantial contribution to achieving the objectives of the European Green Deal and of the Farm To Fork and Biodiversity strategies for a more resilient and sustainable agri-food system.

It was also recognized that the current regulatory system imposes difficult authorization, traceability and labeling controls on competent authorities for NGT products that do not contain foreign genetic material. Furthermore, these controls are disproportionate and often not justified since, as indicated by EFSA, conventional breeding techniques, genome editing and cisgenesis can produce plants with similar risk profiles. The study therefore concludes that it is necessary to overcome the rigidity of current legislation which fails to ensure risk assessment adequate to scientific progress and creates a regulatory disproportion between products obtained with different techniques but with a similar level of risk. The Commission has therefore launched a political initiative to propose a new legal framework for plants obtained by targeted mutagenesis and cisgenesis and for food and feed obtained from them. The initiative must maintain the objectives of the current legislation regarding the high levels of protection of human and animal health and of the environment but also allow the fruits of innovation in the agri-food system to be reaped, allowing the safe products obtained through NGT to contribute to the achievement of the objectives of the Green Deal and the Farm to Fork strategy and to a more competitive economy, which are at the heart of the EU's current priorities.

While awaiting the discussion and completion of the regulatory process at EU level, it is hoped that research will not stop. It should in fact be considered that even if they were not transferable directly to the field due to a regulatory constraint, the results obtained with the use of genome editing could still be useful in genetic improvement programs using more traditional approaches.

Therefore, the knowledge that will be acquired with modern biotechnology will in any case remain in the genetic heritage of Italian agriculture, whatever the legislation that will regulate this sector in the future. It would therefore be advisable to continue research in this field in a decisive manner, allowing experimentation even outside the laboratories, in compliance with current legislation and an approach based on scientific knowledge.

In this context, the Draft Law (A.C.3310) presented to the Agriculture Commission of the Chamber of Deputies on 12/15/2021 is of considerable interest, this law aims to introduce a simplified procedure for experimentation in the open field for scientific and plant research purposes obtained through targeted mutagenesis and cisgenesis by amending Legislative Decree 224 of 2003 which transposes EU Directive 2001/18 in Italy and overcoming the de facto moratorium determined by the failure to identify suitable sites for experimental fields envisaged by the Decree of the Ministry of Agriculture, Food and forestry of 19 January 2005.

1.4.2 CRISPR/Cas9

The most promising NPBTs is currently the CRISPR system associated with the Cas9 enzyme (CRISPR/Cas9), which is simpler than the others. More detailed, the CRISPR/Cas9 technology is based on the use of a guide RNA and a CRISPR-associate (Cas9) protein. The guide RNA is a short fragment, homologous to a target sequence, which vehicles the Cas9 within the genome. Among the Cas9, the first one used in genome editing was isolated from *Streptococcus pyogenes*. It is called genetic scissor for its ability to bind and cleave the target DNA (Mengstie & Wondimu, 2021).

The CRISPR system is an immune mechanism present in many bacteria and most characterized Archaea (Hille & Charpentier, 2016). In general, to perform an immune response following the recognition of nucleic acids (viral DNA/RNA, DNA fragments or plasmids) from the external environment, the mechanism can be divided into three steps:

I. Acquisition phase: DNA fragments from exogenous bacteriophages or plasmids are incorporated into the host CRISPR locus as spacers;

II. In the second phase, Cas proteins are expressed, the CRISPR array containing the acquired spacers is transcribed forming pre-crRNAs, which will in turn be cut and processed by Cas proteins (Deltcheva et al., 2011);

III. In the final step, Cas proteins, carried by crRNAs, recognize the target within the genome. This mechanism then protects the host cell by tracking and cutting exogenous DNA.

The constituents of the CRISPR system in practice are: genes for Cas9s, crRNAs and tracrRNAs; the latter form a complex known as guide RNA, which, together with Protosapcer Adjacent Motif (PAM), determines the recognition of the target in the genome and the cutting specificity by the Cas9 (Barrangou, 2013; Makarova et al., 2015). In practice, the system consists of a defined number of repeats, each of which is followed by short spacer DNA fragments, generated by past exposure of the bacterium to bacteriophage viruses or plasmids. Small gene clusters, encoding nucleases (Cas9), are associated with these sequences. Cas9 nucleases are able to cut DNA at the

sequence complementary to the guide RNA. The CRISPR spacer sequences are basically the guide RNA templates, which drive Cas9 protein to the target DNA to be cut. In the perspective of its full exploitation for genetic engineering purposes, the system was engineered to replace these spacer sequences with target sequences of interest. Specifically, it was thought to fuse the crRNAs with the tracrRNAs generating the well-known single guide RNA sgRNA (Jinek et al., 2012). In particular, the sgRNA consists of a structural region (scaffold) and a variable region, 20 nucleotides long, complementary to the sequence in the genome to be edited. To achieve a precise and efficient cut, the 20 nucleotides on the DNA must be followed by a PAM sequence that varies according to the chosen nuclease (Khatodia et al., 2016; Sander & Joung, 2014).

Due to its simplicity and versatility, the technique of genome editing has been widely used for years alongside traditional breeding methods. The first evidence of genome editing in tomato plants dates back to 2014 (Brooks et al., 2014). Over the years, it has been used to evaluate parameters such as fruit ripeness, resistance to biotic and abiotic stresses, improvement of fruit yield and quality.

Nutritional improvement in cultivated crops is one of the major targets. A great amount of research has been carried out in this field: for example, CRISPR/Cas9 multiplexing was recently used on several genes of the carotenoid metabolic pathway (*stay-green 1* (*SGR1*), *lycopene ε-cyclase* (*LCY-E*), *beta-lycopene cyclase* (*Blc*), *lycopene β-cyclase 1* and *2* (*LCY-B1* and *LCY-B2*)) in tomato plants, cultivar '*Ailsa Craig*', in order to increase the lycopene content of the fruit (X. Li et al., 2018). In spite of the various gene combinations, the authors found that the lines edited for the *SGR1* gene were those that showed a higher lycopene content (approximately 5.1-fold), associated with an overall increase in other carotenoids such as phytoene, prolycopene, α-carotene and lutein. Moreover, in Japan a tomato with a high γ- aminobutyric acid (GABA) content has recently been launched. It accumulates about 4-5 times more GABA than the ordinary tomatoes present in the market (Nonaka et al., 2017). It has also been shown that knock-out of the gene Sl7-DR2 leads to an accumulation of the 7-dehydrocholesterol, known as provitamin D3, in leaves and mature green fruit (J. Li et al., 2022).

Since genome editing mutations are thought to be nearly comparable with spontaneous genetic mutations, and taking into account that it is possible to remove the inducer of the desired mutation from the plant host, the novel varieties could be efficiently generated in the future by genome-edited crops.

1.5 Aim of the work

Tomato is one of the most widely grown crops worldwide and it is also a model species for biological studies as food crop, with particular regard to nutritional aspects. Although tomato fruits represent one of the foods with the highest content of nutritional and functional compounds with antioxidant activity, such as the carotenoids lycopene, β-carotene and lutein, as well as some flavonoids (naringenin chalcone and rutin), phenolic acids and ascorbic acid (Jafari et al., 2017; Odriozola-Serrano et al., 2009), it is also well known as a source of numerous anti-nutritional factors (such as phytates, tannins, SGAs, etc.) and ALs (Kurze et al., 2018; Oyetayo & Ibitoye, 2012).

This PhD project aims to improve the overall nutritional quality of tomato berries through the CRISPR/Cas9 technology.

Therefore, the main goals of this research were:

1: to decrease allergenic and glycoalkaloid content by targeting the *GAME 4* and *Sola l 4* genes with the CRISPR/Cas9 technique;

2: to evaluate through a multi-omics approach (metabolomics volatile and non-volatile and proteomics) potential effect on plant health, fruits qualities like aroma and taste and nutritional traits;

3: to analyze, by 16S rRNA sequencing, a possible shaping of bacterial community structure in the rhizosphere of edited plants as a consequence of the knock-out of *GAME 4* and *Sola l 4* genes;

4: to assess the effect, at metabolic level, of the induced mutations in a crop- and locus-specific manner (*GREENFLESH* locus) in edited plants.

1.6 References

Alseekh, S., Tohge, T., Wendenberg, R., Scossa, F., Omranian, N., Li, J., Kleessen, S., Giavalisco, P., Pleban, T., Mueller-Roeber, B., Zamir, D., Nikoloski, Z., & Fernie, A. R. (2015). Identification and Mode of Inheritance of Quantitative Trait Loci for Secondary Metabolite Abundance in Tomato. *The Plant Cell*, *27*(3), 485–512. https://doi.org/10.1105/tpc.114.132266

Andrade, L. B. da S., Oliveira, A. S., Ribeiro, J. K. C., Kiyota, S., Vasconcelos, I. M., de Oliveira, J. T. A., & de Sales, M. P. (2010). Effects of a Novel Pathogenesis-Related Class 10 (PR-10) Protein from Crotalaria pallida Roots with Papain Inhibitory Activity against Root-Knot Nematode Meloidogyne incognita. *Journal of Agricultural and Food Chemistry*, *58*(7), 4145–4152. https://doi.org/10.1021/jf9044556

Asero, R. (2011). Lipid transfer protein cross-reactivity assessed in vivo and in vitro in the office: Pros and cons. *Journal of Investigational Allergology & Clinical Immunology*, *21*(2), 129–136.

Asero, R. (2013). Tomato allergy: Clinical features and usefulness of current routinely available diagnostic methods. *Journal of Investigational Allergology & Clinical Immunology*, *23*(1), 37–42.

Asero, R., Antonicelli, L., Arena, A., Bommarito, L., Caruso, B., Colombo, G., Crivellaro, M., De Carli, M., Della Torre, E., Della Torre, F., Heffler, E., Lodi Rizzini, F., Longo, R., Manzotti, G., Marcotulli, M., Melchiorre, A., Minale, P., Morandi, P., Moreni, B., … Senna, G. E. (2009). Causes of Food-Induced Anaphylaxis in Italian Adults: A Multi-Centre Study. *International Archives of Allergy and Immunology*, *150*(3), 271–277. https://doi.org/10.1159/000222679

Asero, R., Piantanida, M., Pinter, E., & Pravettoni, V. (2018). The clinical relevance of lipid transfer protein. *Clinical & Experimental Allergy*, *48*(1), 6–12. https://doi.org/10.1111/cea.13053

Ballester, A.-R., Molthoff, J., de Vos, R., Hekkert, B. te L., Orzaez, D., Fernaǔndez-Moreno, J.-P., Tripodi, P., Grandillo, S., Martin, C., Heldens, J., Ykema, M., Granell, A., & Bovy, A. (2009). Biochemical and Molecular Analysis of Pink Tomatoes: Deregulated Expression of the Gene Encoding Transcription Factor SlMYB12 Leads to Pink Tomato Fruit Color. *Plant Physiology*, *152*(1), 71–84. https://doi.org/10.1104/pp.109.147322

Barrangou, R. (2013). CRISPR-Cas systems and RNA-guided interference: CRISPR-Cas systems and RNA-guided interference. *Wiley Interdisciplinary Reviews: RNA*, *4*(3), 267–278. https://doi.org/10.1002/wrna.1159

Bialczyk, J., & Lechowski, Z. (2000). Tannin concentration in tomato fruit at different stages of its development / Tanninkonzentration in verschiedenen Entwicklungsstadien in der Tomatenfrucht. *Zeitschrift Für Pflanzenkrankheiten Und Pflanzenschutz / Journal of Plant Diseases and Protection*, *107*(2), 189–196. JSTOR.

Boileau, T. W.-M., Boileau, A. C., & Erdman, J. W. (2002). Bioavailability of all-trans and cis–Isomers of Lycopene. *Experimental Biology and Medicine*, *227*(10), 914–919. https://doi.org/10.1177/153537020222701012

Borrelli, V. M. G., Brambilla, V., Rogowsky, P., Marocco, A., & Lanubile, A. (2018). The Enhancement of Plant Disease Resistance Using CRISPR/Cas9 Technology. *Frontiers in Plant Science*, *9*, 1245. https://doi.org/10.3389/fpls.2018.01245

Boutrot, F., Guirao, A., Alary, R., Joudrier, P., & Gautier, M.-F. (2005). Wheat non-specific lipid transfer protein genes display a complex pattern of expression in developing seeds. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*, *1730*(2), 114–125. https://doi.org/10.1016/j.bbaexp.2005.06.010

Breiteneder, H., & Mills, C. (2005). Nonspecific lipid-transfer proteins in plant foods and pollens: An important allergen class. *Current Opinion in Allergy & Clinical Immunology*, *5*(3), 275–279. https://doi.org/10.1097/01.all.0000168794.35571.a5

Brooks, C., Nekrasov, V., Lippman, Z. B., & Van Eck, J. (2014). Efficient Gene Editing in Tomato in the First Generation Using the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated9 System. *PLANT PHYSIOLOGY*, *166*(3), 1292–1297. https://doi.org/10.1104/pp.114.247577

Burney, P. G. J., Potts, J., Kummeling, I., Mills, E. N. C., Clausen, M., Dubakiene, R., Barreales, L., Fernandez-Perez, C., Fernandez-Rivas, M., Le, T.-M., Knulst, A. C., Kowalski, M. L., Lidholm, J., Ballmer-Weber, B. K., Braun-Fahlander, C., Mustakov, T., Kralimarkova, T., Popov, T., Sakellariou, A., … van Ree, R. (2014). The prevalence and distribution of food sensitization in European adults. *Allergy*, *69*(3), 365–371. https://doi.org/10.1111/all.12341

Cárdenas, P. D., Sonawane, P. D., Heinig, U., Jozwiak, A., Panda, S., Abebie, B., Kazachkova, Y., Pliner, M., Unger, T., Wolf, D., Ofner, I., Vilaprinyo, E., Meir, S., Davydov, O., Gal-on, A., Burdman, S., Giri, A., Zamir, D., Scherf, T., … Aharoni, A. (2019). Pathways to defense metabolites and evading fruit bitterness in genus Solanum evolved through 2-oxoglutarate-dependent dioxygenases. *Nature Communications*, *10*(1), 5169. https://doi.org/10.1038/s41467-019-13211-4

Cárdenas, P. D., Sonawane, P. D., Pollier, J., Vanden Bossche, R., Dewangan, V., Weithorn, E., Tal, L., Meir, S., Rogachev, I., Malitsky, S., Giri, A. P., Goossens, A., Burdman, S., & Aharoni, A. (2016). GAME9 regulates the biosynthesis of steroidal alkaloids and upstream isoprenoids in the plant mevalonate pathway. *Nature Communications*, *7*(1), 10654. https://doi.org/10.1038/ncomms10654

Carlsson, L., Nyström, L.-E., Sundkvist, I., Markey, F., & Lindberg, U. (1977). Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *Journal of Molecular Biology*, *115*(3), 465–483. https://doi.org/10.1016/0022-2836(77)90166-8

Carrari, F. (2006). Metabolic regulation underlying tomato fruit development. *Journal of Experimental Botany*, *57*(9), 1883–1897. https://doi.org/10.1093/jxb/erj020

Castro-Alba, V., Lazarte, C. E., Bergenståhl, B., & Granfeldt, Y. (2019). Phytate, iron, zinc, and calcium content of common Bolivian foods and their estimated mineral bioavailability. *Food Science & Nutrition*, *7*(9), 2854–2865. https://doi.org/10.1002/fsn3.1127

Chaudhary, P., Sharma, A., Singh, B., & Nagpal, A. K. (2018). Bioactivities of phytochemicals present in tomato. *Journal of Food Science and Technology*, *55*(8), 2833–2849. https://doi.org/10.1007/s13197-018-3221-z

Chen, Z.-Y., Brown, R. L., Damann, K. E., & Cleveland, T. E. (2007). Identification of Maize Kernel Endosperm Proteins Associated with Resistance to Aflatoxin Contamination by *Aspergillus flavus*. *Phytopathology®*, *97*(9), 1094–1103. https://doi.org/10.1094/PHYTO-97-9-1094

Cheng, G., Chang, P., Shen, Y., Wu, L., El-Sappah, A. H., Zhang, F., & Liang, Y. (2020). Comparing the Flavor Characteristics of 71 Tomato (Solanum lycopersicum) Accessions in Central Shaanxi. *Frontiers in Plant Science*, *11*, 586834. https://doi.org/10.3389/fpls.2020.586834

Clark, N. F., & Taylor-Robinson, A. W. (2020). COVID-19 Therapy: Could a Chlorophyll Derivative Promote Cellular Accumulation of Zn2+ Ions to Inhibit SARS-CoV-2 RNA Synthesis? *Frontiers in Plant Science*, *11*, 1270. https://doi.org/10.3389/fpls.2020.01270

Cooperstone, J. L., Ralston, R. A., Riedl, K. M., Haufe, T. C., Schweiggert, R. M., King, S. A., Timmers, C. D., Francis, D. M., Lesinski, G. B., Clinton, S. K., & Schwartz, S. J. (2015). Enhanced bioavailability of lycopene when consumed as *cis* -isomers from *tangerine* compared to red tomato juice, a randomized, cross-over clinical trial. *Molecular Nutrition & Food Research*, *59*(4), 658–669. https://doi.org/10.1002/mnfr.201400658

da Silva, D. C., Andrade, P. B., Valentão, P., & Pereira, D. M. (2017). Neurotoxicity of the steroidal alkaloids tomatine and tomatidine is RIP1 kinase- and caspase-independent and involves

the eIF2α branch of the endoplasmic reticulum. *The Journal of Steroid Biochemistry and Molecular Biology*, *171*, 178–186. https://doi.org/10.1016/j.jsbmb.2017.03.009

D'Agostino, N., Buonanno, M., Ayoub, J., Barone, A., Monti, S. M., & Rigano, M. M. (2019). Identification of non-specific Lipid Transfer Protein gene family members in Solanum lycopersicum and insights into the features of Sola l 3 protein. *Scientific Reports*, *9*(1), 1607. https://doi.org/10.1038/s41598-018-38301-z

Daimon, T., Taguchi, T., Meng, Y., Katsuma, S., Mita, K., & Shimada, T. (2008). β-Fructofuranosidase Genes of the Silkworm, Bombyx mori. *Journal of Biological Chemistry*, *283*(22), 15271–15279. https://doi.org/10.1074/jbc.M709350200

Damon, M., Zhang, N. Z., Haytowitz, D. B., & Booth, S. L. (2005). Phylloquinone (vitamin K1) content of vegetables. *Journal of Food Composition and Analysis*, *18*(8), 751–758. https://doi.org/10.1016/j.jfca.2004.07.004

Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J., & Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, *471*(7340), 602–607. https://doi.org/10.1038/nature09886

Dölle, S., Lehmann, K., Schwarz, D., Weckwert, W., Scheler, C., George, E., Franken, P., & Worm, M. (2011). Allergenic activity of different tomato cultivars in tomato allergic subjects. *Clinical & Experimental Allergy*, *41*(11), 1643–1652. https://doi.org/10.1111/j.1365-2222.2011.03841.x

EFSA Panel on Genetically Modified Organisms (GMO). (2012). Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. *EFSA Journal*, *2012;10(2):2561*. https://doi.org/10.2903/j.efsa.2012.2561

EFSA Panel on Genetically Modified Organisms (EFSA GMO Panel), Naegeli, H., Bresson, J., Dalmay, T., Dewhurst, I. C., Epstein, M. M., Firbank, L. G., Guerche, P., Hejatko, J., Moreno, F. J., Mullins, E., Nogué, F., Sánchez Serrano, J. J., Savoini, G., Veromann, E., Veronesi, F., Casacuberta, J., Gennaro, A., Paraskevopoulos, K., … Rostoks, N. (2020). Applicability of the EFSA Opinion on site-directed nucleases type 3 for the safety assessment of plants developed using site-directed nucleases type 1 and 2 and oligonucleotide-directed mutagenesis. *EFSA Journal*, *18*(11). https://doi.org/10.2903/j.efsa.2020.6299

European Network of GMO Laboratories (ENGL). (2019, March 26). *Detection of food and feed plant products obtained by new mutagenesis techniques*.

Evans, M., Beck, M., Elliott, J., Etheve, S., Roberts, R., & Schalch, W. (2013). Effects of formulation on the bioavailability of lutein and zeaxanthin: A randomized, double-blind, cross-over, comparative, single-dose study in healthy subjects. *European Journal of Nutrition*, *52*(4), 1381–1391. https://doi.org/10.1007/s00394-012-0447-9

Fanghänel, J., & Fischer, G. (2004). Insights into the catalytic mechanism of peptidyl prolyl cis/trans isomerases. *Frontiers in Bioscience*, *9*(1–3), 3453. https://doi.org/10.2741/1494

Fernandes, H., Michalska, K., Sikorski, M., & Jaskolski, M. (2013). Structural and functional aspects of PR-10 proteins. *FEBS Journal*, *280*(5), 1169–1199. https://doi.org/10.1111/febs.12114

Flores, T., Alape-Girón, A., Flores-Díaz, M., & Flores, H. E. (2002). Ocatin. A Novel Tuber Storage Protein from the Andean Tuber Crop Oca with Antibacterial and Antifungal Activities. *Plant Physiology*, *128*(4), 1291–1302. https://doi.org/10.1104/pp.010541

Foetisch, K., Son, D., Altmann, F., Aulepp, H., Conti, A., Haustein, D., & Vieths, S. (2001). Tomato (Lycopersicon esculentum) allergens in pollen-allergic patients. *European Food Research and Technology*, *213*(4–5), 259–266. https://doi.org/10.1007/s002170100343

Friedman, M. (2013). Anticarcinogenic, Cardioprotective, and Other Health Benefits of Tomato Compounds Lycopene, α-Tomatine, and Tomatidine in Pure Form and in Fresh and Processed

Tomatoes. *Journal of Agricultural and Food Chemistry*, *61*(40), 9534–9550. https://doi.org/10.1021/jf402654e

Friedman, M., & Levin, C. E. (2016). Glycoalkaloids and Calystegine Alkaloids in Potatoes. In *Advances in Potato Chemistry and Technology* (pp. 167–194). Elsevier. https://doi.org/10.1016/B978-0-12-800002-1.00007-8

Friedman, M., Levin, C. E., Lee, S.-U., Kim, H.-J., Lee, I.-S., Byun, J.-O., & Kozukue, N. (2009). Tomatine-Containing Green Tomato Extracts Inhibit Growth of Human Breast, Colon, Liver, and Stomach Cancer Cells. *Journal of Agricultural and Food Chemistry*, *57*(13), 5727–5733. https://doi.org/10.1021/jf900364j

Gachon, C. M. M., Langlois-Meurinne, M., & Saindrenan, P. (2005). Plant secondary metabolism glycosyltransferases: The emerging functional analysis. *Trends in Plant Science*, *10*(11), 542–549. https://doi.org/10.1016/j.tplants.2005.09.007

García-Valverde, V., Navarro-González, I., García-Alonso, J., & Periago, M. J. (2013). Antioxidant Bioactive Compounds in Selected Industrial Processing and Fresh Consumption Tomato Cultivars. *Food and Bioprocess Technology*, *6*(2), 391–402. https://doi.org/10.1007/s11947-011-0687-3

Gascuel, Q., Diretto, G., Monforte, A. J., Fortes, A. M., & Granell, A. (2017). Use of Natural Diversity and Biotechnology to Increase the Quality and Nutritional Content of Tomato and Grape. *Frontiers in Plant Science*, *8*, 652. https://doi.org/10.3389/fpls.2017.00652

Gasser, C. S., Gunning, D. A., Budelier, K. A., & Brown, S. M. (1990). Structure and expression of cytosolic cyclophilin/peptidyl-prolyl cis-trans isomerase of higher plants and production of active tomato cyclophilin in Escherichia coli. *Proceedings of the National Academy of Sciences*, *87*(24), 9519–9523. https://doi.org/10.1073/pnas.87.24.9519

Gee, J. M., Wortley, G. M., Johnson, I. T., Price, K. R., Rutten, A. A. J. J. L., Houben, G. F., & Penninks, A. H. (1996). Effects of saponins and glycoalkaloids on the permeability and viability of mammalian intestinal cells and on the integrity of tissue preparations in vitro. *Toxicology in Vitro*, *10*(2), 117–128. https://doi.org/10.1016/0887-2333(95)00113-1

Gonzali, S., & Perata, P. (2020). Anthocyanins from Purple Tomatoes as Novel Antioxidants to Promote Human Health. *Antioxidants*, *9*(10), 1017. https://doi.org/10.3390/antiox9101017

Hille, F., & Charpentier, E. (2016). CRISPR-Cas: Biology, mechanisms and relevance. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1707), 20150496. https://doi.org/10.1098/rstb.2015.0496

Hoagland, R. E. (2009). Toxicity of tomatine and tomatidine on weeds, crops and phytopathogenetic fungi. *Allelopathy Journal*, *23*, 425–443.

Holmes, R. P., Goodman, H. O., & Assimos, D. G. (2001). Contribution of dietary oxalate to urinary oxalate excretion. *Kidney International*, *59*(1), 270–276. https://doi.org/10.1046/j.1523-1755.2001.00488.x

Iablokov, V., Sydora, B. C., Foshaug, R., Meddings, J., Driedger, D., Churchill, T., & Fedorak, R. N. (2010). Naturally Occurring Glycoalkaloids in Potatoes Aggravate Intestinal Inflammation in Two Mouse Models of Inflammatory Bowel Disease. *Digestive Diseases and Sciences*, *55*(11), 3078–3085. https://doi.org/10.1007/s10620-010-1158-9

Isaacson, T., Ronen, G., Zamir, D., & Hirschberg, J. (2002). Cloning of *tangerine* from Tomato Reveals a Carotenoid Isomerase Essential for the Production of β-Carotene and Xanthophylls in Plants. *The Plant Cell*, *14*(2), 333–342. https://doi.org/10.1105/tpc.010303

Itkin, M., Heinig, U., Tzfadia, O., Bhide, A. J., Shinde, B., Cardenas, P. D., Bocobza, S. E., Unger, T., Malitsky, S., Finkers, R., Tikunov, Y., Bovy, A., Chikate, Y., Singh, P., Rogachev, I., Beekwilder, J., Giri, A. P., & Aharoni, A. (2013). Biosynthesis of Antinutritional Alkaloids in

Solanaceous Crops Is Mediated by Clustered Genes. *Science*, *341*(6142), 175–179. https://doi.org/10.1126/science.1240230

Itkin, M., Rogachev, I., Alkan, N., Rosenberg, T., Malitsky, S., Masini, L., Meir, S., Iijima, Y., Aoki, K., de Vos, R., Prusky, D., Burdman, S., Beekwilder, J., & Aharoni, A. (2011). GLYCOALKALOID METABOLISM1 Is Required for Steroidal Alkaloid Glycosylation and Prevention of Phytotoxicity in Tomato. *The Plant Cell*, *23*(12), 4507–4525. https://doi.org/10.1105/tpc.111.088732

Jafari, S. M., Jabari, S. S., Dehnad, D., & Shahidi, S. A. (2017). Effects of thermal processing by nanofluids on vitamin C, total phenolics and total soluble solids of tomato juice. *Journal of Food Science and Technology*, *54*(3), 679–686. https://doi.org/10.1007/s13197-017-2505-z

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*, *337*(6096), 816–821. https://doi.org/10.1126/science.1225829

Karamać, M. (2009). Chelation of Cu(II), Zn(II), and Fe(II) by Tannin Constituents of Selected Edible Nuts. *International Journal of Molecular Sciences*, *10*(12), 5485–5497. https://doi.org/10.3390/ijms10125485

Kazachkova, Y., Zemach, I., Panda, S., Bocobza, S., Vainer, A., Rogachev, I., Dong, Y., Ben-Dor, S., Veres, D., Kanstrup, C., Lambertz, S. K., Crocoll, C., Hu, Y., Shani, E., Michaeli, S., Nour-Eldin, H. H., Zamir, D., & Aharoni, A. (2021). The GORKY glycoalkaloid transporter is indispensable for preventing tomato bitterness. *Nature Plants*, *7*(4), 468–480. https://doi.org/10.1038/s41477-021-00865-6

Khatodia, S., Bhatotia, K., Passricha, N., Khurana, S. M. P., & Tuteja, N. (2016). The CRISPR/Cas Genome-Editing Tool: Application in Improvement of Crops. *Frontiers in Plant Science*, *7*. https://doi.org/10.3389/fpls.2016.00506

Kimura, S., & Sinha, N. (2008). Tomato (*Solanum lycopersicum*): A Model Fruit-Bearing Crop. *Cold Spring Harbor Protocols*, *2008*(11), pdb.emo105. https://doi.org/10.1101/pdb.emo105

Kirsh, V. A., Hayes, R. B., Mayne, S. T., Chatterjee, N., Subar, A. F., Dixon, L. B., Albanes, D., Andriole, G. L., Urban, D. A., & Peters, U. (2006). Supplemental and Dietary Vitamin E, β-Carotene, and Vitamin C Intakes and Prostate Cancer Risk. *JNCI: Journal of the National Cancer Institute*, *98*(4), 245–254. https://doi.org/10.1093/jnci/djj050

Kiyota, K., Yoshimitsu, M., Satsuki-Murakami, T., Akutsu, K., Kajimura, K., & Yamano, T. (2017). Detection of the tomato allergen Sola l 1 and evaluation of its reactivity after heat and papain treatment. *Food and Agricultural Immunology*, *28*(6), 1450–1459. https://doi.org/10.1080/09540105.2017.1347914

Klee, H. J., & Giovannoni, J. J. (2011). Genetics and Control of Tomato Fruit Ripening and Quality Attributes. *Annual Review of Genetics*, *45*(1), 41–59. https://doi.org/10.1146/annurev-genet-110410-132507

Koh, E., Kaffka, S., & Mitchell, A. E. (2013). A long-term comparison of the influence of organic and conventional crop management practices on the content of the glycoalkaloid *α* -tomatine in tomatoes: Cropping system comparisons of *α* -tomatine in tomatoes. *Journal of the Science of Food and Agriculture*, *93*(7), 1537–1542. https://doi.org/10.1002/jsfa.5951

Kurze, E., Lo Scalzo, R., Campanelli, G., & Schwab, W. (2018). Effect of tomato variety, cultivation, climate and processing on Sola l 4, an allergen from Solanum lycopersicum. *PLOS ONE*, *13*(6), e0197971. https://doi.org/10.1371/journal.pone.0197971

Le, L., Mahler, V., Lorenz, Y., Scheurer, S., Biemelt, S., Vieths, S., & Sonnewald, U. (2006). Reduced allergenicity of tomato fruits harvested from Lyc e 1–silenced transgenic tomato plants. *Journal of Allergy and Clinical Immunology*, *118*(5), 1176–1183. https://doi.org/10.1016/j.jaci.2006.06.031

Le, L. Q., Lorenz, Y., Scheurer, S., Fotisch, K., Enrique, E., Bartra, J., Biemelt, S., Vieths, S., & Sonnewald, U. (2006). Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-LTP (Lyc e 3) expression. *Plant Biotechnology Journal*, *4*(2), 231–242. https://doi.org/10.1111/j.1467-7652.2005.00175.x

Li, J., Scarano, A., Gonzalez, N. M., D'Orso, F., Yue, Y., Nemeth, K., Saalbach, G., Hill, L., de Oliveira Martins, C., Moran, R., Santino, A., & Martin, C. (2022). Biofortified tomatoes provide a new route to vitamin D sufficiency. *Nature Plants*, *8*(6), 611–616. https://doi.org/10.1038/s41477-022-01154-6

Li, S., Chen, K., & Grierson, D. (2019). A critical evaluation of the role of ethylene and MADS transcription factors in the network controlling fleshy fruit ripening. *New Phytologist*, *221*(4), 1724–1741. https://doi.org/10.1111/nph.15545

Li, X., Wang, Y., Chen, S., Tian, H., Fu, D., Zhu, B., Luo, Y., & Zhu, H. (2018). Lycopene Is Enriched in Tomato Fruit by CRISPR/Cas9-Mediated Multiplex Genome Editing. *Frontiers in Plant Science*, *9*, 559. https://doi.org/10.3389/fpls.2018.00559

Liu, F., Zhang, X., Lu, C., Zeng, X., Li, Y., Fu, D., & Wu, G. (2015). Non-specific lipid transfer proteins in plants: Presenting new advances and an integrated functional analysis. *Journal of Experimental Botany*, *66*(19), 5663–5681. https://doi.org/10.1093/jxb/erv313

Lorenz, Y., Enrique, E., Lequynh, L., Fotisch, K., Retzek, M., Biemelt, S., Sonnewald, U., Vieths, S., & Scheurer, S. (2006). Skin prick tests reveal stable and heritable reduction of allergenic potency of gene-silenced tomato fruits. *Journal of Allergy and Clinical Immunology*, *118*(3), 711–718. https://doi.org/10.1016/j.jaci.2006.05.014

Luthria, D. L., Mukhopadhyay, S., & Krizek, D. T. (2006). Content of total phenolics and phenolic acids in tomato (Lycopersicon esculentum Mill.) fruits as influenced by cultivar and solar UV radiation. *Journal of Food Composition and Analysis*, *19*(8), 771–777. https://doi.org/10.1016/j.jfca.2006.04.005

Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., Barrangou, R., Brouns, S. J. J., Charpentier, E., Haft, D. H., Horvath, P., Moineau, S., Mojica, F. J. M., Terns, R. M., Terns, M. P., White, M. F., Yakunin, A. F., Garrett, R. A., van der Oost, J., … Koonin, E. V. (2015). An updated evolutionary classification of CRISPR–Cas systems. *Nature Reviews Microbiology*, *13*(11), 722–736. https://doi.org/10.1038/nrmicro3569

Martí, R., Roselló, S., & Cebolla-Cornejo, J. (2016). Tomato as a Source of Carotenoids and Polyphenols Targeted to Cancer Prevention. *Cancers*, *8*(6), 58. https://doi.org/10.3390/cancers8060058

Martín-Pedraza, L., González, M., Gómez, F., Blanca-López, N., Garrido-Arandia, M., Rodríguez, R., Torres, M. J., Blanca, M., Villalba, M., & Mayorga, C. (2016). Two nonspecific lipid transfer proteins (nsLTPs) from tomato seeds are associated to severe symptoms of tomato-allergic patients. *Molecular Nutrition & Food Research*, *60*(5), 1172–1182. https://doi.org/10.1002/mnfr.201500782

Matas, A. J., Gapper, N. E., Chung, M.-Y., Giovannoni, J. J., & Rose, J. K. (2009). Biology and genetic engineering of fruit maturation for enhanced quality and shelf-life. *Current Opinion in Biotechnology*, *20*(2), 197–203. https://doi.org/10.1016/j.copbio.2009.02.015

Mengstie, M. A., & Wondimu, B. Z. (2021). Mechanism and Applications of CRISPR/Cas-9-Mediated Genome Editing. *Biologics: Targets and Therapy*, *Volume 15*, 353–361. https://doi.org/10.2147/BTT.S326422

Mihalache, G., Peres, C. I., Bodale, I., Achitei, V., Gheorghitoaie, M. V., Teliban, G. C., Cojocaru, A., Butnariu, M., Muraru, V., & Stoleru, V. (2020). Tomato Crop Performances under Chemical Nutrients Monitored by Electric Signal. *Agronomy*, *10*(12), 1915. https://doi.org/10.3390/agronomy10121915

Milner, S. E., Brunton, N. P., Jones, P. W., O' Brien, N. M., Collins, S. G., & Maguire, A. R. (2011). Bioactivities of Glycoalkaloids and Their Aglycones from Solanum Species. *Journal of Agricultural and Food Chemistry*, *59*(8), 3454–3484. https://doi.org/10.1021/jf200439q

Naidu, K. A. (2003). Vitamin C in human health and disease is still a mystery? An overview. *Nutrition Journal*, *2*(1), 7. https://doi.org/10.1186/1475-2891-2-7

Nakayasu, M., Akiyama, R., Kobayashi, M., Lee, H. J., Kawasaki, T., Watanabe, B., Urakawa, S., Kato, J., Sugimoto, Y., Iijima, Y., Saito, K., Muranaka, T., Umemoto, N., & Mizutani, M. (2020). Identification of α-Tomatine 23-Hydroxylase Involved in the Detoxification of a Bitter Glycoalkaloid. *Plant and Cell Physiology*, *61*(1), 21–28. https://doi.org/10.1093/pcp/pcz224

Nakayasu, M., Umemoto, N., Ohyama, K., Fujimoto, Y., Lee, H. J., Watanabe, B., Muranaka, T., Saito, K., Sugimoto, Y., & Mizutani, M. (2017). A Dioxygenase Catalyzes Steroid 16α-Hydroxylation in Steroidal Glycoalkaloid Biosynthesis. *Plant Physiology*, *175*(1), 120–133. https://doi.org/10.1104/pp.17.00501

Nonaka, S., Arai, C., Takayama, M., Matsukura, C., & Ezura, H. (2017). Efficient increase of ɣ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis. *Scientific Reports*, *7*(1), 7057. https://doi.org/10.1038/s41598-017-06400-y

Noonan, S. C., & Savage, G. P. (1999). Oxalate content of foods and its effect on humans. *Asia Pacific Journal of Clinical Nutrition*, *8*(1), 64–74.

Odriozola-Serrano, I., Soliva-Fortuny, R., Hernández-Jover, T., & Martín-Belloso, O. (2009). Carotenoid and phenolic profile of tomato juices processed by high intensity pulsed electric fields compared with conventional thermal treatments. *Food Chemistry*, *112*(1), 258–266. https://doi.org/10.1016/j.foodchem.2008.05.087

Oguri, S., Amano, K., Nakashita, H., Nagata, Y., & Momonoki, Y. S. (2008). Molecular Structure and Properties of Lectin from Tomato Fruit. *Bioscience, Biotechnology, and Biochemistry*, *72*(10), 2640–2650. https://doi.org/10.1271/bbb.80310

Oh, K., Ivanchenko, M. G., White, T. J., & Lomax, T. L. (2006). The diageotropica gene of tomato encodes a cyclophilin: A novel player in auxin signaling. *Planta*, *224*(1), 133–144. https://doi.org/10.1007/s00425-005-0202-z

Ohyama, A., Hirai, M., & Nishimura, S. (1992). A novel cDNA clone for acid invertase in tomato fruit. *The Japanese Journal of Genetics*, *67*(6), 491–492. https://doi.org/10.1266/jjg.67.491

Ojo, M. A. (2022). Tannins in Foods: Nutritional Implications and Processing Effects of Hydrothermal Techniques on Underutilized Hard-to-Cook Legume Seeds—A Review. *Preventive Nutrition and Food Science*, *27*(1), 14–19. https://doi.org/10.3746/pnf.2022.27.1.14

Olejnik, P., Mądrzak, C. J., & Nuc, K. (2021). Cyclophilins and Their Functions in Abiotic Stress and Plant–Microbe Interactions. *Biomolecules*, *11*(9), 1390. https://doi.org/10.3390/biom11091390

Oostende, C. van, Widhalm, J. R., & Basset, G. J. C. (2008). Detection and quantification of vitamin K1 quinol in leaf tissues. *Phytochemistry*, *69*(13), 2457–2462. https://doi.org/10.1016/j.phytochem.2008.07.006

Ortolani, C., Ispano, M., Pastorello, E. A., Ansaloni, R., & Magri, G. C. (1989). Comparison of results of skin prick tests (with fresh foods and commercial food extracts) and RAST in 100 patients with oral allergy syndrome. *Journal of Allergy and Clinical Immunology*, *83*(3), 683–690. https://doi.org/10.1016/0091-6749(89)90083-3

Oyetayo, F. L., & Ibitoye, M. F. (2012). Phytochemical and nutrient/antinutrient interactions in cherry tomato (*Lycopersicon esculentum*) fruits. *Nutrition and Health*, *21*(3), 187–192. https://doi.org/10.1177/0260106012467241

Park, C.-J., Kim, K.-J., Shin, R., Park, J. M., Shin, Y.-C., & Paek, K.-H. (2004). Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway: Characterization of the hot pepper PR-10. *The Plant Journal*, *37*(2), 186–198. https://doi.org/10.1046/j.1365-313X.2003.01951.x

Paudel, J. R., Davidson, C., Song, J., Maxim, I., Aharoni, A., & Tai, H. H. (2017). Pathogen and Pest Responses Are Altered Due to RNAi-Mediated Knockdown of *GLYCOALKALOID METABOLISM 4* in *Solanum tuberosum*. *Molecular Plant-Microbe Interactions®*, *30*(11), 876–885. https://doi.org/10.1094/MPMI-02-17-0033-R

Pecker, I., Gabbay, R., Cunningham, F. X., & Hirschberg, J. (1996). Cloning and characterization of the cDNA for lycopene β-cyclase from tomato reveals decrease in its expression during fruit ripening. *Plant Molecular Biology*, *30*(4), 807–819. https://doi.org/10.1007/BF00019013

Perea-Domínguez, X. P., Hernández-Gastelum, L. Z., Olivas-Olguin, H. R., Espinosa-Alonso, L. G., Valdez-Morales, M., & Medina-Godoy, S. (2018). Phenolic composition of tomato varieties and an industrial tomato by-product: Free, conjugated and bound phenolics and antioxidant activity. *Journal of Food Science and Technology*, *55*(9), 3453–3461. https://doi.org/10.1007/s13197-018-3269-9

Peters, R. L., Krawiec, M., Koplin, J. J., & Santos, A. F. (2021). Update on food allergy. *Pediatric Allergy and Immunology*, *32*(4), 647–657. https://doi.org/10.1111/pai.13443

Petersen, A., Vieths, S., Aulepp, H., Schlaak, M., & Becker, W. (1996). Ubiquitous structures responsible for IgE cross-reactivity between tomato fruit and grass pollen allergens. *Journal of Allergy and Clinical Immunology*, *98*(4), 805–815. https://doi.org/10.1016/S0091-6749(96)70130-6

Petroski, W., & Minich, D. M. (2020). Is There Such a Thing as "Anti-Nutrients"? A Narrative Review of Perceived Problematic Plant Compounds. *Nutrients*, *12*(10), 2929. https://doi.org/10.3390/nu12102929

Petruk, G., Raiola, A., Del Giudice, R., Barone, A., Frusciante, L., Rigano, M. M., & Monti, D. M. (2016). An ascorbic acid-enriched tomato genotype to fight UVA-induced oxidative stress in normal human keratinocytes. *Journal of Photochemistry and Photobiology B: Biology*, *163*, 284–289. https://doi.org/10.1016/j.jphotobiol.2016.08.047

Petry, N., Egli, I., Zeder, C., Walczyk, T., & Hurrell, R. (2010). Polyphenols and Phytic Acid Contribute to the Low Iron Bioavailability from Common Beans in Young Women. *The Journal of Nutrition*, *140*(11), 1977–1982. https://doi.org/10.3945/jn.110.125369

Peumans, W. J., Rougé, P., & van DAMME, E. J. M. (2003). The tomato lectin consists of two homologous chitin-binding modules separated by an extensin-like linker. *Biochemical Journal*, *376*(3), 717–724. https://doi.org/10.1042/bj20031069

Porteus, M. (2011). Seeing the light: Integrating genome engineering with double-strand break repair. *Nature Methods*, *8*(8), 628–630. https://doi.org/10.1038/nmeth.1656

Porteus, M. (2016). Genome Editing: A New Approach to Human Therapeutics. *Annual Review of Pharmacology and Toxicology*, *56*(1), 163–190. https://doi.org/10.1146/annurev-pharmtox-010814-124454

Preedy, V. R., & Watson, R. R. (2008). *Tomatoes and tomato products: Nutritional, medicinal and therapeutic properties*. Science publ.

Raiola, A., Rigano, M. M., Calafiore, R., Frusciante, L., & Barone, A. (2014). Enhancing the Health-Promoting Effects of Tomato Fruit for Biofortified Food. *Mediators of Inflammation*, *2014*, 1–16. https://doi.org/10.1155/2014/139873

Remington, B. C., Westerhout, J., Meima, M. Y., Blom, W. M., Kruizinga, A. G., Wheeler, M. W., Taylor, S. L., Houben, G. F., & Baumert, J. L. (2020). Updated population minimal eliciting

dose distributions for use in risk assessment of 14 priority food allergens. *Food and Chemical Toxicology*, *139*, 111259. https://doi.org/10.1016/j.fct.2020.111259

Rick, C. M., Uhlig, J. W., & Jones, A. D. (1994). High alpha-tomatine content in ripe fruit of Andean Lycopersicon esculentum var. cerasiforme: Developmental and genetic aspects. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(26), 12877–12881. https://doi.org/10.1073/pnas.91.26.12877

Riso, P., Visioli, F., Erba, D., Testolin, G., & Porrini, M. (2004). Lycopene and vitamin C concentrations increase in plasma and lymphocytes after tomato intake. Effects on cellular antioxidant protection. *European Journal of Clinical Nutrition*, *58*(10), 1350–1358. https://doi.org/10.1038/sj.ejcn.1601974

Rodríguez del Río, P., Díaz-Perales, A., Sánchez-García, S., Escudero, C., Ibáñez, M., Méndez-Brea, P., & Barber, D. (2018). Profilin, a Change in the Paradigm. *Journal of Investigational Allergology and Clinical Immunology*, *28*(1), 1–12. https://doi.org/10.18176/jiaci.0193

Romano, P. G. N., Horton, P., & Gray, J. E. (2004). The Arabidopsis Cyclophilin Gene Family. *Plant Physiology*, *134*(4), 1268–1282. https://doi.org/10.1104/pp.103.022160

Ronen, G., Carmel-Goren, L., Zamir, D., & Hirschberg, J. (2000). An alternative pathway to β-carotene formation in plant chromoplasts discovered by map-based cloning of *Beta* and *old-gold* color mutations in tomato. *Proceedings of the National Academy of Sciences*, *97*(20), 11102–11107. https://doi.org/10.1073/pnas.190177497

Ronen, G., Cohen, M., Zamir, D., & Hirschberg, J. (1999). Regulation of carotenoid biosynthesis during tomato fruit development: Expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutantDelta. *The Plant Journal*, *17*(4), 341–351. https://doi.org/10.1046/j.1365-313X.1999.00381.x

Roth, N., Klimesch, J., Dukowic-Schulze, S., Pacher, M., Mannuss, A., & Puchta, H. (2012). The requirement for recombination factors differs considerably between different pathways of homologous double-strand break repair in somatic plant cells: *Recombination factors and pathways of homologous double-strand break repair*. *The Plant Journal*, *72*(5), 781–790. https://doi.org/10.1111/j.1365-313X.2012.05119.x

Rudolf, K., & Rudolf, E. (2016). Antiproliferative effects of α-tomatine are associated with different cell death modalities in human colon cancer cells. *Journal of Functional Foods*, *27*, 491–502. https://doi.org/10.1016/j.jff.2016.10.005

Saini, R. K., Nile, S. H., & Park, S. W. (2015). Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities. *Food Research International*, *76*, 735–750. https://doi.org/10.1016/j.foodres.2015.07.047

Salminen, T. A., Blomqvist, K., & Edqvist, J. (2016). Lipid transfer proteins: Classification, nomenclature, structure, and function. *Planta*, *244*(5), 971–997. https://doi.org/10.1007/s00425-016-2585-4

Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature Biotechnology*, *32*(4), 347–355. https://doi.org/10.1038/nbt.2842

Savary, S., Ficke, A., Aubertot, J.-N., & Hollier, C. (2012). Crop losses due to diseases and their implications for global food production losses and food security. *Food Security*, *4*(4), 519–537. https://doi.org/10.1007/s12571-012-0200-5

Sawai, S., Ohyama, K., Yasumoto, S., Seki, H., Sakuma, T., Yamamoto, T., Takebayashi, Y., Kojima, M., Sakakibara, H., Aoki, T., Muranaka, T., Saito, K., & Umemoto, N. (2014). Sterol Side Chain Reductase 2 Is a Key Enzyme in the Biosynthesis of Cholesterol, the Common Precursor of Toxic Steroidal Glycoalkaloids in Potato. *The Plant Cell*, *26*(9), 3763–3774. https://doi.org/10.1105/tpc.114.130096

Schaart, J. G., van de Wiel, C. C. M., Lotz, L. A. P., & Smulders, M. J. M. (2016). Opportunities for Products of New Plant Breeding Techniques. *Trends in Plant Science*, *21*(5), 438–449. https://doi.org/10.1016/j.tplants.2015.11.006

Silva-Beltrán, N. P., Ruiz-Cruz, S., Cira-Chávez, L. A., Estrada-Alvarado, M. I., Ornelas-Paz, J. de J., López-Mata, M. A., Del-Toro-Sánchez, C. L., Ayala-Zavala, J. F., & Márquez-Ríos, E. (2015). Total Phenolic, Flavonoid, Tomatine, and Tomatidine Contents and Antioxidant and Antimicrobial Activities of Extracts of Tomato Plant. *International Journal of Analytical Chemistry*, *2015*, 1–10. https://doi.org/10.1155/2015/284071

Singh, B., Singh, J. P., Kaur, A., & Singh, N. (2016). Bioactive compounds in banana and their associated health benefits – A review. *Food Chemistry*, *206*, 1–11. https://doi.org/10.1016/j.foodchem.2016.03.033

Singh, B., Singh, J. P., Kaur, A., & Singh, N. (2018). Phenolic compounds as beneficial phytochemicals in pomegranate (Punica granatum L.) peel: A review. *Food Chemistry*, *261*, 75–86. https://doi.org/10.1016/j.foodchem.2018.04.039

Sinha, M., Singh, R. P., Kushwaha, G. S., Iqbal, N., Singh, A., Kaushik, S., Kaur, P., Sharma, S., & Singh, T. P. (2014). Current Overview of Allergens of Plant Pathogenesis Related Protein Families. *The Scientific World Journal*, *2014*, 1–19. https://doi.org/10.1155/2014/543195

Sonawane, P. D., Heinig, U., Panda, S., Gilboa, N. S., Yona, M., Kumar, S. P., Alkan, N., Unger, T., Bocobza, S., Pliner, M., Malitsky, S., Tkachev, M., Meir, S., Rogachev, I., & Aharoni, A. (2018). Short-chain dehydrogenase/reductase governs steroidal specialized metabolites structural diversity and toxicity in the genus *Solanum*. *Proceedings of the National Academy of Sciences*, *115*(23). https://doi.org/10.1073/pnas.1804835115

Sonawane, P. D., Jozwiak, A., Panda, S., & Aharoni, A. (2020). 'Hijacking' core metabolism: A new panache for the evolution of steroidal glycoalkaloids structural diversity. *Current Opinion in Plant Biology*, *55*, 118–128. https://doi.org/10.1016/j.pbi.2020.03.008

Sonawane, P. D., Pollier, J., Panda, S., Szymanski, J., Massalha, H., Yona, M., Unger, T., Malitsky, S., Arendt, P., Pauwels, L., Almekias-Siegl, E., Rogachev, I., Meir, S., Cárdenas, P. D., Masri, A., Petrikov, M., Schaller, H., Schaffer, A. A., Kamble, A., … Aharoni, A. (2017). Plant cholesterol biosynthetic pathway overlaps with phytosterol metabolism. *Nature Plants*, *3*(1), 16205. https://doi.org/10.1038/nplants.2016.205

Sucha, L., & Tomsik, P. (2016). The Steroidal Glycoalkaloids from Solanaceae: Toxic Effect, Antitumour Activity and Mechanism of Action. *Planta Medica*, *82*(05), 379–387. https://doi.org/10.1055/s-0042-100810

Szymański, J., Bocobza, S., Panda, S., Sonawane, P., Cárdenas, P. D., Lashbrooke, J., Kamble, A., Shahaf, N., Meir, S., Bovy, A., Beekwilder, J., Tikunov, Y., Romero de la Fuente, I., Zamir, D., Rogachev, I., & Aharoni, A. (2020). Analysis of wild tomato introgression lines elucidates the genetic basis of transcriptome and metabolome variation underlying fruit traits and pathogen response. *Nature Genetics*, *52*(10), 1111–1121. https://doi.org/10.1038/s41588-020-0690-6

Tham, E. H., & Leung, D. Y. M. (2018). How Different Parts of the World Provide New Insights Into Food Allergy. *Allergy, Asthma & Immunology Research*, *10*(4), 290. https://doi.org/10.4168/aair.2018.10.4.290

Thapar, R. (2015). Roles of Prolyl Isomerases in RNA-Mediated Gene Expression. *Biomolecules*, *5*(2), 974–999. https://doi.org/10.3390/biom5020974

van de Wiel, C. C. M., Schaart, J. G., Lotz, L. A. P., & Smulders, M. J. M. (2017). New traits in crops produced by genome editing techniques based on deletions. *Plant Biotechnology Reports*, *11*(1), 1–8. https://doi.org/10.1007/s11816-017-0425-z

Van Loon, L. C., & Van Strien, E. A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology*, *55*(2), 85–97. https://doi.org/10.1006/pmpp.1999.0213

Verheul, M. J., Slimestad, R., & Tjøstheim, I. H. (2015). From Producer to Consumer: Greenhouse Tomato Quality As Affected by Variety, Maturity Stage at Harvest, Transport Conditions, and Supermarket Storage. *Journal of Agricultural and Food Chemistry*, *63*(20), 5026–5034. https://doi.org/10.1021/jf505450j

Vermeer, C., Shearer, M. J., Zittermann, A., Bolton-Smith, C., Szulc, P., Hodges, S., Walter, P., Rambeck, W., Stocklin, E., & Weber, P. (2004). Beyond Deficiency: Potential benefits of increased intakes of vitamin K for bone and vascular health. *European Journal of Nutrition*, *43*(6), 325–335. https://doi.org/10.1007/s00394-004-0480-4

Volpicella, M., Leoni, C., Fanizza, I., Rinalducci, S., Placido, A., & Ceci, L. R. (2015). Expression and characterization of a new isoform of the 9 kDa allergenic lipid transfer protein from tomato (variety San Marzano). *Plant Physiology and Biochemistry*, *96*, 64–71. https://doi.org/10.1016/j.plaphy.2015.07.019

Wangorsch, A., Jamin, A., Foetisch, K., Malczyk, A., Reuter, A., Vierecke, S., Schülke, S., Bartel, D., Mahler, V., Lidholm, J., Vieths, S., & Scheurer, S. (2015). Identification of Sola l 4 as Bet v 1 homologous pathogenesis related-10 allergen in tomato fruits. *Molecular Nutrition & Food Research*, *59*(3), 582–592. https://doi.org/10.1002/mnfr.201300620

Wei, M.-M., & Gregory, J. F. (1998). Organic Acids in Selected Foods Inhibit Intestinal Brush Border Pteroylpolyglutamate Hydrolase in Vitro: Potential Mechanism Affecting the Bioavailability of Dietary Polyglutamyl Folate. *Journal of Agricultural and Food Chemistry*, *46*(1), 211–219. https://doi.org/10.1021/jf970662g

Welter, S., Dölle, S., Lehmann, K., Schwarz, D., Weckwerth, W., Worm, M., & Franken, P. (2013). Pepino mosaic virus Infection of Tomato Affects Allergen Expression, but Not the Allergenic Potential of Fruits. *PLoS ONE*, *8*(6), e65116. https://doi.org/10.1371/journal.pone.0065116

Wensing, M., Akkerdaas, J. H., van Leeuwen, W. A., Stapel, S. O., Bruijnzeel-Koomen, C. A. F. M., Aalberse, R. C., Bast, B. J. E. G., Knulst, A. C., & van Ree, R. (2002). IgE to Bet v 1 and profilin: Cross-reactivity patterns and clinical relevance. *Journal of Allergy and Clinical Immunology*, *110*(3), 435–442. https://doi.org/10.1067/mai.2002.126380

Westphal, S., Kempf, W., Foetisch, K., Retzek, M., Vieths, S., & Scheurer, S. (2004). Tomato profilin Lyc e 1: IgE cross-reactivity and allergenic potency. *Allergy*, *59*(5), 526–532. https://doi.org/10.1046/j.1398-9995.2003.00413.x

Westphal, S., Kolarich, D., Foetisch, K., Lauer, I., Altmann, F., Conti, A., Crespo, J. F., Rodriguez, J., Enrique, E., Vieths, S., & Scheurer, S. (2003). Molecular characterization and allergenic activity of Lyc e 2 (beta-fructofuranosidase), a glycosylated allergen of tomato. *European Journal of Biochemistry*, *270*(6), 1327–1337. https://doi.org/10.1046/j.1432-1033.2003.03503.x

Willerroider, M., Fuchs, H., Ballmer-Weber, B. K., Focke, M., Susani, M., Thalhamer, J., Ferreira, F., Wüthrich, B., Scheiner, O., Breiteneder, H., & Hoffmann-Sommergruber, K. (2003). Cloning and Molecular and Immunological Characterisation of Two New Food Allergens, Cap a 2 and Lyc e 1, Profilins from Bell Pepper *(Capsicum annuum)* and Tomato *(Lycopersicon esculentum)*. *International Archives of Allergy and Immunology*, *131*(4), 245–255. https://doi.org/10.1159/000072136

Włodarczyk, K., Smolińska, B., & Majak, I. (2022). Tomato Allergy: The Characterization of the Selected Allergens and Antioxidants of Tomato (Solanum lycopersicum)—A Review. *Antioxidants*, *11*(4), 644. https://doi.org/10.3390/antiox11040644

Xie, Y.-R., Chen, Z.-Y., Brown, R. L., & Bhatnagar, D. (2010). Expression and functional characterization of two pathogenesis-related protein 10 genes from Zea mays. *Journal of Plant Physiology*, *167*(2), 121–130. https://doi.org/10.1016/j.jplph.2009.07.004

Yoo, H., Park, W., Lee, G.-M., Oh, C.-S., Yeam, I., Won, D.-C., Kim, C., & Lee, J. (2017). Inferring the Genetic Determinants of Fruit Colors in Tomato by Carotenoid Profiling. *Molecules*, *22*(5), 764. https://doi.org/10.3390/molecules22050764

Yu, L.-X., Nasrallah, J., Valenta, R., & Parthasarathy, M. V. (1998). Molecular cloning and mRNA localization of tomato pollen profilin. *Plant Molecular Biology*, *36*(5), 699–707. https://doi.org/10.1023/A:1005971327353

Yu, L.-X., & Parthasarathy, M. V. (2014). Molecular and Cellular Characterization of the Tomato Pollen Profilin, LePro1. *PLoS ONE*, *9*(1), e86505. https://doi.org/10.1371/journal.pone.0086505

Zacharisen, M. C., Elms, N. P., & Kurup, V. P. (2002). Severe tomato allergy (Lycopersicon esculentum). *Allergy and Asthma Proceedings*, *23*(2), 149–152.

Zhang, M., Yuan, B., & Leng, P. (2009). The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *Journal of Experimental Botany*, *60*(6), 1579–1588. https://doi.org/10.1093/jxb/erp026

Zhao, D.-K., Zhao, Y., Chen, S.-Y., & Kennelly, E. J. (2021). *Solanum* steroidal glycoalkaloids: Structural diversity, biological activities, and biosynthesis. *Natural Product Reports*, *38*(8), 1423–1444. https://doi.org/10.1039/D1NP00001B

Chapter 2. Editing tomato for low allergenicity and improved nutritional composition

Note: This chapter contains data included in a publication that will be submitted to Plant Biotechnology Journal in collaboration with the groups listed below.

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2.1 Introduction

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops cultivated around the world, and its fruit is a reserve of pro-nutritional compounds like carotenoids, vitamins, flavonoids (Chaudhary et al., 2018). For this reason, tomato is a very popular fruit crop grown and consumed by people all around the world and it is considered as an important component of healthy diet; in fact, the consumption of tomatoes is known to promote a series of anti-inflammatory and anti-oxidants effects (Raiola et al., 2014). However, the tomato fruit is a source also of some antinutritional compounds including glycoalkaloids (SGAs), tannins and phytates (Oyetayo & Ibitoye, 2012); in particular, SGAs are a group of compounds that show toxic effects in humans causing gastrointestinal and neurological disorders, disruption of membranes and inhibition of acetylcholine esterase activity (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2020; Mensinga et al., 2005). The compound α-tomatine is the most abundant form of SGAs present in both tomato leaves and green fruits, where they take part in the protection against plant pathogens including insects, bacteria, viruses, and fungi (Altesor et al., 2014; Campbell & Duffey, 1979; Chowański et al., 2016; Kaup et al., 2005; Sandrock & VanEtten, 1998; Seipke & Loria, 2008). On the contrary, in red ripe tomato fruit the content of α -tomatine is low (5 mg/kg) if compared to green fruit (500 mg/kg), due to the conversion to esculeoside A (Friedman, 2013).

The SGAs biosynthetic pathway starts from cholesterol and involves multiple enzymatic reactions encoded by *GLYCOALKALOID METABOLISM* (*GAME*) genes (Itkin et al., 2011, 2013; Sonawane et al., 2018), most of them are well known and have been functionally characterized: for instance, the oxidase *GAME 4*, the transaminase *GAME 12*, the dehydrogenase/reductase *GAME 25*, the glycosyltransferases *GAME 1*, *GAME 17*, *GAME 18*, *GAME 2* and *GAME 5* and finally the dioxygenase *GAME 31* (Alseekh et al., 2015; Cárdenas et al., 2019; Itkin et al., 2011, 2013; Nakayasu et al., 2017, 2020; Sonawane et al., 2018, 2020). More in detail, we focused our attention on the *GAME4* gene that is located in the branching point of the pathway leading to the formation of steroidal alkaloids on one hand, and steroidal saponins on the other hand. It has been previously shown that RNA interference of *GAME 4* gene determines a reduction of α-tomatine by a factor of ~100 (Itkin et al., 2013), therefore potentially the knock-out of this gene could lead to the complete lack of glycoalkaloids in the fruit.

Another very important aspect regarding the consumption of tomatoes for a healthy diet is the allergenicity of their fruits. Tomato is one of the most prevalent allergenic vegetables and, in fact, it is known that 16% of the population in Italy is affected by allergy towards this fruit (Asero R., 2013). Tomato allergy is associated to oral allergy syndrome (OAS), rhinitis, abdominal pain, dermatitis, urticaria and anaphylactic reactions (Zacharisen et al., 2002). Referring to the

Allergome site (https://www.allergome.org), the *Sola l* genes code for the seven most important allergens in tomato, most of which have been characterized at function, protein structure and allergenicity level: *Sola l 1* (profilin), *Sola l 2* (a fructofuranosidase), *Sola l 3* (lipid transfer protein), *Sola l 4* (phatogenesis-related-10), *Sola l 5* (cyclophilin), *Sola l 6* (lipid transfer protein) and *Sola l 7* (lipid transfer protein) (Włodarczyk et al., 2022).

In this study, taking into consideration previous evidences showing that cross reactivity among tomato and pollen is common (Asero, 2013), we focused on the Sola l 4 allergen, that is the homologous of the *Bet v 1* gene, the major allergen in birch pollen (Wangorsch et al., 2015). From an enzymatic point of view, Sola l 4 is a pathogenesis-related (PR) protein, belonging to class 10 (Wangorsch et al., 2015). Two different isoforms of the PR-protein Sola l 4 have been identified in tomato, Sola l 4.01 (Acc. KF682291) and Sola l 4.02 (Acc. KF682292) (Wangorsch et al., 2015). We chose to target the Sola l 4.02 isoform due to the fact that this isoform is the most expressed in fruit compared with the isoform Sola l 4.01 (Wangorsch et al., 2015), and its allergenicity has been previously demonstrated in different tomato cultivars (Kurze et al., 2018).

The purpose of this work has been to get eliminate and/or decrease the accumulation of some of these antinutritional and/or allergenic molecules in fruit using the new plant breeding technology (NPBT), and in particular relying on the CRISPR/Cas9 genome editing system. This approach has rapidly become the most powerful genome editing system in genetic engineering of crop (Zhang et al., 2020). In this study, we designed specific gRNAs for two genes of interest, *GAME4* to knock-out a key gene involved in the biosynthetic pathway of glycoalkaloids, and *Sola l 4*, one of the major allergens in tomato. In this way, we proposed an innovative NPBT-based strategy, in which we simultaneously targeted two different types of antinutritional components (a protein and a small metabolite), in order to obtain plants with an improved nutritional balance.

2.2 Materials and methods

2.2.1 sgRNA design, plasmid construction and plant transformation

Benchling gRNA Design Tool (https://benchling.com/signup) was used to design the gRNAs (Supplementary Table 1) targeting the *GAME4* (Solyc12g006460) and *Sola l 4* (Solyc09g090980) genes in the second and first exon, respectively (Figure 1A). GoldenBraid (GB) CRISPR domesticaror (https://gbcloning.upv.es/) tool was used to link the two small guide sequences with the appropriate construct harboring *Cas9* gene and other transcriptional units according with the Golden Braid technology (Vázquez-Vilar et al., 2016). Each cloning step was confirmed by both restriction enzyme analysis (RE) and sequencing. Finally, the pDGB3ω1 Tnos:nptII:Pnos—U6-26:gRNA (*GAME 4*):scaffold—U6-26:gRNA (*Sola l 4*):scaffold —P35S:hCas9:Tnos plasmid was

transferred to *Agrobacterium tumefaciens* (strain EHA105) electrocompetent cells and *in vitro* tomato transformation was performed as previously described (Gianoglio et al., 2022).

2.2.2 Plant materials and growth conditions

Solanum lycopersicum cv. MoneyMaker (MM) and CRISPR/Cas9-edited transgenic tomato plants were grown in a containment greenhouse under the following photoperiod: 16 h of lighting at 24 °C, 8 h of darkness at 18 °C as previously described (Diretto et al., 2020). Mature seeds were collected from the edited plants at T0 generation. They were then sterilized in 70% ethanol for 30 seconds and immersed in sodium hypochlorite (1%) for 10 minutes at room temperature with gentle shaking. The seeds were finally washed in sterile water for at least thirty minutes and placed, in dark condition, in petri dishes for germination. For both T1 and T2 generations, the germinated seeds were planted in the soil, and seedlings were grown under the above-mentioned growth conditions. Concerning all the -omic analysis, leaf samples were collected two months after transplantation, while fruits were harvested at the mature green (MG) and red ripe (RR) stages. For each line, three biological replicates were collected, each one consisting of a pool of five leaves or fruits of uniform size and color. Once sampled, the tissues were immediately frozen in liquid nitrogen, ground and stored at -80°C.

2.2.3 Molecular screening and sequence analysis

Genomic DNA was extracted from finely ground powder of WT and edited leaf tissues using the Plant/Fungi Isolation kit (NorgenBiotek Corp.), following the manufacturer's instructions. Specific primers (Supplementary Table 1) flanking the mutation site were used to amplify the target region of *GAME 4* (Solyc12g006460) and *Sola l 4* (Solyc09g090980) genes, in order to detect Cas9 induced mutations. The amplicons were Sanger-sequenced and compared with the WT reference sequence both using CodonCode Aligner program (ver. 9.0, www.codoncode.com/aligner/), which performs a multiple alignment of sequences, and TIDE (https://tide.deskgen.com/) (Brinkman et al., 2014) tool, which aligns specifically two chromatograms (WT and the specific line), thus allowing to identity and confirm mutation types and frequencies (insertions and/ or deletions) on different lines.

2.2.4 Detection of potential OFF-target activity

OFF-target events were predicted using Benchling tool according with the small guides design; the program, before finding sequences similar to our small guides in the tomato genome, generates a list of putative genes in which off-target events could potentially have occurred. From the list

(Supplementary Table 2), we selected off-targets containing "NGG" PAM sequence and less than four mismatched nucleotides. Since most of mismatches were located in intergenic regions, we decided to analyze only those present in the coding regions. The screening was performed as described above for edited plant selection.

2.2.5 Cloning, expression and purification of *Sola l 4* from *Solanum Lycopersicum*

The cDNA encoding Sola l 4 from *S. Lycopersicum* (GenBank NM_001288651.2) was amplified via PCR with the forward and reverse oligonucleotides (Supplementary Table 1) containing respectively the recognition sites for NcoI and XhoI restriction enzymes. The product of the amplification was cloned in the pETM11 vector (a kind gift from EMBL, Heidelberg), which allows for the expression of proteins with a N-terminus His tag, a long linker and a sequence recognized by Tobacco Etch Virus (TEV) protease. The recombinant protein was expressed in *E. coli* BL21 (DE3) cells, grown in LB broth and induced by adding 0.5 mM IPTG (Isopropil-b-D-1-tiogalattopiranoside) at OD_{600} of 0.6 and making a further growth for 16 h at 22°C. Cells were harvested by centrifugation at 4°C and resuspended in lysis buffer (20 mM Tris-HCl, 20 mM imidazole, 500 mM NaCl, pH 8.0), in presence of 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mg/ml DNaseI, 0.1 mg/ml lysozyme and 1 µg/mL Aprotinin, 1 µg/mL Leupeptin, 1 µg/mL Pepstatin protease inhibitors (Sigma-Aldrich). After sonication on ice and centrifugation of the lysate, the supernatant was loaded onto a 1 ml His Trap FF column (Cytiva), and the purification performed stepwise by FPLC. Finally, the protein was purified by Size Exclusion Chromatography (SEC) using a Superdex 75 column (Cytiva) in 20 mM Tris-HCl, 150 mM NaCl, pH 8.0. Protein purity was assessed by SDS-PAGE, on a 15% gel, using Euroclone Sharp Mass VII (6.5–240 kDa) as molecular mass marker.

2.2.6 Proteins extraction and Western blot analysis

Firstly, based on the data present in bibliography, several extraction buffers, different in salts composition and pH, were tested for their efficiency in protein extraction from tomato fruits, preserving soluble fractions. The McIlvaine buffer (pH 2.8) (Granell et al., 1987), a buffer described in the paper of Kurze et al., 2018 (pH 6.5), the Phosphate Buffered Saline (pH 7.5), and the Glycerol Buffer, GB (pH 8.5) (Donald et al., 1993) were analyzed, evaluating the protein content after the extraction through a Bradford Assay, and the proteins profile through an SDS-PAGE Coomassie Blue stained. The results obtained (data not shown) allowed us to identify the GB buffer as the more suitable for our purposes to be used in the following experiment.

Total protein extracts were obtained from tomato fruits by grinding into a fine powder 300 mg of tissue in liquid nitrogen and by suspending it 1:1 w/v in GB extraction buffer (100 mM TrisHCl, 10% glycerol, 14% sucrose, 5 mM MgCl2, 10 mM KCl, 10 mM 2-mercaptoethanol, pH 8.1). Samples were centrifuged at 20000 x g, for 30 min, at 4°C, then quantified using the Bradford Assay (Bio-Rad). Ten µg of red-ripe tomato extracts, six µg of mature green tomato extracts and 50 ng of the recombinant protein were separated on 12% (w/v) mini-protean TGX precast gel (Bio-Rad) in reducing and denaturing conditions, before transferring onto PVDF membranes by using the Trans-Blot Turbo Transfer System (Bio-Rad). A Coomassie blue stained gel was used to assess normalization (Supplementary figure 2B). The membrane was blocked with ECL™ Advance Blocking Reagent (2%) in PBS containing 0.1% (v/v) Tween-20 (PBS-T), 1 h at room temperature (RT) by gentle agitation, then over night at 4°C. After washing three times with PBS-T, the membrane was incubated for 2 h at RT with 1:1000 dilution in 2% ECL™ Advance Blocking Reagent (Thermo Fischer) in PBS-T, of a rabbit polyclonal antibody specific for the wild-type Sola l 4 protein (gently provided by Dr. Elisabeth Kurze, Biotechnology of Natural Products, Technische Universitat Munchen, Freising, Germany). After further washings (as described above), the membrane was incubated for 1 h at RT with 1:2500 dilution in 2% ECL™ Advance Blocking Reagent (Thermo Fischer) in PBS-T, of Sheep Anti-Rabbit IgG (H+L) Secondary Antibody, HRP (Thermo Fisher Scientific), as indicated by the manufacturers. The signal development was obtained on washed membranes by enhanced chemiluminescence (Amersham ECL Prime, Cytiva) and the images acquired through the iBright Imaging System (Thermo Fischer).

2.2.7 Non-volatile metabolite extraction and LC-HRMS analysis

For semi-polar analysis, tomato fruit and leaves were freeze-dried and 10mg of powder were ground with tungsten beads at 25 Hz for 3 min with the tissuelyser (mixermill MM 300, Retsch). Samples were resuspended in 500µl or 1 ml, respectively for fruit or leaves, of 75% (v/v) cold methanol with 0.1% Formic Acid (Sigma-Aldrich, Cat. 5630-50ML-F), spiked with 0.5 mg/mL formononetin (Sigma–Aldrich, Cat. No. 47752-25MG-F) and metabolites were extracted at RT by orbital shaker (VDRL MOD. 711+ Asal S.r.l.) at 20 Hz for 20 min two times; subsequently, samples were centrifuged at 20,000g for 20 min and 400 µl of supernatant was collected and filtered with HPLC filter tubes (0.45 mm pore size, Whatmann, Cat. 514-8110). Liquid Chromatography coupled to High-Resolution Mass Spectrometry (LC-HRMS) analysis was carried out as previously reported (Noronha et al., 2022). Full scan MS with data-dependent MS/MS fragmentation in both positive and negative ionization mode was used for metabolite

identification, through the comparison with authentic standards, when available, and on the basis of the accurate masses obtained from the Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) for native compounds or from the Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/) for adducts. For the identification of the de novo accumulated saponins, in silico (X. Chen et al., 2022; Kaunda & Zhang, 2019) coupled to manual curation of MS chromatograms was carried out, and tentative ions were validated as aforementioned. Data were quantified as fold internal standard. Non polar analysis was performed as reported previously (Frusciante et al., 2022) with slight modification: 5 and 3mg DW of tomato fruit and leaves were used for metabolites extractions.

2.2.8 Volatile metabolite extraction and GC-MS analysis

Fruit samples were processed similarly as described in (Rambla et al., 2016). Roughly, five hundred milligrams of frozen tomato powder were introduced in a 15 mL glass vial, the cap closed and incubated at 37ºC for 10 min in a water bath. Five hundred mL of an EDTA 100 mM, pH 7.5 solution and 1.1 g of $CaCl_2.2H_2O$ were added, gently mixed and sonicated for 5 min. One mL of the resulting paste was transferred to a 10 mL screw cap headspace vial with silicon/PTFE septum and analyzed within 10 hours. Tomato leaves were flash-frozen in liquid nitrogen and ground with mortar and pestle. One hundred mg of this frozen powder were introduced in a 10 mL screw cap headspace vial with silicon/PTFE septum. Then, 1 mL of a 5M $CaCl_2$ solution and 150 µL of 500 mM EDTA pH 7.5 were added. Then the vial was closed and sonicated for 5 min. Samples were analyzed within 3 hours after preparation. Volatile compounds were extracted from the headspace by first preincubating the vials at 50°C for 10 min under 500 rpm agitation. A 65 µm PDMS/DVB SPME fiber (SUPELCO) was then introduced in the vial and exposed to the headspace for 20 min, with identical conditions of agitation and temperature. The volatile compounds adsorbed in the fiber were desorbed in the injection port of the gas chromatograph at 250°C for 1 min in splitless mode. The fiber was then cleaned at 250ºC for an additional 5 min in an SPME Fiber Conditioning Module (CTC Analytics) to prevent cross-contamination between samples. Incubation, extraction, injection and fiber cleaning were performed by means of a CombiPAL autosampler (CTC Analytics). Chromatography was performed on a 6890N gas chromatograph (Agilent) with a DB-5ms (60 m, 0.25 mm, 1.00 µm) capillary column (J&W) with a constant helium flow of 1.2 mL/min. Oven ramp conditions were: 40°C for 2 min, 5°C/min ramp until 250°C and a final hold at 250°C for 5 min. GC interface and MS source temperatures were 260°C and 230°C, respectively. Detection was performed in a 5975B mass spectrometer (Agilent) in the 35–300 m/z range at 5.2 scans/s, with 70 eV electron impact ionization. Data were recorded and processed with the

MassHunter software (Agilent). Unequivocal identification of volatile compounds was performed by comparison of both mass spectra and retention time with those of pure standards (Sigma). For quantitation, one specific ion was selected for each compound and the corresponding peak from the extracted ion chromatogram was integrated. An admixture reference sample was prepared for each season by mixing thoroughly equal amounts of each sample. An aliquot of the admixture was analyzed every six samples and processed as any other sample as part of the injection series. This admixture was used as a reference to normalize for temporal variation and fiber aging.

2.2.9 Proteomic analysis

Proteins from powder prepared from *S. lycopersicum* leaves and fruits were extracted in SDT buffer (4% SDS, 0.1M DTT, 0.1M Tris/HCl, pH 7.6) in a thermomixer (Eppendorf ThermoMixer C, 60 min, 95°C, 750 rpm). After that, all samples were centrifuged (15 min, 20,000 x g) and the supernatants (ca 50 µg of total protein) used for filter-aided sample preparation (FASP) as described elsewhere (Mikulášek et al., 2021) using 1 µg of trypsin (sequencing grade; Promega). Resulting peptides were extracted into LC-MS vials by 2.5% formic acid (FA) in 50% acetonitrile (ACN) and 100% ACN with addition of polyethylene glycol (final concentration 0.001%) (REF) and concentrated in a SpeedVac concentrator (Thermo Fisher Scientific).

Resulting peptides were analysed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) performed using UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) on-line coupled with Orbitrap Exploris 480 spectrometer (Thermo Fisher Scientific). See supplemental material section for full details regarding the analyses and data evaluation. Data were acquired in a data-independent acquisition mode (DIA). The survey scan covered m/z range of 350-1400 at resolution of 60,000 (at m/z 200) and maximum injection time of 55 ms (normalized AGC target 300%). HCD MS/MS (27% relative fragmentation energy) were acquired in the range of m/z 200-2000 at 30,000 resolutions (maximum injection time 55 ms, normalized AGC target 1000%). Overlapping windows scheme in the precursor m/z range from 400 to 800 were used as isolation window placements. DIA data were processed in DIA-NN3 (version 1.8) in library free mode against modified cRAP database (based on http://www.thegpm.org/crap/; 111 sequences in total) and UniProtKB protein database for *Solanum lycopersicum* sapiens (https://ftp.uniprot.org/pub/databases/uniprot/current_release/knowledgebase/reference_proteomes/Eukaryota/UP000004994/UP000004994_4081.fasta.gz; version 2022/03, number of protein sequences: 34,655). No optional, carbamide methylation as fixed modification and trypsin/P enzyme with 1 allowed missed cleavages and peptide length 6-30 were set during the library preparation. False discovery rate (FDR) control was set to 1% FDR. MS1 and MS2 accuracies as

well as scan window parameters were set based on the initial test searches (median value from all samples ascertained parameter values). Protein MaxLFQ intensities reported in the DIA-NN main report file were further processed using the software container environment (https: //github.com/OmicsWorkflows), version 4.1.3a.

Processing workflow consists on: a) removal of low-quality precursors and contaminant protein groups, b) protein group MaxLFQ intensities log2 transformation, c) filtering out of protein groups not quantified in more than half of the replicates of at least one sample type, d) imputation of the missing values from the random distribution around the global minimal value, e) differential expression analysis using LIMMA statistical test. Proteins with adjusted p-value <0.05 and fold change >|1.5| were considered as significantly changing ones. Finally, Gene Ontology Enrichment Analysis (GOEA) by using Panther with Accession protein ID was performed.

2.2.10 Fruit phenotypic trait measurements

In order to evaluate the ripening attitude of the edited fruits, a series of phenotypic parameters were considered. More in detail, blooming flowers were tagged in order to evaluate possible alterations during fruit maturation. Subsequently, water loss determination was performed on at least 10 berries for each genotype from four different plants. Fruits were harvested and weighted immediately after detachment at MG stage, and were kept at constant temperature and relative humidity (22 ± 2 °C, 60 ± 5%), by measuring fruit weight reduction at five different ripening stages: Br, Br+4, Br+7, Br+10 and Br+15. The same experimental conditions were used in order to evaluate fruit firmness with a 4502 Instron Testing machine (Instron). Fruit compression resistance (CR) was measured positioning tomato berries on load cell. A stainless-steel probe, after establishing zero-force contact with the fruit, was used to compress of 2.5 mm two points of the equator of the fruit. Firmness was expressed at the maximum force in grams-force (gf) per surface area. Finally, a total of 5 tomato fruits at RR stage were used for Brix analysis. Briefly, four replicates (~ 500 mg) for each genotype were homogenized in bi-distilled water (1:1 w/v). Samples were centrifuged 15,000 x g, for 5 min and 100 µl of supernatant were distributed on the refractometer.

2.3 Results

2.3.1 CRISPR/Cas-9-targeted editing of *GAME 4* and *Sola l 4* and screening of mutants

To generate mutants of the tomato *GAME 4* and *Sola l 4* genes, a single CRISPR/Cas-9 construct containing one sgRNA for each gene was designed. In Figure 1A, the position of the two sgRNAs in the *GAME 4* and *Sola l 4* genes structure can be seen. Totally, 38 primary transformants (T0)

were obtained through Agrobacterium-mediated transformation and plants containing mutations simultaneously within the *GAME 4* and *Sola l 4* genes were selected *via* PCR amplification and sequencing by Sanger method (Supplementary Figure 1A). Two different primer combinations were designed, respectively flanking GAME 4-sgRNA and Sola l 4-sgRNA (Figure 1 and Supplementary Table 1). The screening was performed by comparing, using TIDE tool, wild-type gene sequence with gene sequence of edited plant (Figure 1B). Subsequently, edited lines carrying homozygous or heterozygous mutations indifferently were selected and subsequently self-pollinated to produce T1 and T2 progenies. T2 progenies were analyzed by PCR and individual T2 plants homozygous for the mutation were selected to produce T3 progenies to facilitate further characterization of plants. Also, in the homozygous T3 lines, the presence of mutant alleles was verified by repeating the PCR amplification. Overall, we identified seven different mutation events for *GAME 4* gene and six for *Sola l 4* gene (Supplementary Figure 1B). All the mutation events are predicted to generate premature translation termination codons (PTTCs) in the transcript (Supplementary Figure 2A) with resulting truncated protein. For all further characterizations we chose two lines: the line 1.2, carrying a deletion of -10 nucleotides in the *Sola l 4* gene and a deletion of -4 nucleotides in the *GAME 4* gene, and the line 13A14 carrying an insertion of +1 nucleotides in the *Sola l 4* gene and a deletion of -8 nucleotides in the *GAME 4* gene. The resulting truncated proteins, with a deletion of 75,6-76,4% of aa content for GAME 4 and of 62,5-66,9% of aa content for Sola l 4, are totally misfolded and not able to maintain any secondary/tertiary structures similar to WT proteins (Figure 1C).

2.3.2 OFF-target analysis

In order to investigate if CRISPR/Cas9 editing system could affect genomic regions different from target genes, we analyzed putative off-target genes based on predicted Benchling tool. The tool found sequences in the tomato genome sharing similarity with the sequence of our small guides. We screened a total of four potential OFF-target sites, two for each gene to determine whether OFF-target occurred (Supplementary Table 2). Sequence analysis of putative genes in edited lines compared with wild type showed no differences, indicating no OFF-target mutation had occurred in the *GAME 4* and *Sola l 4* edited lines.

2.3.3 Protein extraction and Western Blot analysis

A western blot experiment has been performed to verify the presence/absence of the protein in the edited transgenic lines and its immunogenicity. Total soluble proteins were extracted from tomato fruits of the wild-type, 1.2 line and 13A line plants, as described in the Materials and Methods

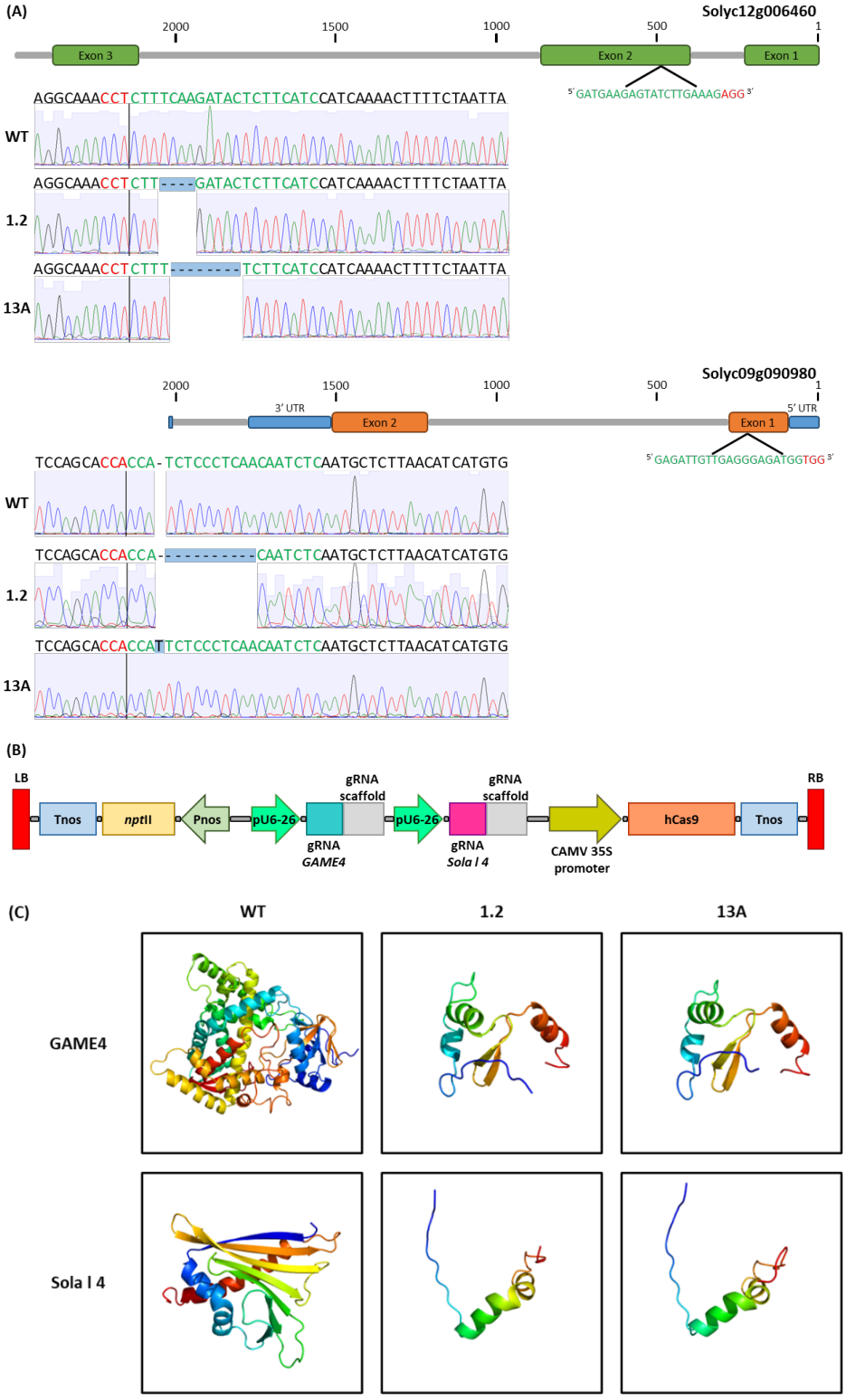


Figure 1. *(A) Structure and genome editing mutations of target genes. Schematic architecture of GAME 4 (Solyc12g006460) and Sola l 4 (Solyc09g090980) genes. The gRNA sequences and the protospacer-adjacent motif sequences are shown in green and red, respectively. Electropherograms show the WT sequence and the mutated sequences of target genes; mutations in each line are highlighted in light blue. (B) Gene editing construct. T-DNA structure utilized to create tomato edited mutants. Two Arabidopsis PolIII U6-26 promoters drive the expression of GAME 4 and Sola l 4 gRNAs, the CaMV 35S promoter drives the expression of human Cas9 endonuclease; the nos promoter drives the production of the nptII selection marker for kanamycin resistance. (C) Structure of GAME 4 and Sola l 4 WT proteins and putative reconstruction of edited proteins. Structural models were constructed using Phyre 2 (Kelley et al., 2015).*

section. Extracts, quantified and normalized, were loaded on an SDS-PAGE together with the recombinant protein produced in *E. coli*, and Sola l 4 was detected by using a polyclonal antibody specific for the protein produced in bacteria. As shown in Figure 2, the antibody was able to identify the positive control, *i.e.,* the recombinant protein produced in *E. coli*, at the expected molecular size of 23 kDa, due to the presence of the tags, as well as the protein in wild-type tissues at the expected molecular size of approximately 17.4 kDa. Moreover, western blot showed a higher presence of the allergen in the fruit at the MG stage than in the RR, suggesting that Sola l 4 content decreases during ripening process. On the contrary, no signal was revealed in both edited transgenic lines, confirming that the protein was no longer expressed in its native conformation, and its expected truncated form, if present, was however no longer capable of being recognized by the antibody, losing its immunogenicity.

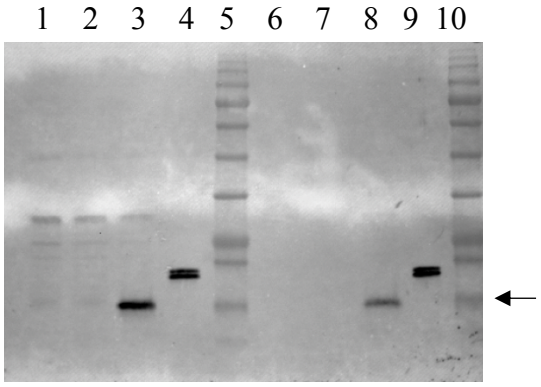


Figure 2. Western Blot analysis of Sola l 4 protein in fruit protein extracts. Proteins were extracted, quantified, normalized and separated on a reducing SDS-PAGE. Detection of Sola l 4 was performed with a rabbit anti-Sola l 4 polyclonal antibody, revealed by a horseradish peroxidase-conjugated anti-rabbit polyclonal antibody produced in mouse. ***1-3****: 6 µg of total soluble proteins extracted from tomato mature green fruits of 13A and 1.2 edited lines and WT, respectively.* ***6-8****: 10 µg of total soluble proteins extracted from red-ripe fruits of 13A and 1.2 edited lines and WT, respectively.* ***4*** *and* ***9****: 50 ng of recombinant protein produced in E. coli.* ***5*** *and* ***10****: Molecular weight marker (BLUeye prestained Protein Ladder 245–11 kDa, Sigma-Aldrich). Arrow indicated the band of 18 kDa.*

2.3.4 Non-volatile metabolome of the edited plants

In order to better understand the effects of the genetic editing at the primary and secondary metabolic level, a semi-polar and non-polar targeted profiling was carried out by Liquid Chromatography coupled to High-Resolution Mass Spectrometry (LC-HRMS). The observed alterations will be described as follows, firstly at local metabolism and subsequently to unrelated pathways. At the glycoalkaloid level, our data confirmed previous studies showing that the content of total SGAs was higher in green tissues of tomato WT plants, particularly in leaves, while their abundance decrease during fruit ripening (Friedman, 2013; Iijima et al., 2009). These changes were either qualitative and quantitative, since the distribution of glycoalkaloids was also affected: whereas in the leaves, indeed, α-tomatine and its dehydro- form were the most abundant

metabolites (representing more than 99.7 % of total SGAs), in the fruits the composition is more heterogeneous: more in detail, in the MG fruit, α-tomatine remained the most abundant SGA, together with hydroxy-tomatine and acetoxy-tomatine, contributing to more than 96 % of the total. In RR fruit, on the contrary, almost all α-tomatine was converted in the less bitter and less toxic esculeosides A, the most represented, and B. These compounds, with an α- and hydroxy- tomatine residues, constituted more than 98% of total SGAs. In both edited lines 1.2 and 13A *GAME 4* knock-out, SGA levels reached a drastic decrease (greater than 99.7%) (Figure 3).

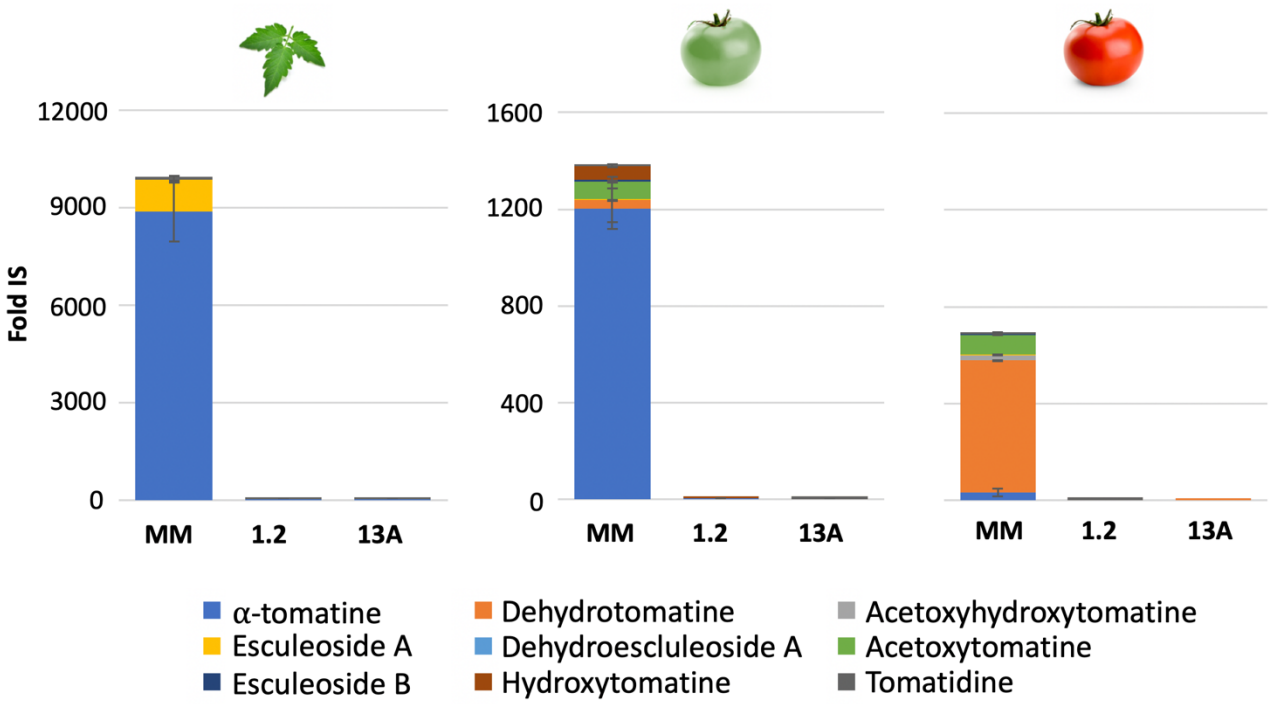


Figure 3: LC-HRSM analysis of glycoalkaloids in tomato leaves and fruits of WT (cv. MM) and edited lines. Data are shown as Fold IS (Internal Standard). See Materials and Methods for details.

Subsequently, since *GAME 4* is located in a branching point in the pathway that leads to the formation of glycoalkaloids on one hand and, on the other, to the formation of steroidal saponins (Itkin et al., 2013), we also considered to evaluate their contents. In accordance with previous reports, uttroside B, absent in WT leaves and fruits, was detected only in tissues of edited lines ranging from 47-fold IS in fruit at both ripening stages and 189-fold IS in leaves (Table 1 and Supplementary Table 3). However, and unexpectedly, we identified tigonin as the most abundant saponin in all the tissues of edited lines. More specifically, this molecule accumulated 64 times in edited leaves, 272-310 times in the MG and about 328-331 up-fold in the RR of line 1.2 and 13A, respectively, compared to WT (Table 1 and Supplementary Table 3). This evidence provided hints about the existence of a broader extent of changes in the saponin group; for this reason, an

additional effort based on in silico search of *Solanum* and non-*Solanum* species coupled to targeted metabolomics identification and validation (for more details, see materials and methods) was carried out. In this way, several additional saponins were found, all resulting up-accumulated compared to WT counterparts, or only being detected in the edited lines. In particular, 16 differentially accumulated saponins were found in tomato leaves (Supplementary Table 3), whereas 11 in both MG and RR fruits were detected (Table 1). Notably, some of these changes were observed only in leaves: for instance, solanigroside M, solanigroside Y6, solanigroside Y7, solanigroside Y8, degalactotigonin rhamnoside, (25R)-26-O-β-D-glucopyranosylfurost-5(6)-ene-3β,22α,26-triol-3-O-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside, officinalisnin I and voghieroside B1-C1. On the contrary, three were only detected in the fruits of the edited lines (nuatigenin 3-beta-D-glucopyranoside, digitogenin and capsicoside A). Finally, a last sub-group of saponins was found in both tissues: abutiloside L, degalactotigonin , diosgenin glucoside, indioside D, , solanigroside Y5, torvoside H, (25S)-26-(beta-D-Glucopyranosyloxy)-22alpha-methoxyfurosta-5-ene-3beta-yl]2-O-alpha-L-rhamnopyranosyl-3-O-(4-O-beta-D-glucopyranosyl-alpha-L-rhamnopyranosyl)-beta-D-glucopyranoside and in particular , tigogenin 3-O-β-D-glucopyranoside, which resulted the most up-accumulated compound in the comparison edited vs WT plants (around 93 up-fold in leaves and 250-370 in MG and RR fruits, respectively).

By moving from the epicenter of the genetic modification outwards, we focused our attention on phytosterols, which shares common intermediates with SGA pathway, and like them, have cholesterol as common precursor (Sonawane et al., 2017). More in detail, in leaves and fruits at MG stage of both 1.2 and 13A edited lines, an accumulation was observed for β-sitosterol (2.23/2.23 and 3.26/3.27, respectively), while in RR berries, it significantly decreased (0.61 for line 1.2 and 0.57 for line 13A, respectively). Notably, stigmasterol, previously reported to over-accumulate in *GAME 4*-KO tissues (Itkin et al., 2013), remained unaltered in our experimental conditions (Figure 4 B and C).

A series of additional, alterations were revealed, either at primary and secondary metabolite levels; in some cases, these changes were only detected in one line, suggesting the occurrence of stochastic rather editing-based effects. However, numerous compounds were also found to be up- and down-accumulated in GAME 4-Sola l 4 vs WT lines, albeit often with tissue-specific patterns. This finding, at leaf level, was particularly evident for amino acids: indeed, an up-accumulation of glutamine and valine (~ 1.2-1.5) and a decrease in tryptophan content of approximately 0.67 and 0.73 in 1.2 and 13A lines were observed. However, it is noteworthy noticing that the total aminoacidic content in leaf remained unchanged in the edited lines. Interestingly, the ratio between

sucrose and hexoses was also affected in GAME 4-Sola l 4 leaves, being over- and down-accumulated, respectively.

Table 1. LC-HRMS analysis of the saponins composition in MG and RR tomato fruits. The symbol - indicates the lack of saponin in MM samples. Only saponins with p-value <0.05 were shown.

Metabolite	Chemical formula	RT	MG FC MM		RR FC MM	
			1.2	13A	1.2	13A
Abutiloside L	C51H82O23	11.05	-	-	-	-
Capsicoside A	C63H106O35	10.45	-	-	-	-
Degalactotigonin	C50H82O22	11.22	53.78	62.38	26.00	27.80
Digitonin	C56H92O29	13.75	-	-	-	-
Diosgenin glucoside	C33H52O8	11.09	-	-	-	-
Indioside D	C51H84O23	11.18	10.80	14.55	77.25	83.92
Nuatigenin 3-β-D-glucopyranoside	C33H52O9	11.03	3.37	5.38	1.24	2.00
Solanigroside Y5	C57H94O28	11.00	-	-	-	-
Tigogenin 3-*O*-β-D-glucopyranoside	C33H54O8	11.22	253.47	283.65	369.21	375.83
Tigonin	C56H92O27	10.61	272.31	309.81	327.83	331.45
Torvoside H	C45H74O18	10.46	48.42	47.63	199.59	177.11
Uttroside B	C56H94O28	11.74	-	-	-	-
(25S)-26-(β-D-Glucopyranosyloxy)-22α-methoxyfurosta-5-ene-3β-yl]2-*O*- α -L-rhamnopyranosyl-3-*O*-(4-*O*-β-D-glucopyranosyl- α -L-rhamnopyranosyl)-β-D-glucopyranoside	C58H96O27	11.24	-	-	-	-

Another group showing a great extent of alteration, mostly down-accumulated, was the one of acids, with benzoic and coumaric acids, only found in WT samples, and quinic acid equal to 0.77 in edited vs WT lines; the only exception was represented by succinic acid, which increased (1.54 and 1.74 in lines 1.2 and 13A, respectively). Furthermore, a strong decrease in the content of several polyphenols, including either flavonoid (quercetin, naringenin, Kaempferol 7-O-glucoside, kaempferol-3-*O*-rutinoside7-*O*-glucoside) and anthocyanins (delphinidin, cyanidin 3-O-galactoside), as well as of α-tocopherol (vitamin E) was found, whereas a significant over-accumulation of ascorbic acid (vitamin C), more than 50 times, and phylloquinone (vitamin K) was observed. Finally, some key-compounds in photosynthesis were also altered: chlorophyll *a* and plastoquinone, involved in the electron transport chain in the light-dependent reactions (Figure 4A).

Some of these perturbations were conserved in ripening fruits, whereas others were exclusive of the latter: for instance, a reduction in the amine content, especially spermidine (~0.6-0.8), at both

stages and, limited to RR berries, spermine and tyramine; as well as amides, such as dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid, and the phenolic gallic acid, down-accumulated in MG as well as in RR fruits. At the opposite, some acids were also characterized by a significant decrease specific for a single ripening stage (benzoic acid, undetectable in GAME 4-Sola l 4 lines at MG, and caffeic acid-hexose in RR fruits). Interestingly, an increase in shikimic acid was observed in edited MG fruits (1.4 and 1.3 for lines 1.2 and 13A, respectively).

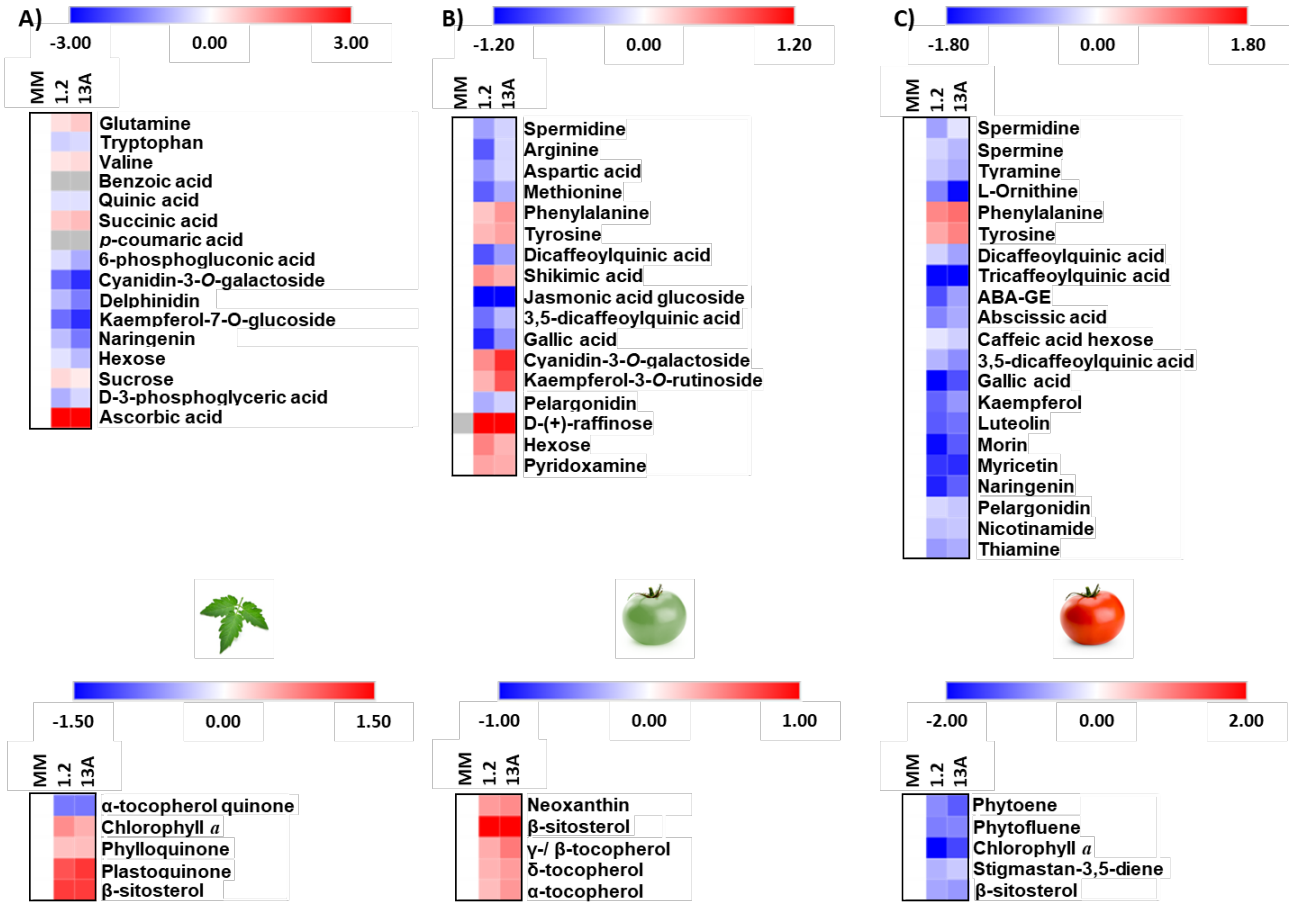


Figure 4. Semi polar and non-polar metabolomics analysis of WT and edited tomato leaves (A) and fruits at MG (B) and RR (C) ripening stages. All values are normalized with respect to the WT (MM) and are log2 adjusted. In light grey are showed all the metabolites not detected. Only consensus data in both edited lines in the comparison with the WT were considered.

Overall, a conserved trend in the two ripening stages was also observed in the amino acid content: more in detail, an up-accumulation of two aromatic aa, the essential amino acid phenylalanine (1.2-1.4 in MG and 1.9-2.0 in RR) and tyrosine (1.2-1.3 in MG and 1.2-1.4 in RR), for lines 1.2 and 13A, respectively, was found. Instead, a set of other aa showed significant decrease: in the green fruit, methionine, aspartic acid and arginine, while in the red fruit L-ornithine. Furthermore, the contents of some sugars increased in the edited lines: D-raffinose, absent in WT, was indeed

found in the green fruit, and a significant increase in hexoses was observed. The latter also displayed increments in the red fruit, but significantly only in line 1.2. On the contrary, a different trend was observed for the vitamin B content: in the green tomato, in fact, an increase in pyridoxamine was observed, whereas in the red fruit significant reductions in pyridoxine, thiamine and nicotinamide were detected.

Concerning fruit secondary metabolism, polyphenols displayed a reduction in the total content during ripening. Although most of them were not detected at the MG stage, few differences were observed for those ones identified. The only significant differences regarded cyanidin 3-*O*-galactoside and kaempferol-3-*O*-rutinoside, both of which were up-accumulated in the edited lines with respect to the WT (1.4-2.0 and 1.3-1.7 up-folded, respectively); whereas a small decrease in pelargonidin content was also detected (down-fold 0.8-0.9). The same trend was also observed in RR stage with a significant increment of cyanidin 3-*O*-galactoside and kaempferol-3-*O*-rutinoside only in line 13A, as well as a decrease in pelargonidin. However, several other polyphenols decreased in RR fruit such as kaempferol, luteolin, morin, myricetin and naringenin.

At the opposite, a distinct attitude in terms of isoprenoid accumulation was observed according the ripening stage under study: in fact, while at the earlier stage, a significant increase in neoxanthin and tocochromanols (α-, β-/γ- and δ- tocopherols) was found, a consistent reduction in both GAME 4-Sola l 4 lines was observed at chlorophyll a, phytoene and phytofluene levels (Figure 4 B and C).

2.3.5 Volatile metabolite profiles

Overall, fifty-seven different volatile compounds (VOCs) were unequivocally or putative identified in the tomato leaves, with glandular-trichome terpenoids being the main represented compounds in the dataset and the metabolic class with the highest number (11/22) of significantly reduced fold-values in both the edited vs WT lines (Figure 5A). Specifically, the most altered compounds were: α-pinene, 2-carene and α-phellandrene. More in general, in line 1.2 all compounds showed a reduction below 0.36-fold, whereas in line 13A it was 0.24. Among the non-terpenoids compounds, only the fatty acid derivatives, (E)-2-heptenal, octanal and nonanal, were characterized by a slight reduction in both transgenic lines.

Sixty-seven and eighty-two different VOCs were unequivocally or putative identified in the MG and RR fruit samples, respectively (Figure 5 B and C). Although the production of volatile compounds is much lower in green fruits than in ripe ones, both in terms of variety and relative abundance, the results showed essentially the same profile. Among them, the main VOCs characteristic of tomato flavor, which most influence consumer liking, were also identified

(Martina et al., 2021). Similarly to what shown above, also edited fruits were characterized by a strong reduction in a series of terpene VOCs, with (Z)-linalool oxide, (E)-linalool oxide and 2-caren-10-al being affected at both ripening stages, and p-cymene exclusively decreasing in MG berries. Notably, in the edited lines, an increase in branched-chain amino acids group (BCAA VOCs), often associated to positive aromatic notes in tomato, was observed. In this group, the most up-regulated compound was 3-methylbutanal for both MG and RR stage, with an up-fold of 4.05 for both edited lines in the green fruit, and an up-fold of 2.04 and 2.49 for 1.2 and 13A lines in the RR, respectively. A significant increase was observed also for 2-methylbutanal (> 2.5-fold), but only at MG stage, and 3-methylbutanol and 3-methylbutanoic acid, limited to RR fruits. While, in addition, at the RR stage, 2-isobutyl-thiazole over-accumulated (> 1.5-fold), whereas it was absent in the MG. Of interest, a distinct alteration pattern of differentially accumulated volatiles was found in phenolic/phenylpropanoid-derived volatiles (Phe VOCs) according the ripening stage: indeed, 1-nitro-2-phenylethane and benzylnitrile, providing floral odor type, increased at MG, while 1-nitro-2-phenylethane showed higher levels in RR edited fruits. The phenolic VOC phenylacetaldehyde also differed between edited and WT lines: it was especially up-regulated in MG (> 5.15 and 4.30 for 1.2 and 13A lines), while at the RR stage an up-fold of 2.35 and 1.82, respectively, was observed. Whereas, the main phenylpropanoid VOCs in tomato guaiacol, methyl salicylate and eugenol decreased in the edited lines at both ripening stages, with the exception of eugenol which resulted up-accumulated only in MG fruits. Finally, an overall decrease in fatty acids derived volatiles (FA VOCs), particularly linolenic acid derivatives (Z)-3-hexenal and (E)-2-hexenal at MG, and (E,E)-2,4-hexadienal in RR berries, was shown, with 1-hexanol representing the only compound from this class displaying an opposite tendency (1.72 and 1.52 in lines 1.2 and 13A, respectively) (Figure 5B and C).

2.3.6 Proteomic analysis

Proteomic characterization of tomato leaves was achieved by LC-HRMS profiling of proteins extracted from three replicates for each line. A total of 7,780 peptides were identified and 9,271 proteins were detected. Among those proteins, 245 resulted differentially abundant proteins (DAPs). More in detail, only proteins present in the same regulation (UP- or DW-) in both edited lines with a fold change greater than 1.5 or lower than 0.66 and with adjusted P-value less than 0.05 were considered (Supplementary Table 4). Specifically, 116 proteins resulted DW-regulated with the following peptides being the most down-regulated in both lines: a non-specific lipid-transfer protein 1(Acc. A0A3Q7HZ96), highly expressed in leaf and flower tissues (D'Agostino et al., 2019), and the Cytochrome P450 (Acc. A0A3Q7J325) GAME 4, which was one of the

chosen targets (Itkin et al., 2013). In addition, some key enzymes involved in lignin and flavonoid synthesis, such as three different phenylalanine ammonia-lyase (PAL) (Acc. P26600, A0A3Q7IPA2, A0A3Q7GQY4), a caffeoyl-CoA *O*-methyltransferase (Acc. A0A3Q7ESA3),

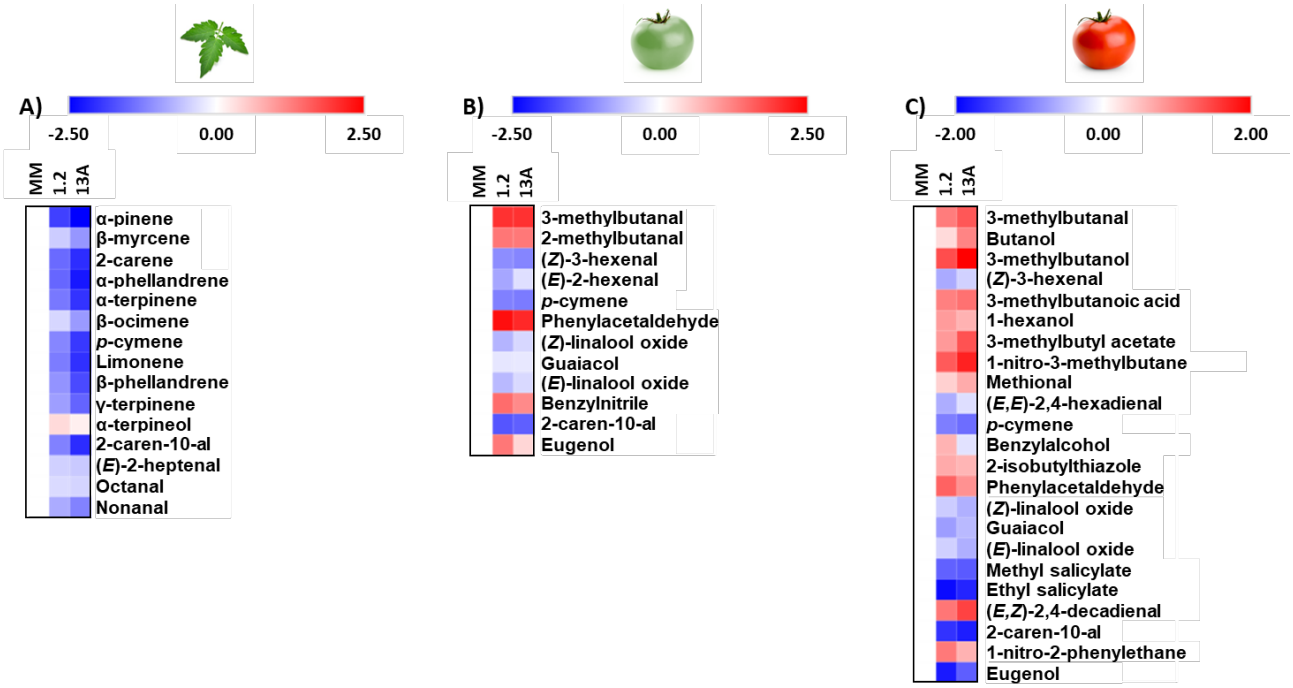


Figure 5. Volatile metabolite profile of WT and edited tomato leaves (A) and fruits at MG (B) and RR (C) ripening stages. All values are normalized with respect to the WT (MM) and are log2 adjusted. Only consensus data in both edited lines in the comparison with the WT were considered.

a chalcone synthase 2 (P23419) and a chalcone-flavanone isomerase family protein (Acc. A0A3Q7HFP3) were also DW-regulated. Regarding the UP-regulated proteins, 129 hits were found. Among those, the most accumulated in both edited lines were two PKS_ER domain-containing proteins (Acc. A0A3Q7I48 showed an up-fold of 87.63 and 99.55; Acc. A0A3Q7I1T6 un up-fold of 34.32 and 49.83 for lines 1.2 and 13A respectively); as well as numerous proteins involved in defense mechanisms against both biotic and abiotic stresses: e.g., peroxidases, superoxide dismutase, chitinase and additional PR-proteins.

At berry level, proteomics analyses were carried out on samples of WT and edited lines at either MG and RR ripening stages with, respectively, a total number of 7,629 and 5,913 proteins. Among them, 23 and 27 resulted stage-specific (Table 2 and 3). Moreover, for both ripening stages, 6 DAPs were DW-regulated, while 17 were UP-regulated in MG and 21 in RR berries. Notably, it was possible to identify 11 proteins consensually UP-regulated in fruits at both ripening stages under study. Of these, those with a higher UP-fold were PKS_ER domain-containing proteins, belonging to the zinc-type alcohol dehydrogenase-like protein family following Panther's classification. Interestingly, also three proteins involved in carbohydrate metabolism belonged to

this group: in particular, acid beta-fructofuranosidase (Acc. P29000) (Elliott et al., 1993), which showed the higher UP-fold in the edited lines (~ 2.2 in green fruits in both lines; 3.55 and 2.54 in red fruits in 1.2 and 13A lines); and Alpha-1,4 glucan phosphorylase (Acc. A0A3Q7I2P2) and Glyco_transf_28 domain-containing protein (A0A3Q7I2U8), both with glycosyltransferase activity, which resulted UP-regulated about 2.2-fold in the MG fruit and about 3-fold in the RR fruit of edited lines.

Table 2: List of differentially abundant proteins up- or down- regulated in mature green fruits of edited lines. Only proteins with adjusted p-value <0.05 and fold change (FC) >|1.5| were shown.

Description	Accessions	FC 1.2-WT	FC 13A-WT
Uncharacterized protein	A0A3Q7HZN3	0,11	0,15
Uncharacterized protein	A0A3Q7F523	0,21	0,33
40S ribosomal protein S8	A0A3Q7IY72	0,37	0,32
Uncharacterized protein	A0A3Q7HJ32; A0A3Q7I3Y2	0,40	0,30
PKS_ER domain-containing protein	A0A3Q7I2C5; A0A3Q7I3U9	0,42	0,40
40S ribosomal protein S8	A0A3Q7ICF4; A0A3Q7IY72	0,63	0,57
Uncharacterized protein	A0A3Q7HZK1	1,65	1,59
WPP domain-containing protein	A0A3Q7IZX3	1,73	1,76
Peptidylprolyl isomerase (EC 5.2.1.8)	A0A3Q7I412	1,78	1,89
CS domain-containing protein	A0A3Q7JX37	1,78	1,67
Wheel domain-containing protein	A0A3Q7I353	1,79	1,60
Uncharacterized protein	A0A3Q7I0E1	2,08	2,24
Glyco_transf_28 domain-containing protein	A0A3Q7I2U8	2,11	2,05
Alpha-1,4 glucan phosphorylase (EC 2.4.1.1)	A0A3Q7I2P2	2,14	2,48
Acid beta-fructofuranosidase (EC 3.2.1.26)	P29000	2,20	2,16
Uncharacterized protein	A0A3Q7I4B1	2,54	3,40
Uncharacterized protein	A0A3Q7IZK4	2,65	2,03
Non-specific serine/threonine protein kinase (EC 2.7.11.1)	A0A3Q7GXR3	2,72	2,65
Uncharacterized protein	A0A3Q7HRY7	5,81	4,23
PKS_ER domain-containing protein	A0A3Q7I1T6	6,84	6,34
PKS_ER domain-containing protein	A0A3Q7I1T6; A0A3Q7I2C5	7,77	8,94
PKS_ER domain-containing protein	A0A3Q7I2C5	18,25	18,40
Uncharacterized protein	A0A3Q7I482	74,34	99,63

Table 3: List of differentially abundant proteins up- or down- regulated in red ripe fruits of edited lines. Only proteins with adjusted p-value <0.05 and fold change (FC) >|1.5| were shown.

Description	Accessions	FC 1.2-WT	FC 13A-WT
Uncharacterized protein	A0A3Q7J2P9	0,04	0,03
Uncharacterized protein	A0A3Q7I3M9	0,11	0,05
Uncharacterized protein	A0A3Q7HJ32; A0A3Q7I3Y2	0,26	0,22
DLH-domain-containing protein	A0A3Q7I516	0,27	0,30
40S ribosomal protein S8	A0A3Q7IY72	0,44	0,40
RNA helicase (EC 3.6.4.13)	A0A3Q7IHN6	0,61	0,65
PKS_ER domain-containing protein	A0A3Q7I3U9	1,84	1,92
Thioredoxin domain containing protein	A0A3Q7IVV1	1,88	2,02
Uncharacterized protein	A0A3Q7I0E1	1,89	2,04
WPP domain-containing protein	A0A3Q7IZX3	1,93	2,10
Uncharacterized protein	A0A3Q7FYI5	2,04	2,34
Uncharacterized protein	A0A3Q7FH11	2,11	2,37
DAO domain-containing protein	A0A3Q7I3J1	2,20	2,02
Uncharacterized protein	A0A3Q7IF17	2,44	2,02
Alpha-1,4 glucan phosphorylase (EC 2.4.1.1)	A0A3Q7I2P2	2,54	2,90
Glyco_transf_28 domain-containing protein	A0A3Q7I2U8	2,96	3,13
Uncharacterized protein	A0A3Q7FBF2	3,16	2,73
Uncharacterized protein	A0A3Q7HZK1	3,18	3,22
Acid beta-fructofuranosidase (EC 3.2.1.26)	P29000	3,55	2,54
Peptidylprolyl isomerase (EC 5.2.1.8)	A0A3Q7I412	5,03	4,98
R3H-assoc domain-containing protein	A0A3Q7I3V5	5,23	5,20
Uncharacterized protein	A0A3Q7FJA5	8,11	6,90
PKS_ER domain-containing protein	A0A3Q7I2C5	13,25	13,26
PKS_ER domain-containing protein	A0A3Q7I1T6	14,72	14,47
Uncharacterized protein	A0A3Q7HJ32; A0A3Q7IYI8	18,21	16,88
PKS_ER domain-containing protein	A0A3Q7I1T6; A0A3Q7I2C5	56,95	73,45
Uncharacterized protein	A0A3Q7I482	171,78	169,66

2.3.7 Evaluation of fruit phenotypic traits

In order to verify if edited fruits were affected in developmental and ripening stages, a series of phenotypic parameters were evaluated. First of all, we calculated the average day post anthesis (dpa) for each line to reach the breaker stage, which resulted unaltered between edited and control fruits (data not shown). Subsequently, the firmness of the fruit, which is fundamental parameter to assess the ripening attitude, was also evaluated on "off the vine" samples. At this aim, at least 10 fruits per line were harvested at the MG stage and analyzed. To evaluate the CR, a non-destructive test was carried out for six experimental points up to the Br+15 stage. As shown in Figure 6A, no statistically significant differences were observed with the exception of the Br+7 stage in which the edited lines showed a lower CR, which was restored in the subsequent ripening stages. Similarly, average weight and water loss were also evaluated, showing no alteration between WT and edited berries (Figure 6B). Finally, soluble solids (Brix) content of ripe fruits was measured using a refractometer, resulting in Brix values in the edited line tomatoes (Line 1.2 = 4.48 ± 0.18;

Line 13A = 4.16 ± 0.31) which did not differ from those measured for the WT berries (4.26 ± 0.14) (Supplementary Figure 3).

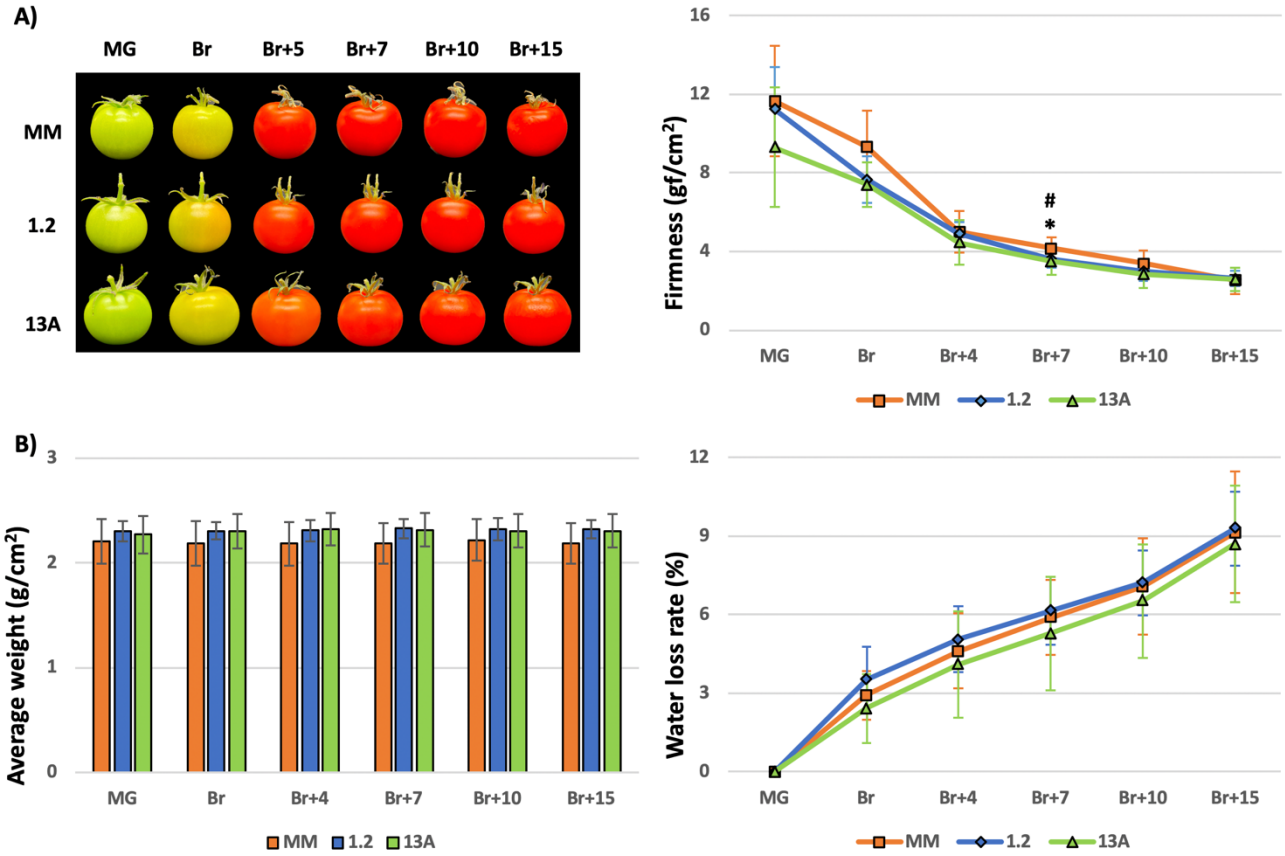


*Figure 6. A) «Off the vine» ripening analysis of tomato fruits. Change of fruit firmness in WT and edited lines. The fruit firmness, expressed as gf, was normalized for longitudinal and transversal diameter. B) Physiological loss of water in WT and edited fruits. All the analysis were measured at six different ripening stages and were calculated at least for 10 fruits per line. All data were expressed as mean ± SD. * and # indicate significant differences respectively between MM - 1.2 fruits, and MM – 13A fruits, with P < 0.05, as determined by t-test.*

2.4 Discussion

It is known that tomato fruit is a source of many pro-nutritional compounds (Chaudhary et al., 2018); however, at the same time and albeit not frequently considered, it also contains some antinutritional compounds like SGAs and allergens (Friedman, 2002; Włodarczyk et al., 2022). However, while the former has been subjected to intensive efforts by classic breeding to achieve "low SGA-trait" varieties (Johns & Alonso, 1990), the latter has been largely neglected for long time, and only more recently, also due to the increase in allergy rates, its relevance has strongly increased (Melotto et al., 2020). In this perspective, NPBTs offer innovative and more efficient and rapid opportunities to generate plant idiotypes yielding desired and improved traits.

In the present study, using the CRISPR/Cas9 system, we generated tomato plants edited in two target genes for both small and large molecules: *GAME 4,* a key gene involved in the biosynthetic pathway of SGAs, and *Sola l 4*, one of the major allergens in tomato. T3 progenies were first analyzed to verify mutation homozygosity, absence of Cas9 gene and of OFF-target events. The two chosen lines 1.2 and 13A were subsequently analyzed at a "omics" multi-level approach (proteomic, metabolomic, volatile).

As a result of knock-out of *Sola l 4* gene, we expected, by in silico prediction, a non-functional truncated protein in edited lines. Accordingly, analysis of the tomato extracts through western blot with a Sola l 4 specific polyclonal antibody, we did not observe any signal in both edited lines, confirming that the protein was absent in its native conformation, and its truncated form, if present, was no recognized by the antibody, suggesting to be not immunogenic anymore. Moreover, comparing Sola l 4 with Bet v1, although there are T-cell epitopes along the entire sequence of Bet v 1, one of the major t-cell epitopes is located at the C-terminus of the protein (Kungl et al., 1996). Moreover, in general, allergens of the Bet v 1 protein family mostly bind IgE only in their native protein conformation (Husslik et al., 2015; Kahlert et al., 2008; Wallner et al., 2011). Since the truncated Sola l 4 lacked 2/3 of the sequence and was totally misfolded, it was reasonably to hypothesize that lost also its allergenicity.

In order to investigate the effects of *GAME 4* and *Sola l 4* gene editing at the metabolic level, a semi-polar and non-polar targeted characterization was carried out. As expected, a very strong reduction of total SGAs was achieved, greater than 99% in leaves and fruits, thus confirming the effectiveness of GAME 4 as key enzyme in SGAs accumulation.

In parallel, a marked increase of some saponins, such as tigonin and tigogenin 3-O-β-D-glucopyranoside, was observed in the edited lines. At the same time, we also detected saponins that, to our knowledge, are not usually present in tomato plants. In addition to uttroside B, already identified in a previous study (Itkin et al., 2013), eight and six new saponins, usually found in wild species belonging to the *Solanaceae* family, were found in MG and RR leaves and fruits of edited lines, respectively. It is known that these classes of compounds confer protection against plant pathogens like insects, bacteria, viruses, and fungi (Altesor et al., 2014; Chowański et al., 2016); therefore, an accumulation of these molecules could potentially balance the almost total absence of SGAs being compounds involved in plant defense mechanisms. Moreover, several compounds of these metabolic class showed various biological activities and pharmaceutical effects such as: antitumor, hepatoprotective, anti-inflammatory and antiviral (Sharma et al., 2023). As these compounds are usually not accumulated in tomato or are present in traces, from a molecular farming-perspective, the GAME 4-Sola l 4 lines could also be considered as a novel production

platform of these valuable metabolites. Another aspect of note is that the biosynthetic pathways of uttroside B and tigonin have not yet been elucidated, paving the way to the possibility to use these edited lines to perform a de novo discovery of candidate genes involved in the synthesis of these rare saponins, trying to understand the epigenetic mechanisms in the silencing of certain genes in the tomato genome.

Moving onto the pathway of phytosterols, which share a common precursor with the pathway of SGAs (Itkin et al., 2013), we observed few variations in the edited plants. The most significant was the accumulation of β-sitosterol in leaves and MG fruits. Notably, β-sitosterol is one of the most widespread phytosterols in the plant kingdom and therefore one of the most widely consumed in the human diet, and it is known to have numerous biological activities such as immunomodulatory, anti-inflammatory, lipid lowering effect and anti-diabetic activity (Babu & Jayaraman, 2020). However, a different trend was observed during fruit ripening. Indeed, the β-sitosterol content decreased in the RR fruits. This could be explained by the higher concentration of SGAs in the MG; therefore, a blockage in their pathway would provide greater flux to neighboring pathways. Furthermore, a negative feedback mechanism could be hypothesized; once a threshold in β-sitosterol content was reached, its synthesis was blocked, leading to its decrease in the RR.

We also evaluated the occurrence of GAME 4-Sola l 4 KO-derived pleiotropic effects, either at metabolomics, proteomic levels, in leaves and ripening fruits. In some cases, the two lines under study evidenced the presence of genotype-specific alterations, likely due to stochastic events, or to the occurrence of unintended genomic changes, which cannot be excluded although the analysis of the off targets did not reveal any significant perturbation at the expenses of the other tested genes. For this reason, only consensus data in both edited lines in the comparison with the WT were considered for further investigation.

Some other metabolic perturbations could be associated to the sensorial properties of tomato: for instance, a significant increase of D-raffinose, absent in WT, was found in the MG of the edited lines, and a significant increase in hexoses (glucose, fructose etc.) was also observed in RR of the 1.2 line; notably, this latter result can find a partial explanation in the increase, in MG/RR fruits of both lines, of the acid beta-fructofuranosidase protein (Acc. P29000) (Elliott et al., 1993), which converts sucrose in glucose e fructose. Simultaneously the decrease of some polyamines (e.g., spermine, spermidine), often associated to unpleasant smell, was found.

Volatile analyses in edited tissues also revealed the presence of noteworthy metabolic changes: specifically, for instance, MG and RR fruits emitted higher levels of compounds often associated with positive aromatic notes in tomato (3-methylbutanal, phenylacetaldehyde, 3-methylbutanol

and 3-methylbutanoic acid) (D. M. Tieman et al., 2007). The increase in phenylacetaldehyde content could be explained by the accumulation of phenylalanine in edited fruits at both stages. This hypothesis is reinforced by the presence, among the up-regulated proteins in the MG fruits, of aromatic amino acid decarboxylase (Acc. A0A3Q7IZK4), a key enzyme class in the synthesis of flavor volatiles (D. Tieman et al., 2006).

At the same time, a significant decrease of aromatic molecules considered unpalatable by consumers was observed especially in RR fruit (guaicol, methyl salicylate and eugenol) (Distefano et al., 2022; D. Tieman et al., 2010).

In addition, an increase in several pro-nutritional compounds was observed in fruits at the MG stage. Among the class of polyphenols an increase in the flavonoid kamferol-3-rutinoside content, with antioxidant and anti-inflammatory activities (Hua et al., 2021), and in the anthocyanin cyanidin 3-O-galactoside content, with several positive health effects, including antioxidant, anti-inflammatory, anticancer, antidiabetic, cardiovascular (Liang et al., 2021), were highlighted. Finally, a higher level of certain vitamins of group B and E was also detected. Although tocopherols and tocotrienols have different bioavailability and bioactivity, it is well known how all isoforms of vitamin E exert neuroprotective and anti-tumoral activities (Ghosh et al., 2020). In particular, an up-accumulation of all α-, β-, γ-, δ- tocopherol was observed in edited fruits. In the B group, more specifically among the B6 compounds, involved in protective mechanisms against various diseases (Stach et al., 2021), an increase in pyridoxamine has been shown.

These results, together with the dramatic reduction in SGAs which confer a bitter taste, might foresee an alteration in the sensorial properties of the edited fruits, aspect that need to be better analyzed in future investigations, also considering that our effort resulted in the production of green berries devoid in SGAs, and which might so consider as new source of healthy food.

A last point of interest regards the possibility to exploit the novel metabolic remodeling achieved in the GAME 4-Sola l 4 leaves in the perspective of the generation of novel products for human and animal diets: indeed, tomato leaves cannot be used for edible purposes mainly due to the accumulation in glycoalkaloids: thus, the edited leaves might pave the way to the generation of novel food sources, also taking into consideration the beneficial side metabolic changes detected following GAME 4 and Sola l 4 knock-out. Furthermore, and in the same context, this alternative use of tomato leaves, normally considered a byproduct, would perfectly fit the current needs in terms of sustainability and circular bio-economy, by converting a waste in a novel resource. It is noteworthy mentioning that only limited efforts have been carried on the use of tomato leaves for alternative purposes (e.g., extraction of bioactive compounds as rutin, quercetin, phenolic acids etc. in pharmacological and nutraceutical studies (Bazzarelli et al., 2022; Figueiredo-González et

al., 2016; Junker-Frohn et al., 2019; Silva-Beltrán, Ruiz-Cruz, Chaidez, et al., 2015)), and never, at our knowledge, for the constitution of edible products. For this reason, future studies to evaluate the potential bioactivity/safety of the edited tissues by in vitro animal systems will be needed to assess the feasibility of this approach.

Since we obtained plants edited for GAME 4 and Sola l 4, two genes both involved, directly or indirectly, in plant defense mechanisms, and having observed at the metabolic level several compounds which could strongly impact on plant fitness facing biotic stressors, the two lines were subsequently tested in terms of their biotic stress responses (in collaboration with Professor Pennacchio; Laboratory of Entomology, Naples Federico II, Italy). Preliminary results (data not shown) revealed an unchanged susceptibility to attack by two different types of insects. However, reduced fertility was observed in insects fed on the leaves of the edited lines. The global proteome studies of leaf tissues may provide an explanation. Interestingly, proteomic data showed the presence, among the up-regulated proteins, of numerous hits involved in defense mechanisms against biotic stresses, such as several peroxidases, chitinases and PR-proteins. In particular, these analyses allowed to observe how the plant metabolism tends to remodel itself and replace missing molecules; indeed, we hypothesize that edited plant counterpoise the absence of both glycoalkaloids and one PR-protein with different compounds like saponins and other protein involved in the same functions.

2.5 References

Alseekh, S., Tohge, T., Wendenberg, R., Scossa, F., Omranian, N., Li, J., Kleessen, S., Giavalisco, P., Pleban, T., Mueller-Roeber, B., Zamir, D., Nikoloski, Z., & Fernie, A. R. (2015). Identification and Mode of Inheritance of Quantitative Trait Loci for Secondary Metabolite Abundance in Tomato. *The Plant Cell*, *27*(3), 485–512. https://doi.org/10.1105/tpc.114.132266

Altesor, P., García, Á., Font, E., Rodríguez-Haralambides, A., Vilaró, F., Oesterheld, M., Soler, R., & González, A. (2014). Glycoalkaloids of Wild and Cultivated Solanum: Effects on Specialist and Generalist Insect Herbivores. *Journal of Chemical Ecology*, *40*(6), 599–608. https://doi.org/10.1007/s10886-014-0447-8

Asero, R. (2013). Tomato allergy: Clinical features and usefulness of current routinely available diagnostic methods. *Journal of Investigational Allergology & Clinical Immunology*, *23*(1), 37–42.

Brinkman, E. K., Chen, T., Amendola, M., & van Steensel, B. (2014). Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Research*, *42*(22), e168–e168. https://doi.org/10.1093/nar/gku936

Babu, S., & Jayaraman, S. (2020). An update on β-sitosterol: A potential herbal nutraceutical for diabetic management. Biomedicine & Pharmacotherapy, 131, 110702. https://doi.org/10.1016/j.biopha.2020.110702

Bazzarelli, F., Mazzei, R., Papaioannou, E., Giannakopoulos, V., Roberts, M. R., & Giorno, L. (2022). Biorefinery of Tomato Leaves by Integrated Extraction and Membrane Processes to Obtain Fractions That Enhance Induced Resistance against Pseudomonas syringae Infection. Membranes, 12(6), 585. https://doi.org/10.3390/membranes12060585

Campbell, B. C., & Duffey, S. S. (1979). Tomatine and Parasitic Wasps: Potential Incompatibility of Plant Antibiosis with Biological Control. *Science*, *205*(4407), 700–702. https://doi.org/10.1126/science.205.4407.700

Cárdenas, P. D., Sonawane, P. D., Heinig, U., Jozwiak, A., Panda, S., Abebie, B., Kazachkova, Y., Pliner, M., Unger, T., Wolf, D., Ofner, I., Vilaprinyo, E., Meir, S., Davydov, O., Gal-on, A., Burdman, S., Giri, A., Zamir, D., Scherf, T., … Aharoni, A. (2019). Pathways to defense metabolites and evading fruit bitterness in genus Solanum evolved through 2-oxoglutarate-dependent dioxygenases. *Nature Communications*, *10*(1), 5169. https://doi.org/10.1038/s41467-019-13211-4

Chaudhary, P., Sharma, A., Singh, B., & Nagpal, A. K. (2018). Bioactivities of phytochemicals present in tomato. *Journal of Food Science and Technology*, *55*(8), 2833–2849. https://doi.org/10.1007/s13197-018-3221-z

Chen, X., Dai, X., Liu, Y., Yang, Y., Yuan, L., He, X., & Gong, G. (2022). Solanum nigrum Linn.: An Insight into Current Research on Traditional Uses, Phytochemistry, and Pharmacology. Frontiers in Pharmacology, 13, 918071. https://doi.org/10.3389/fphar.2022.918071

Chowański, S., Adamski, Z., Marciniak, P., Rosiński, G., Büyükgüzel, E., Büyükgüzel, K., Falabella, P., Scrano, L., Ventrella, E., Lelario, F., & Bufo, S. (2016). A Review of Bioinsecticidal Activity of Solanaceae Alkaloids. *Toxins*, *8*(3), 60. https://doi.org/10.3390/toxins8030060

D'Agostino, N., Buonanno, M., Ayoub, J., Barone, A., Monti, S. M., & Rigano, M. M. (2019). Identification of non-specific Lipid Transfer Protein gene family members in Solanum lycopersicum and insights into the features of Sola l 3 protein. *Scientific Reports*, *9*(1), 1607. https://doi.org/10.1038/s41598-018-38301-z

Diretto, G., Frusciante, S., Fabbri, C., Schauer, N., Busta, L., Wang, Z., Matas, A. J., Fiore, A., K.C. Rose, J., Fernie, A. R., Jetter, R., Mattei, B., Giovannoni, J., & Giuliano, G. (2020). Manipulation of β-carotene levels in tomato fruits results in increased ABA content and extended shelf life. *Plant Biotechnology Journal*, *18*(5), 1185–1199. https://doi.org/10.1111/pbi.13283

Distefano, M., Mauro, R. P., Page, D., Giuffrida, F., Bertin, N., & Leonardi, C. (2022). Aroma Volatiles in Tomato Fruits: The Role of Genetic, Preharvest and Postharvest Factors. *Agronomy*, *12*(2), 376. https://doi.org/10.3390/agronomy12020376

Donald, R. G. K., Zhou, H., & Jackson, A. O. (1993). Serological Analysis of Barley Stripe Mosaic Virus-Encoded Proteins in Infected Barley. *Virology*, *195*(2), 659–668. https://doi.org/10.1006/viro.1993.1417

EFSA Panel on Contaminants in the Food Chain (CONTAM), Schrenk, D., Bignami, M., Bodin, L., Chipman, J. K., del Mazo, J., Hogstrand, C., Hoogenboom, L. (Ron), Leblanc, J., Nebbia, C. S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Schwerdtle, T., Vleminckx, C., Wallace, H., Brimer, L., Cottrill, B., … Grasl-Kraupp, B. (2020). Risk assessment of glycoalkaloids in feed and food, in particular in potatoes and potato-derived products. *EFSA Journal*, *18*(8). https://doi.org/10.2903/j.efsa.2020.6222

Elliott, K. J., Butler, W. O., Dickinson, C. D., Konno, Y., Vedvick, T. S., Fitzmaurice, L., & Mirkov, T. E. (1993). Isolation and characterization of fruit vacuolar invertase genes from two tomato species and temporal differences in mRNA levels during fruit ripening. *Plant Molecular Biology*, *21*(3), 515–524. https://doi.org/10.1007/BF00028808

Figueiredo-González, M., Valentão, P., & Andrade, P. B. (2016). Tomato plant leaves: From by-products to the management of enzymes in chronic diseases. Industrial Crops and Products, 94, 621–629. https://doi.org/10.1016/j.indcrop.2016.09.036

Friedman, M. (2002). Tomato Glycoalkaloids: Role in the Plant and in the Diet. *Journal of Agricultural and Food Chemistry*, *50*(21), 5751–5780. https://doi.org/10.1021/jf020560c

Friedman, M. (2013). Anticarcinogenic, Cardioprotective, and Other Health Benefits of Tomato Compounds Lycopene, α-Tomatine, and Tomatidine in Pure Form and in Fresh and Processed Tomatoes. *Journal of Agricultural and Food Chemistry*, *61*(40), 9534–9550. https://doi.org/10.1021/jf402654e

Frusciante, S., Demurtas, O. C., Sulli, M., Mini, P., Aprea, G., Diretto, G., Karcher, D., Bock, R., & Giuliano, G. (2022). Heterologous expression of *Bixa orellana* cleavage dioxygenase 4–3 drives crocin but not bixin biosynthesis. *Plant Physiology*, *188*(3), 1469–1482. https://doi.org/10.1093/plphys/kiab583

Ghosh, N., Das, A., & Khanna, S. (2020). Vitamin E: Tocopherols and tocotrienol and their role in health and disease. In Essential and Toxic Trace Elements and Vitamins in Human Health (pp. 283–293). Elsevier. https://doi.org/10.1016/B978-0-12-805378-2.00020-6

Gianoglio, S., Comino, C., Moglia, A., Acquadro, A., García-Carpintero, V., Diretto, G., Sevi, F., Rambla, J. L., Dono, G., Valentino, D., Moreno-Giménez, E., Fullana-Pericàs, M., Conesa, M. A., Galmés, J., Lanteri, S., Mazzucato, A., Orzáez, D., & Granell, A. (2022). In-Depth Characterization of greenflesh Tomato Mutants Obtained by CRISPR/Cas9 Editing: A Case Study With Implications for Breeding and Regulation. *Frontiers in Plant Science*, *13*, 936089. https://doi.org/10.3389/fpls.2022.936089

Giovannoni, J., Nguyen, C., Ampofo, B., Zhong, S., & Fei, Z. (2017). The Epigenome and Transcriptional Dynamics of Fruit Ripening. *Annual Review of Plant Biology*, *68*(1), 61–84. https://doi.org/10.1146/annurev-arplant-042916-040906

Granell, A., Bellés, J. M., & Conejero, V. (1987). Induction of pathogenesis-related proteins in tomato by citrus exocortis viroid, silver ion and ethephon. *Physiological and Molecular Plant Pathology*, *31*(1), 83–90. https://doi.org/10.1016/0885-5765(87)90008-7

Hua, F., Zhou, P., Liu, P., & Bao, G. (2021). Rat plasma protein binding of kaempferol-3- O -rutinoside from Lu'an GuaPian tea and its anti-inflammatory mechanism for cardiovascular protection. Journal of Food Biochemistry, 45(7). https://doi.org/10.1111/jfbc.13749

Husslik, F., Hanschmann, K.-M., Krämer, A., Seutter von Loetzen, C., Schweimer, K., Bellinghausen, I., Treudler, R., Simon, J. C., Vogel, L., Völker, E., Randow, S., Reuter, A., Rösch, P., Vieths, S., Holzhauser, T., & Schiller, D. (2015). Folded or Not? Tracking Bet v 1 Conformation in Recombinant Allergen Preparations. *PLOS ONE*, *10*(7), e0132956. https://doi.org/10.1371/journal.pone.0132956

Iijima, Y., Fujiwara, Y., Tokita, T., Ikeda, T., Nohara, T., Aoki, K., & Shibata, D. (2009). Involvement of Ethylene in the Accumulation of Esculeoside A during Fruit Ripening of Tomato (*Solanum lycopersicum*). *Journal of Agricultural and Food Chemistry*, *57*(8), 3247–3252. https://doi.org/10.1021/jf8037902

Itkin, M., Heinig, U., Tzfadia, O., Bhide, A. J., Shinde, B., Cardenas, P. D., Bocobza, S. E., Unger, T., Malitsky, S., Finkers, R., Tikunov, Y., Bovy, A., Chikate, Y., Singh, P., Rogachev, I., Beekwilder, J., Giri, A. P., & Aharoni, A. (2013). Biosynthesis of Antinutritional Alkaloids in Solanaceous Crops Is Mediated by Clustered Genes. *Science*, *341*(6142), 175–179. https://doi.org/10.1126/science.1240230

Itkin, M., Rogachev, I., Alkan, N., Rosenberg, T., Malitsky, S., Masini, L., Meir, S., Iijima, Y., Aoki, K., de Vos, R., Prusky, D., Burdman, S., Beekwilder, J., & Aharoni, A. (2011). GLYCOALKALOID METABOLISM1 Is Required for Steroidal Alkaloid Glycosylation and Prevention of Phytotoxicity in Tomato. *The Plant Cell*, *23*(12), 4507–4525. https://doi.org/10.1105/tpc.111.088732

Johns, T., & Alonso, J. G. (1990). Glycoalkaloid change during the domestication of the potato, Solanum Section Petota. *Euphytica*, *50*(3), 203–210. https://doi.org/10.1007/BF00023646

Junker-Frohn, L. V., Lück, M., Schmittgen, S., Wensing, J., Carraresi, L., Thiele, B., Groher, T., Reimer, J. J., Bröring, S., Noga, G., Jupke, A., Schurr, U., Usadel, B., Wiese-Klinkenberg, A., & Wormit, A. (2019). Tomato's Green Gold: Bioeconomy Potential of Residual Tomato Leaf Biomass as a Novel Source for the Secondary Metabolite Rutin. ACS Omega, 4(21), 19071–19080. https://doi.org/10.1021/acsomega.9b01462

Kahlert, H., Suck, R., Weber, B., Nandy, A., Wald, M., Keller, W., Cromwell, O., & Fiebig, H. (2008). Characterization of a Hypoallergenic Recombinant Bet v 1 Variant as a Candidate for Allergen-Specific Immunotherapy. *International Archives of Allergy and Immunology*, *145*(3), 193–206. https://doi.org/10.1159/000109288

Kaunda, J. S., & Zhang, Y.-J. (2019). The Genus Solanum: An Ethnopharmacological, Phytochemical and Biological Properties Review. Natural Products and Bioprospecting, 9(2), 77–137. https://doi.org/10.1007/s13659-019-0201-6

Kaup, O., Gräfen, I., Zellermann, E.-M., Eichenlaub, R., & Gartemann, K.-H. (2005). Identification of a Tomatinase in the Tomato-Pathogenic Actinomycete *Clavibacter michiganensis* subsp. *Michiganensis* NCPPB382. *Molecular Plant-Microbe Interactions®*, *18*(10), 1090–1098. https://doi.org/10.1094/MPMI-18-1090

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. E. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols*, *10*(6), 845–858. https://doi.org/10.1038/nprot.2015.053

Kungl, A. J., Susani, M., Lindemann, A., Machius, M., Visser, A. J. W. G., Scheiner, O., Kraft, D., Breitenbach, M., & Auer, M. (1996). Evidence for an Alpha Helical T Cell Epitope in the C-Terminus of the Main Birch Pollen Allergen Bet V 1. *Biochemical and Biophysical Research Communications*, *223*(1), 187–192. https://doi.org/10.1006/bbrc.1996.0867

Liang, Z., Liang, H., Guo, Y., & Yang, D. (2021). Cyanidin 3-O-galactoside: A Natural Compound with Multiple Health Benefits. International Journal of Molecular Sciences, 22(5), 2261. https://doi.org/10.3390/ijms22052261

Martina, M., Tikunov, Y., Portis, E., & Bovy, A. G. (2021). The Genetic Basis of Tomato Aroma. *Genes*, *12*(2), 226. https://doi.org/10.3390/genes12020226

Melotto, M., Brandl, M. T., Jacob, C., Jay-Russell, M. T., Micallef, S. A., Warburton, M. L., & Van Deynze, A. (2020). Breeding Crops for Enhanced Food Safety. *Frontiers in Plant Science*, *11*, 428. https://doi.org/10.3389/fpls.2020.00428

Mensinga, T. T., Sips, A. J. A. M., Rompelberg, C. J. M., van Twillert, K., Meulenbelt, J., van den Top, H. J., & van Egmond, H. P. (2005). Potato glycoalkaloids and adverse effects in humans: An ascending dose study. *Regulatory Toxicology and Pharmacology*, *41*(1), 66–72. https://doi.org/10.1016/j.yrtph.2004.09.004

Mikulášek, K., Konečná, H., Potěšil, D., Holánková, R., Havliš, J., & Zdráhal, Z. (2021). SP3 Protocol for Proteomic Plant Sample Preparation Prior LC-MS/MS. *Frontiers in Plant Science*, *12*, 635550. https://doi.org/10.3389/fpls.2021.635550

Nakayasu, M., Akiyama, R., Kobayashi, M., Lee, H. J., Kawasaki, T., Watanabe, B., Urakawa, S., Kato, J., Sugimoto, Y., Iijima, Y., Saito, K., Muranaka, T., Umemoto, N., & Mizutani, M. (2020). Identification of α-Tomatine 23-Hydroxylase Involved in the Detoxification of a Bitter Glycoalkaloid. *Plant and Cell Physiology*, *61*(1), 21–28. https://doi.org/10.1093/pcp/pcz224

Nakayasu, M., Umemoto, N., Ohyama, K., Fujimoto, Y., Lee, H. J., Watanabe, B., Muranaka, T., Saito, K., Sugimoto, Y., & Mizutani, M. (2017). A Dioxygenase Catalyzes Steroid 16α-Hydroxylation in Steroidal Glycoalkaloid Biosynthesis. *Plant Physiology*, *175*(1), 120–133. https://doi.org/10.1104/pp.17.00501

Noronha, H., Silva, A., Silva, T., Frusciante, S., Diretto, G., & Gerós, H. (2022). VviRafS5 Is a Raffinose Synthase Involved in Cold Acclimation in Grapevine Woody Tissues. *Frontiers in Plant Science*, *12*, 754537. https://doi.org/10.3389/fpls.2021.754537

Oyetayo, F. L., & Ibitoye, M. F. (2012). Phytochemical and nutrient/antinutrient interactions in cherry tomato (*Lycopersicon esculentum*) fruits. *Nutrition and Health*, *21*(3), 187–192. https://doi.org/10.1177/0260106012467241

Raiola, A., Rigano, M. M., Calafiore, R., Frusciante, L., & Barone, A. (2014). Enhancing the Health-Promoting Effects of Tomato Fruit for Biofortified Food. *Mediators of Inflammation*, *2014*, 1–16. https://doi.org/10.1155/2014/139873

Rambla, J. L., Medina, A., Fernández-del-Carmen, A., Barrantes, W., Grandillo, S., Cammareri, M., López-Casado, G., Rodrigo, G., Alonso, A., García-Martínez, S., Primo, J., Ruiz, J. J., Fernández-Muñoz, R., Monforte, A. J., & Granell, A. (2016). Identification, introgression, and validation of fruit volatile QTLs from a red-fruited wild tomato species. *Journal of Experimental Botany*, erw455. https://doi.org/10.1093/jxb/erw455

Sandrock, R. W., & VanEtten, H. D. (1998). Fungal Sensitivity to and Enzymatic Degradation of the Phytoanticipin α-Tomatine. *Phytopathology®*, *88*(2), 137–143. https://doi.org/10.1094/PHYTO.1998.88.2.137

Seipke, R. F., & Loria, R. (2008). *Streptomyces scabies* 87-22 Possesses a Functional Tomatinase. *Journal of Bacteriology*, *190*(23), 7684–7692. https://doi.org/10.1128/JB.01010-08

Sharma, K., Kaur, R., Kumar, S., Saini, R. K., Sharma, S., Pawde, S. V., & Kumar, V. (2023). Saponins: A concise review on food related aspects, applications and health implications. Food Chemistry Advances, 2, 100191. https://doi.org/10.1016/j.focha.2023.100191

Silva-Beltrán, N. P., Ruiz-Cruz, S., Chaidez, C., Ornelas-Paz, J. de J., López-Mata, M. A., Márquez-Ríos, E., & Estrada, M. I. (2015). Chemical constitution and effect of extracts of tomato plants byproducts on the enteric viral surrogates. International Journal of Environmental Health Research, 25(3), 299–311. https://doi.org/10.1080/09603123.2014.938030

Sonawane, P. D., Heinig, U., Panda, S., Gilboa, N. S., Yona, M., Kumar, S. P., Alkan, N., Unger, T., Bocobza, S., Pliner, M., Malitsky, S., Tkachev, M., Meir, S., Rogachev, I., & Aharoni, A. (2018). Short-chain dehydrogenase/reductase governs steroidal specialized metabolites structural diversity and toxicity in the genus *Solanum*. *Proceedings of the National Academy of Sciences*, *115*(23). https://doi.org/10.1073/pnas.1804835115

Sonawane, P. D., Jozwiak, A., Panda, S., & Aharoni, A. (2020). 'Hijacking' core metabolism: A new panache for the evolution of steroidal glycoalkaloids structural diversity. *Current Opinion in Plant Biology*, *55*, 118–128. https://doi.org/10.1016/j.pbi.2020.03.008

Sonawane, P. D., Pollier, J., Panda, S., Szymanski, J., Massalha, H., Yona, M., Unger, T., Malitsky, S., Arendt, P., Pauwels, L., Almekias-Siegl, E., Rogachev, I., Meir, S., Cárdenas, P. D., Masri, A., Petrikov, M., Schaller, H., Schaffer, A. A., Kamble, A., … Aharoni, A. (2017). Plant cholesterol biosynthetic pathway overlaps with phytosterol metabolism. *Nature Plants*, *3*(1), 16205. https://doi.org/10.1038/nplants.2016.205

Stach, K., Stach, W., & Augoff, K. (2021). Vitamin B6 in Health and Disease. Nutrients, 13(9), 3229. https://doi.org/10.3390/nu13093229

Tieman, D., Taylor, M., Schauer, N., Fernie, A. R., Hanson, A. D., & Klee, H. J. (2006). Tomato aromatic amino acid decarboxylases participate in synthesis of the flavor volatiles 2-phenylethanol and 2-phenylacetaldehyde. Proceedings of the National Academy of Sciences, 103(21), 8287–8292. https://doi.org/10.1073/pnas.0602469103

Tieman, D. M., Loucas, H. M., Kim, J. Y., Clark, D. G., & Klee, H. J. (2007). Tomato phenylacetaldehyde reductases catalyze the last step in the synthesis of the aroma volatile 2-phenylethanol. *Phytochemistry*, *68*(21), 2660–2669. https://doi.org/10.1016/j.phytochem.2007.06.005

Tieman, D., Zeigler, M., Schmelz, E., Taylor, M. G., Rushing, S., Jones, J. B., & Klee, H. J. (2010). Functional analysis of a tomato salicylic acid methyl transferase and its role in synthesis of the flavor volatile methyl salicylate: *Tomato transferase and synthesis of methyl salicylate*. *The Plant Journal*, *62*(1), 113–123. https://doi.org/10.1111/j.1365-313X.2010.04128.x

Wallner, M., Hauser, M., Himly, M., Zaborsky, N., Mutschlechner, S., Harrer, A., Asam, C., Pichler, U., van Ree, R., Briza, P., Thalhamer, J., Bohle, B., Achatz, G., & Ferreira, F. (2011). Reshaping the Bet v 1 fold modulates TH polarization. *Journal of Allergy and Clinical Immunology*, *127*(6), 1571-1578.e9. https://doi.org/10.1016/j.jaci.2011.01.064

Wangorsch, A., Jamin, A., Foetisch, K., Malczyk, A., Reuter, A., Vierecke, S., Schülke, S., Bartel, D., Mahler, V., Lidholm, J., Vieths, S., & Scheurer, S. (2015). Identification of Sola l 4 as Bet v 1 homologous pathogenesis related-10 allergen in tomato fruits. *Molecular Nutrition & Food Research*, *59*(3), 582–592. https://doi.org/10.1002/mnfr.201300620

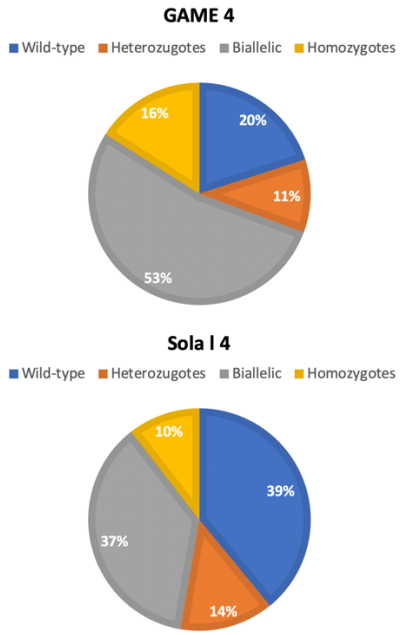
Włodarczyk, K., Smolińska, B., & Majak, I. (2022). Tomato Allergy: The Characterization of the Selected Allergens and Antioxidants of Tomato (Solanum lycopersicum)—A Review. *Antioxidants*, *11*(4), 644. https://doi.org/10.3390/antiox11040644

Zacharisen, M. C., Elms, N. P., & Kurup, V. P. (2002). Severe tomato allergy (Lycopersicon esculentum). *Allergy and Asthma Proceedings*, *23*(2), 149–152.

Zhang, Y., Pribil, M., Palmgren, M., & Gao, C. (2020). A CRISPR way for accelerating improvement of food crops. *Nature Food*, *1*(4), 200–205. https://doi.org/10.1038/s43016-020-0051-8

2.6 Supplemental figures

A)

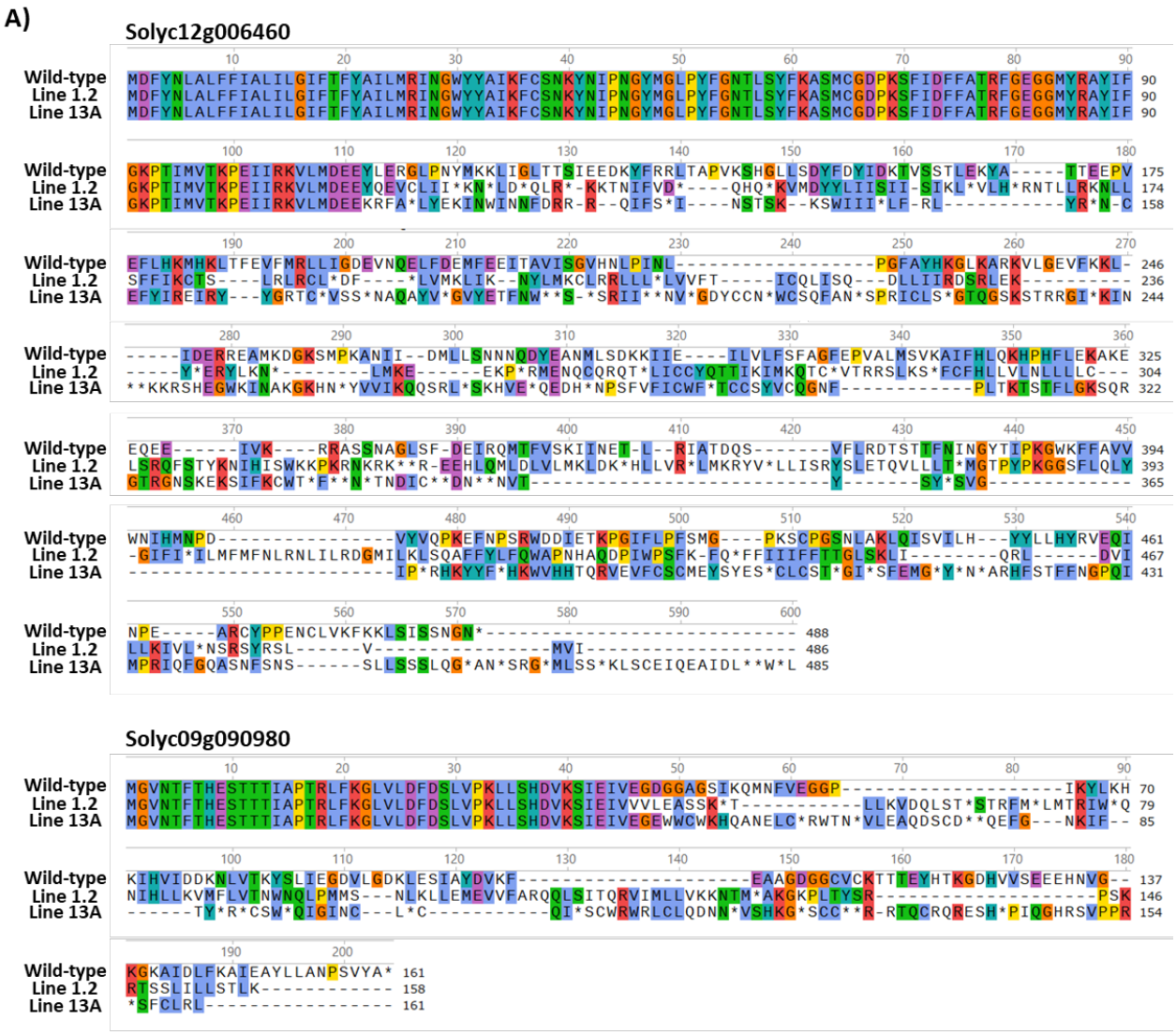


B)

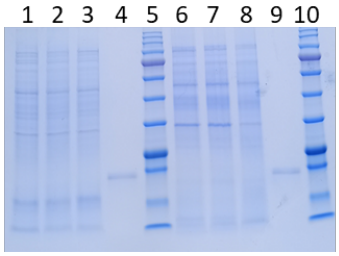
Gene	Mutation Type
GAME 4	Deletion -33
	Deletion -13
	Deletion -8
	Deletion -6
	Deletion -4
	Deletion -1
	Insertion +1
Sola l 4	Deletion -10
	Deletion -5
	Deletion -3
	Deletion -2
	Deletion -1
	Insertion +1

Supplementary Figure 1. Evaluation of editing events: frequency of genome editing events (A) and mutation types (B). Pie charts represent the frequency of each allelic configuration (WT, heterozygous, biallelic, or homozygous) in T0 population.

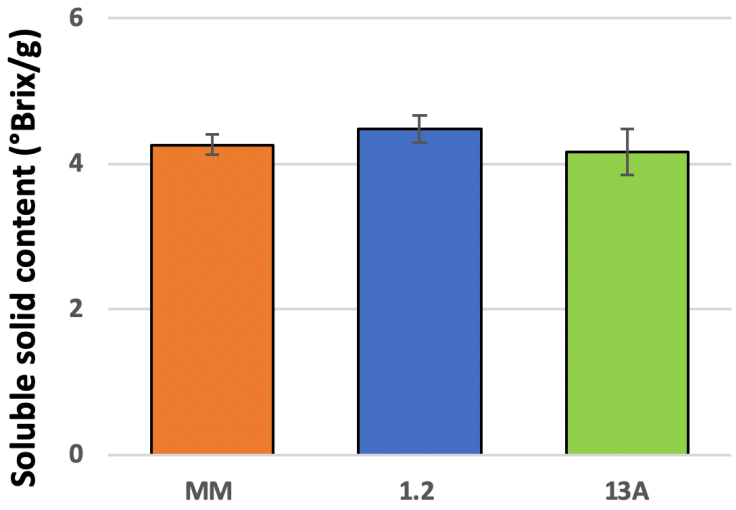
A)



B)



Supplementary Figure 2. A) WT and edited protein sequences. Alignment of the native and CRISPR-generated mutants of lines 1.2 and 13A GAME 4 (Solyc12g006460) and Sola l 4 (Solyc09g090980) proteins. Alignments were obtained with Clustal X and visualized with SnapGene. B) SDS-PAGE analysis of tomato protein extracts. Proteins were visualized by Coomassie Brilliant Blue G250 ***1-3****: 6 µg of total soluble proteins extracted from tomato mature green fruits of 13A and 1.2 edited lines and WT, respectively.* ***6-8****: 10 µg of total soluble proteins extracted from red-ripe fruits of 13A and 1.2 edited lines and WT, respectively.* ***4*** *and* ***9****: 500 ng of recombinant protein produced in E. coli.* ***5*** *and* ***10****: Molecular weight marker (BLUeye prestained Protein Ladder 245–11 kDa, Sigma-Aldrich).*



Supplementary Figure 3. °Brix of MM and edited tomatoes. Analysis was measured on four biological replicates for each line. Data were expressed as mean±SD.

Supplementary Table 1: GoldenBraid parts and list of primers used in this work

GB parts and vectors

GB database ID	Name
GB1001	pUPD U6-26
GB1103	pEGB2omega2 SF-35s:hCas9:tNos
GB0226	pEGB Tnos:NptII:Pnos
GB0475	pDGB1_alpha1
GB0476	pDGB1_alpha2
GB0479	pDGB1_omega1

Primers

GAME 4 gRNA strand +	ATTGATGAAGAGTATCTTGAAAG
GAME 4 gRNA strand -	AAACCTTTCAAGATACTCTTCAT
Sola l 4 gRNA strand +	ATTGAGATTGTTGAGGGAGATGG
Sola l 4 gRNA strand -	AAACCCATCTCCCTCAACAATCTCT
GAME 4 forward	GCTTCAATGTGTGGTGATCC
GAME 4 reverse	ATTCTTGATTAACTTCATCACC
Sola l 4 forward	CACCTTTACTCATGAGTCC
Sola l 4 reverse	ATACAATTTCCAAGTTACAC
hCas9 forward	GAACCTGTCAGACGCCATTC
hCas9 reverse	AGTCATCCACGCGAATCTGG
OFF-target 1 forward	CTAGTCTATGATGTGAGGAG
OFF-target 1 reverse	AGGGAAGCATAACGAAATGC
OFF-target 2 forward	TTGAGGTCAGTGGGCAACC
OFF-target 2 reverse	ATAGGTGAATGTAGAAATGCC
OFF-target 3 forward	ACATTATGGGTGTGAATACCT
OFF-target 3 reverse	GTTACTTGGGTTGAACTAGTC
OFF-target 4 forward	CGGTAGTGTTTGATTTGATGC
OFF-target 4 reverse	AGAAGTCCCTTACAGAGTCC
Actin forward	CTCTGGTGATGGTGTCAG
Actin reverse	AGCCAAGATAGAGCCTCC
GAME 4 forward cDNA	CGCGCGCCATGGGTGTAAACACCTTTACTCATG
GAME 4 reverse cDNA	CGCGCGCTCGAGTTAAGCGTAGACAGAAGGATTAGCGAG

Supplementary Table 2: OFF-target list: in yellow those selected according to the features described in the Materials and Methods section

ID	Gene	gRNA	OFF-target sequence	PAM	Score	Gene	Locus
	GAME4	GATGAAGAGTATCTTGAAAG	G**G**TGAAGAG**A**ATCTTGAAA**T**	TAG	1.7	Solyc01g110700.2	chr1: -97311090
	GAME4	GATGAAGAGTATCTTGAAAG	**TAGGT**AGAG**C**ATCTTGAAAG	AAG	1.4	Solyc02g086500.2	chr2: +49175646
1	*GAME4*	GATGAAGAGTATCTTGAAAG	G**T**TA**A**AGAGTATA**T**TGAAAG	GGG	1.2	Solyc12g099540.1	chr12: -66655485
2	*GAME4*	GATGAAGAGTATCTTGAAAG	GAT**C**AAGAG**G**AT**G**TTGAAAG	TGG	1.1	Solyc03g113500.2	chr3: +63633671
	GAME4	GATGAAGAGTATCTTGAAAG	G**T**TGAAG**TT**TATCTTGAAAG	TAG	1.7		chr7: +56478328
	GAME4	GATGAAGAGTATCTTGAAAG	GAT**CT**AG**T**G**A**ATCTTGAAAG	GAG	1.4		chr6: -6387834
	GAME4	GATGAAGAGTATCTTGAAAG	GAT**TT**AGAGTAT**A**TTGAAAG	GAG	1.2		chr12: -65972658
3	*Sola l 4*	GAGATTGTTGAGGGAGATGG	GAGA**C**TA**T**TGAGGGAGATGG	TGG	3.7	Solyc09g090990.2	chr9: -70356819
	Sola l 4	GAGATTGTTGAGGGAGATGG	G**GA**ATTG**GT**C**A**GGGAGATGG	AAG	1.4	Solyc04g009730.2	chr4: +3056455
	Sola l 4	GAGATTGTTGAGGGAGATGG	**A**AGATTG**G**TGAGGGAGAT**A**G	GAG	1.4	Solyc02g080210.2	chr2: -44494990
4	*Sola l 4*	GAGATTGTTGAGGGAGATGG	GAGA**C**TGTTA**A**G**C**GAGATGG	AGG	1.0	Solyc04g015440.2	chr4: +5629283
	Sola l 4	GAGATTGTTGAGGGAGATGG	GAGA**G**TG**G**TGAGGGAGATGG	GAG	5.7		chr6: +46048870
	Sola l 4	GAGATTGTTGAGGGAGATGG	GAGA**GTT**TTGAGGGAGATGG	GAG	3.7		chr1: +26920116
	Sola l 4	GAGATTGTTGAGGGAGATGG	**TT**GATTGTTGAGGGAGATG**A**	AAG	2.0		chr3: +67674206
	Sola l 4	GAGATTGTTGAGGGAGATGG	GAGATTGT**G**G**T**GGGAGATGG	AAG	1.9		chr4: -55738653
	Sola l 4	GAGATTGTTGAGGGAGATGG	GAGATTGT**G**G**T**GGGAGATGG	AAG	1.9		chr4: +45675734
	Sola l 4	GAGATTGTTGAGGGAGATGG	GA**AT**TTGTTGAG**A**GAGATGG	AGG	1.2		chr1: -91978455
	Sola l 4	GAGATTGTTGAGGGAGATGG	GAGA**A**TG**G**TGAG**A**GAGATGG	GAG	1.1		chr7: -2954473
	Sola l 4	GAGATTGTTGAGGGAGATGG	GAGA**G**TG**G**TGAG**T**GAGATGG	GAG	1.1		chr12: +9246634
	Sola l 4	GAGATTGTTGAGGGAGATGG	GAGA**G**TG**A**TGAG**C**GAGATGG	GAG	1.1		chr9: -60715133
	Sola l 4	GAGATTGTTGAGGGAGATGG	GAGA**G**TG**A**TGAG**T**GAGATGG	GAG	1.1		chr5: +29777302
	Sola l 4	GAGATTGTTGAGGGAGATGG	**AAA**A**T**A**T**TGAGGGAGATGG	AGG	1.0		chr5: -65313289

Supplementary Table 4: List of differentially abundant proteins up- or down- regulated in leaves of edited lines. Only proteins with adjusted p-value <0.05 and fold change (FC) >|1.5| were shown.

Description	Acessions	FC 1.2-WT	FC 13A-WT
Non-specific lipid-transfer protein	A0A3Q7HZ96	0,00	0,01
Uncharacterized protein	A0A3Q7J325	0,01	0,01
Superoxide dismutase [Cu-Zn], chloroplastic (EC 1.15.1.1)	P14831	0,04	0,06
MFS domain-containing protein	A0A3Q7I036	0,04	0,04
Lipoxygenase (EC 1.13.11.-)	A0A3Q7JSV3	0,05	0,16
L-ascorbate oxidase (EC 1.10.3.3)	A0A3Q7IYF8	0,08	0,14
Uncharacterized protein	A0A3Q7FEJ3	0,14	0,16
Uncharacterized protein	A0A3Q7EPL0	0,15	0,12
Exostosin domain-containing protein	A0A3Q7FDL5	0,16	0,23
Uncharacterized protein	A0A3Q7GPG8	0,17	0,20
Uncharacterized protein	A0A3Q7HQ62	0,18	0,25
Xyloglucan endotransglucosylase/hydrolase (EC 2.4.1.207)	A0A3Q7IYQ5	0,19	0,40
AAI domain-containing protein	A0A3Q7FIV4	0,21	0,22
Chalcone synthase 2 (EC 2.3.1.74)	P23419	0,21	0,11
Uncharacterized protein	A0A3Q7GG14	0,22	0,21
Uncharacterized protein	A0A3Q7FP18	0,22	0,20
Laccase (EC 1.10.3.2)	A0A3Q7H0Q4;A0A3Q7I0R7;A0A3Q7IUX9	0,22	0,25
CarbpepA_inh domain-containing protein	A0A3Q7EG84	0,23	0,41
Uncharacterized protein	A0A3Q7ERA8	0,23	0,31
Uncharacterized protein	A0A3Q7HJ32;A0A3Q7I3Y2	0,26	0,25
Bet_v_1 domain-containing protein	A0A3Q7IGD0	0,27	0,34
Glyco_trans_2-like domain-containing protein	A0A3Q7H2X8	0,27	0,29
Phytocyanin domain-containing protein	A0A3Q7FAM3	0,27	0,14
Superoxide dismutase [Cu-Zn] 1 (EC 1.15.1.1)	P14830;Q43779	0,28	0,16
Bet_v_1 domain-containing protein	A0A3Q7FV86	0,29	0,37
Uncharacterized protein	A0A3Q7I304	0,29	0,33
Uncharacterized protein	A0A3Q7FEJ3;A0A3Q7FH23	0,29	0,23
Uncharacterized protein	A0A3Q7FSK9	0,31	0,23
Terpene cyclase/mutase family member (EC 5.4.99.-)	A0A3Q7H8X4	0,33	0,35
M20_dimer domain-containing protein	A0A3Q7F0T1	0,33	0,32
Glyco_hydro_19_cat domain-containing protein	A0A3Q7JDI6	0,34	0,29
MFS domain-containing protein	A0A3Q7ERH4	0,35	0,35
Caffeoyl-CoA O-methyltransferase (EC 2.1.1.104)	A0A3Q7ESA3	0,35	0,32

Uncharacterized protein	A0A3Q7G1C2	0,35	0,26
Pectinesterase (EC 3.1.1.11)	A0A3Q7EL18	0,36	0,38
Uncharacterized protein	A0A3Q7FB62	0,38	0,55
Uncharacterized protein	A0A3Q7HGQ3	0,39	0,46
Uncharacterized protein	A0A3Q7I2I8	0,39	0,54
Str_synth domain-containing protein	A0A3Q7J8Q2	0,40	0,34
Uncharacterized protein	A0A3Q7F4I2	0,40	0,30
Uncharacterized protein	A0A3Q7H5D1;A0A3Q7I4I8	0,40	0,44
Cytokinin riboside 5'-monophosphate phosphoribohydrolase (EC 3.2.2.n1)	A0A3Q7JPP8	0,41	0,42
Uncharacterized protein	A0A3Q7FQH2	0,41	0,37
Peroxidase (EC 1.11.1.7)	A0A3Q7GMA4	0,41	0,40
Uncharacterized protein	A0A3Q7FP18;A0A3Q7FR78	0,41	0,45
AB hydrolase-1 domain-containing protein	A0A3Q7HZK2	0,42	0,36
Uncharacterized protein	A0A3Q7HX29	0,43	0,37
4-coumarate--CoA ligase (EC 6.2.1.12)	A0A3Q7G3R4	0,43	0,39
Cas1_AcylT domain-containing protein	A0A3Q7F295;A0A3Q7G1A6	0,44	0,36
Uncharacterized protein	A0A3Q7EJ56	0,47	0,37
Caffeoyl-CoA O-methyltransferase (EC 2.1.1.104)	A0A3Q7ESA3;A0A3Q7IGY7	0,47	0,44
Uncharacterized protein	K4CWU7	0,47	0,27
Fructose-bisphosphate aldolase (EC 4.1.2.13)	A0A3Q7HX95	0,47	0,45
Uncharacterized protein	A0A3Q7I8W9	0,48	0,31
Uncharacterized protein	A0A3Q7FCM8	0,48	0,36
Cytochrome c oxidase subunit 2	A0A3Q7HZ70	0,48	0,34
Fe2OG dioxygenase domain-containing protein	A0A3Q7JJW7	0,48	0,32
Uncharacterized protein	A0A3Q7FM96	0,48	0,39
Uncharacterized protein	A0A3Q7F2S6	0,49	0,48
Fructokinase-2 (EC 2.7.1.4)	Q42896	0,49	0,55
Plastocyanin, chloroplastic	P17340	0,50	0,31
PMR5N domain-containing protein	A0A3Q7JEU2	0,50	0,49
Cellulose synthase (EC 2.4.1.12)	A0A3Q7G6I1	0,50	0,40
Amino_oxidase domain-containing protein	A0A3Q7HB95	0,51	0,38
Ribosomal_L12 domain-containing protein	A0A3Q7FD59	0,51	0,55
Uncharacterized protein	A0A3Q7HXS6	0,51	0,54
Tonoplast dicarboxylate transporter	K4D641	0,51	0,57
Polyphenol oxidase B, chloroplastic (PPO) (EC 1.10.3.1)	Q08304;Q08307	0,52	0,26
AB hydrolase-1 domain-containing protein	A0A3Q7HZ62	0,52	0,58
40S ribosomal protein S8	A0A3Q7IY72	0,52	0,57
Uncharacterized protein	A0A3Q7J9M4	0,52	0,38
Cellulose synthase (EC 2.4.1.12)	A0A3Q7EJE4	0,53	0,45
L04	B6CG42	0,53	0,42
Phenylalanine ammonia-lyase (EC 4.3.1.24)	A0A3Q7GQY4;A0A3Q7IPA2	0,53	0,34

Cytochrome c oxidase subunit 5C	A0A3Q7EHH2	0,53	0,45
Transmembrane 9 superfamily member	A0A3Q7EQC8;A0A3Q7IDU9	0,54	0,45
Pectin acetylesterase (EC 3.1.1.-)	A0A3Q7EQS7	0,54	0,54
Non-specific serine/threonine protein kinase (EC 2.7.11.1)	A0A3Q7EMM2;A0A3Q7FP82;A0A3Q7H173;A0A3Q7J1Y5	0,54	0,47
Uncharacterized protein	A0A3Q7I9V2	0,54	0,37
Methylenetetrahydrofolate reductase (EC 1.5.1.20)	A0A3Q7GLC6	0,54	0,57
Phenylalanine ammonia-lyase (EC 4.3.1.24)	A0A3Q7IPA2	0,54	0,47
Uncharacterized protein	A0A3Q7H2V7	0,55	0,44
Polyphenol oxidase E, chloroplastic (PPO) (EC 1.10.3.1)	Q08307	0,55	0,31
DCD domain-containing protein	A0A3Q7GNT7	0,56	0,61
Uncharacterized protein	A0A3Q7H1P2	0,56	0,58
Uncharacterized protein	A0A3Q7GLR8	0,57	0,51
Sugar-porter family protein 5	A0A0A8JCK7	0,57	0,60
Uncharacterized protein	A0A3Q7GAQ5	0,57	0,61
Uncharacterized protein	A0A3Q7H3Z1	0,58	0,34
COX2_CUA domain-containing protein	A0A3Q7JMU8	0,58	0,44
PRA1 family protein	A0A3Q7JE05	0,59	0,45
Uncharacterized protein	A0A3Q7I868	0,59	0,43
Uncharacterized protein	A0A3Q7IST6;B6CG42	0,59	0,56
Uncharacterized protein	A0A3Q7IMY2	0,59	0,66
Uncharacterized protein	A0A3Q7GPG7	0,59	0,61
Uncharacterized protein	A0A3Q7INI5	0,59	0,56
Uncharacterized protein	A0A3Q7I0U7	0,59	0,56
Uncharacterized protein	A0A3Q7F628	0,59	0,49
Uncharacterized protein	A0A3Q7HBA1	0,60	0,66
Phenylalanine ammonia-lyase (PAL) (EC 4.3.1.24)	P26600	0,60	0,43
RRM domain-containing protein	A0A3Q7I5J4	0,61	0,48
Uncharacterized protein	A0A3Q7J2G7	0,62	0,55
DUF1421 domain-containing protein	A0A3Q7IB76	0,62	0,63
Uncharacterized protein	A0A3Q7HMK3	0,63	0,59
Uncharacterized protein	A0A3Q7G9R5	0,63	0,58
Bet_v_1 domain-containing protein	A0A3Q7GDF2	0,63	0,47
Uncharacterized protein	A0A3Q7G6Z4	0,63	0,62
MSP domain-containing protein	A0A3Q7HUF7	0,64	0,66
Uncharacterized protein	A0A3Q7GF56	0,64	0,56
Chalcone-flavonone isomerase family protein	A0A3Q7HFP3	0,64	0,51
Uncharacterized protein	A0A3Q7IST6	0,64	0,62
Cellulose synthase (EC 2.4.1.12)	A0A3Q7EJE4;A0A3Q7F4R8;A0A3Q7G6I1;A0A3Q7IID5	0,65	0,56
Uncharacterized protein	A0A3Q7J405	0,65	0,55
40S ribosomal protein S8	A0A3Q7ICF4;A0A3Q7IY72	0,65	0,57

Uncharacterized protein	A0A3Q7FSR2	0,66	0,44
Uncharacterized protein	A0A3Q7E890;A0A3Q7JFW9	0,66	0,61
Uncharacterized protein	A0A3Q7FVM3	1,51	2,13
Uncharacterized protein	A0A3Q7EL73	1,52	1,53
Malic enzyme	A0A3Q7EKQ6	1,52	1,54
Phospholipase A1 (EC 3.1.1.-)	A0A3Q7F3J5;A0A3Q7F5D0	1,52	2,34
DUF1338 domain-containing protein	A0A3Q7GE36	1,52	1,64
Uncharacterized protein	A0A3Q7FWJ6;A0A3Q7FYZ5	1,53	1,76
Superoxide dismutase (EC 1.15.1.1)	A0A3Q7FLN4;A0A3Q7GUW9	1,55	1,76
Basic 30 kDa endochitinase (EC 3.2.1.14)	Q05538	1,55	2,49
Isocitrate lyase (ICL) (EC 4.1.3.1)	P49297	1,55	1,67
Uncharacterized protein	A0A3Q7FAT8	1,55	1,76
Uncharacterized protein	A0A3Q7FCP6	1,56	1,67
Uncharacterized protein	A0A3Q7ETE6	1,57	1,53
Coproporphyrinogen oxidase (EC 1.3.3.3)	A0A3Q7I9A2	1,57	1,58
Peroxidase (EC 1.11.1.7)	A0A3Q7FU03	1,59	1,85
Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	A0A3Q7HAH2	1,59	1,61
Uncharacterized protein	A0A3Q7JX18	1,59	1,62
Rhodanese domain-containing protein	A0A3Q7F7E7	1,61	1,85
Poly [ADP-ribose] polymerase (PARP) (EC 2.4.2.-)	A0A3Q7E9G4	1,62	1,52
Bis(5'-adenosyl)-triphosphatase (EC 3.6.1.29)	A0A3Q7I1X1	1,64	1,68
CASP-like protein	A0A3Q7GZC6	1,64	1,76
Lipoxygenase (EC 1.13.11.-)	A0A3Q7ENA4	1,64	1,82
Pectinesterase (EC 3.1.1.11)	A0A3Q7F4W8;A0A3Q7F5K0	1,64	2,42
Diadenosine tetraphosphate synthetase (EC 6.1.1.14)	A0A3Q7FU99	1,64	1,69
Malic enzyme	A0A3Q7HIS1	1,64	1,64
Gamma aminobutyrate transaminase 3, chloroplastic (EC 2.6.1.96)	Q84P52	1,65	1,87
Uncharacterized protein	A0A3Q7JYU2	1,67	1,60
Uncharacterized protein	A0A3Q7IK89	1,68	1,97
Glutamate synthase (NADH) (EC 1.4.1.14)	A0A3Q7FKX4	1,68	1,92
Calreticulin	A0A3Q7G3G6	1,69	1,73
Uncharacterized protein	Q40129	1,70	1,56
Phosphoenolpyruvate carboxykinase (ATP) (EC 4.1.1.49)	A0A3Q7G9B3	1,70	1,55
R3H-assoc domain-containing protein	A0A3Q7I3V5	1,70	1,67
Uncharacterized protein	A0A3Q7I6I0	1,72	1,73
Peroxidase (EC 1.11.1.7)	A0A3Q7FZA3	1,72	1,73
50S ribosomal protein L36, chloroplastic	Q2MI67	1,73	1,79
NADPH-protochlorophyllide oxidoreductase (EC 1.3.1.33)	A0A3Q7J565	1,73	1,86
Uncharacterized protein	A0A3Q7FQM8	1,74	1,70

Phospholipase A1 (EC 3.1.1.-)	A0A3Q7F5D0	1,75	2,65
40S ribosomal protein S12	A0A3Q7FAA3;A0A3Q7FN30;A0A3Q7JAB8	1,78	2,04
Uncharacterized protein	A0A3Q7GX38	1,78	1,78
Uncharacterized protein	A0A3Q7HTM2;A0A3Q7HVI4	1,78	2,63
NADPH-protochlorophyllide oxidoreductase (EC 1.3.1.33)	A0A3Q7IBI0;A0A3Q7J565	1,78	1,76
Superoxide dismutase (EC 1.15.1.1)	A0A3Q7GUW9	1,79	1,89
Flotillin-like	A0A3Q7FRU4	1,80	1,75
Aldo_ket_red domain-containing protein	A0A3Q7I8W0;A0A3Q7I912	1,81	1,66
Chitinase (EC 3.2.1.14)	A0A3Q7GMW3	1,85	1,89
Aamy domain-containing protein	A0A3Q7I1R5	1,86	2,30
HMA domain-containing protein	A0A3Q7HPP7	1,86	1,50
Uncharacterized protein	A0A3Q7FWJ6	1,86	2,29
Uncharacterized protein	A0A3Q7HZK1	1,86	1,87
Phosphoenolpyruvate carboxykinase (ATP) (EC 4.1.1.49)	A0A3Q7G9B3;A0A3Q7JCG7	1,87	1,61
4HBT domain-containing protein	A0A3Q7H1N1	1,88	2,17
Cryptochrome DASH, chloroplastic/mitochondrial (Cryptochrome-3)	Q38JU2	1,88	1,61
Uncharacterized protein	A0A3Q7HVI4	1,91	2,84
Uncharacterized protein	A0A3Q7FY27	1,91	1,72
AB hydrolase-1 domain-containing protein	A0A3Q7GPY6	1,92	2,02
Amidase domain-containing protein	A0A3Q7J0X9	1,93	2,06
Uncharacterized protein	A0A3Q7HVI0;A0A3Q7HVI4	1,93	2,74
Peroxidase (EC 1.11.1.7)	A0A3Q7F1C1	1,94	2,28
Uncharacterized protein	A0A3Q7HHX1;A0A3Q7HKM1	1,94	2,50
Uncharacterized protein	A0A3Q7JQ05	1,95	1,62
Uncharacterized protein	A0A3Q7HTM2;A0A3Q7HVI0;A0A3Q7HVI4	1,97	2,83
Elongation factor 1-alpha	A0A3Q7J0Z4	2,03	2,43
Uncharacterized protein	A0A3Q7EMC4	2,09	2,85
Aamy domain-containing protein	A0A3Q7I3T7	2,10	2,42
Cellulase domain-containing protein	A0A3Q7JX67	2,11	1,92
Uncharacterized protein	A0A3Q7FCJ1	2,12	1,74
Alpha-1,4 glucan phosphorylase (EC 2.4.1.1)	A0A3Q7I2P2	2,13	2,25
PEROXIDASE_4 domain-containing protein	A0A3Q7HLJ4	2,13	1,98
Uncharacterized protein	A0A3Q7HJT2	2,15	1,99
Biotin synthase (EC 2.8.1.6)	A0A3Q7HVL1	2,16	1,88
Uncharacterized protein	A0A3Q7H012	2,16	3,13
Uncharacterized protein	A0A3Q7H6C9	2,16	2,05
Uncharacterized protein	A0A3Q7FNP8	2,17	2,10
Uncharacterized protein	A0A3Q7E8V7	2,19	2,44
Carboxypeptidase (EC 3.4.16.-)	A0A3Q7H1H4	2,19	2,33

Thioredoxin domain-containing protein	A0A3Q7EXD9;A0A3Q7F545	2,19	2,41
Cytochrome b5 heme-binding domain-containing protein	A0A3Q7I4T1	2,24	1,97
Thioredoxin domain-containing protein	A0A3Q7F545	2,24	2,59
Uncharacterized protein	A0A3Q7FG96	2,27	2,29
O-fucosyltransferase family protein	A0A3Q7G8Z0	2,31	1,98
Peptidylprolyl isomerase (EC 5.2.1.8)	A0A3Q7I412	2,31	2,15
Uncharacterized protein	A0A3Q7HT24	2,41	3,06
DCD domain-containing protein	A0A3Q7I6I1	2,47	2,03
M20_dimer domain-containing protein	A0A3Q7HRC7;A0A3Q7HS08;A0A3Q7HTY1	2,60	2,45
Uncharacterized protein	A0A3Q7HT55	2,64	2,56
O-fucosyltransferase family protein	Q949J8	2,66	2,40
DAO domain-containing protein	A0A3Q7I3J1	2,68	2,58
Sod_Fe_C domain-containing protein	A0A3Q7GS80;A0A3Q7GUW9	2,69	2,86
AvrRpt-cleavage domain-containing protein	A0A3Q7IYL7	2,70	2,39
Uncharacterized protein	A0A3Q7I0E1	2,74	2,55
Uncharacterized protein	A0A3Q7HVV0;A0A3Q7IQ03	2,77	3,26
Glucan endo-1,3-beta-glucosidase A (EC 3.2.1.39)	Q01412	2,79	5,19
Pathogenesis-related protein P2	P32045	2,79	3,65
Uncharacterized protein	A0A3Q7HVQ8;A0A3Q7IM48	2,84	3,14
Uncharacterized protein	A0A3Q7I748	2,84	2,90
Peroxidase (EC 1.11.1.7)	A0A3Q7ER05;A0A3Q7F1C1	2,91	3,04
Chitin-binding type-1 domain-containing protein	A0A3Q7IHS3	2,97	3,69
M20_dimer domain-containing protein	A0A3Q7HRC7	3,04	3,03
M20_dimer domain-containing protein	A0A3Q7HRC7;A0A3Q7HS08	3,05	2,90
Uncharacterized protein	A0A3Q7JS81	3,11	4,66
Aldo_ket_red domain-containing protein	A0A3Q7I912	3,20	2,26
Peroxidase (EC 1.11.1.7)	A0A3Q7ER05	3,21	3,24
Uncharacterized protein	A0A3Q7J2P9	3,23	3,58
Uncharacterized protein	A0A3Q7EK65	3,52	4,16
Peroxidase (EC 1.11.1.7)	A0A3Q7FFR5	3,53	4,04
Uncharacterized protein	A0A3Q7JR76	3,66	3,70
AAI domain-containing protein	A0A3Q7IJL2	3,80	8,41
Carboxypeptidase (EC 3.4.16.-)	A0A3Q7H1H4;A0A3Q7H4N0	3,82	4,93
Protein NP24 (Pathogenesis-related protein PR P23)	P12670;Q01591	4,02	6,14
Glucan endo-1,3-beta-glucosidase B (EC 3.2.1.39)	Q01413	4,30	12,21
Uncharacterized protein	A0A3Q7H8G2	4,39	4,35
Inducible plastid-lipid associated protein	A0RZD0	4,60	5,12
SGNH_hydro domain-containing protein	A0A3Q7IJ05	4,66	4,92
Leucine aminopeptidase 1, chloroplastic (EC 3.4.11.1)	Q10712	4,90	6,37

Uncharacterized protein	A0A3Q7HBX4	5,07	3,63
PKS_ER domain-containing protein	A0A3Q7I2C5	5,31	5,89
Acidic 26 kDa endochitinase (EC 3.2.1.14)	Q05539	7,53	14,00
Wound-induced proteinase inhibitor 2 (Wound-induced proteinase inhibitor II)	P05119	7,94	9,98
Patatin (EC 3.1.1.-)	A0A3Q7HK00	8,56	14,31
Peroxidase (EC 1.11.1.7)	A0A3Q7GKS4	13,72	11,20
Wound-induced proteinase inhibitor 1 (Wound-induced proteinase inhibitor I)	P05118	14,03	15,46
Threonine dehydratase 2 biosynthetic, chloroplastic (EC 4.3.1.17)	P25306	14,75	19,31
PKS_ER domain-containing protein	A0A3Q7I1T6	16,70	18,16
Uncharacterized protein	A0A3Q7I877	18,19	24,97
Uncharacterized protein	A0A3Q7FP33	22,24	33,94
Uncharacterized protein	A0A3Q7FX57;A0A3Q7HZV3	31,70	30,38
PKS_ER domain-containing protein	A0A3Q7I1T6;A0A3Q7I2C5	34,32	49,83
Uncharacterized protein	A0A3Q7I482	87,63	99,55

Chapter 3. CRISPR/Cas9 knockout of antinutritional *GAME 4* and allergenic *Sola l 4* genes shape bacterial community in tomato rhizosphere.

Note: This chapter contains data included in a publication that will be submitted to Frontiers in Microbiology in collaboration with the groups listed below.

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3.1 Introduction

Plants live in close association with soil microbes, which play a central role in plant health and productivity (Mendes et al., 2011; Philippot et al., 2013). Soil contains a huge and highly diverse microorganisms such as bacteria, archaea, fungus, oomycetes, viruses, and protists, representing the largest reservoir of biological diversity (Buée et al., 2009; Torsvik et al., 2002). Soil microorganisms can be beneficial, neutral or harmful (J. Li et al., 2021). The latter, such as soil-borne pathogens, affect crop health, development and plant growth, resulting in yield loss (Yin et al., 2021). On the other hand, beneficial microbes can be involved in the stimulation of plant growth producing plant hormones, improving tolerance to various stresses and enhancing nutrients availability (Haney et al., 2015; R. Jacoby et al., 2017; Yin et al., 2021). So, all these species are engaged in intricate trophic exchange networks with each other and with plant root system (Compant et al., 2019; Fitzpatrick et al., 2020). In fact, plants release a series of organic and inorganic, soluble and insoluble compounds (including up to 40% of photosynthates) into the soil, changing the characteristics of the surrounding soil and the microbial community (Zhalnina et al., 2018). This process known as rhizodeposition varies according to plant species, root age, light intensity, soil characteristics and stress conditions (Nguyen, 2003; Patra et al., 2021). The rhizosphere represents the portion of soil in close contact with the plant roots. It is also the area where the plant's root exudates accumulate, acting as nutrients for soil microorganisms (Bulgarelli et al., 2013). That explains the different concentration and composition of the microbial population with the surrounding bulk soil, that is the soil located very far from roots. In fact, rhizospheric soil can contain up to 10^{11} microbial cells per gram of roots, approximately 10-100 times more than bulk soil (Egamberdieva et al., 2007). The genetic components of the rhizosphere community taken all together, known as the rhizosphere microbiome, are much larger than the plant genome; for this reason, it is also called the second plant genome (Berendsen et al., 2012). However, the microbial communities that live in the rhizosphere, despite its great abundance, show lower biodiversity respect to the bulk soil. Interestingly, plant root exudates would seem to exert a true selection of the microorganisms present in the bulk soil according to the temporary environmental situations (Berendsen et al., 2012). There is therefore a real interaction, a kind of mutualism, between plants and the rhizospheric microbiome. In fact, microbial populations in the rhizosphere, in exchange for food and energy sources from the plant's organic compounds in the root system, help plants growth and function by increasing their resistance to pathogens, retaining more water and, absorbing and utilizing more nutrients (Lakshmanan et al., 2014).

Tomato (*Solanum lycopersicum*) is one of the most economically important vegetable crops and one of the major products of the agri-food industry, with more than 189 million tons worldwide

(FAO 2021). It is largely consumed in the Mediterranean diet, due to its high nutritional value and sensorial traits. Additionally, this species represents a model for both basic and applied research on plant-microbe interactions (Dong et al., 2019). In this context, numerous metagenomics studies have allowed a better understanding of the formation and modulation of the rhizospheric microbiome. In particular, it has been found that the microbial community is influenced, among other factors, by tomato genotypes (French et al., 2020), soil type (Dong et al., 2019), soil-borne diseases (Colagiero et al., 2020; J.-G. Li et al., 2014) and root exudates (Nakayasu, Ohno, et al., 2021). The root exudates of the tomato plant include primary metabolites such as certain sugars, amino acids and organic acids, and plant specialized metabolites (PSMs) such as flavonoids, strigolactones and steroidal glycoalkaloids (SGAs) (Nakayasu et al., 2022). Among these, SGAs exhibit a toxic effect against numerous microorganisms; in particular, α-tomatine shows antibiotic activity against many pathogens, including the tomato Fusarium wilt (*Fusarium oxysporum f. sp. lycopersici*) (Sandrock & VanEtten, 1998). Recently, α-tomatine and tomatidine have been found to alter the microbial community, increasing the abundance of the *Sphingomonadaceae* family in tomato plant rhizosphere (Nakayasu, Ohno, et al., 2021). However, in the same work, the authors also observed a decrease in α-diversity, leading to the hypothesis that their antimicrobial activity could inhibit the growth of certain microorganisms (Nakayasu, Ohno, et al., 2021). However, further investigations are needed to better understand the effect of SGAs in modulating the microbial community.

In our laboratory, we recently generated and characterized CRISPR/Cas9 edited tomato plants in target genes involved in glycoalkaloid metabolism (*GAME 4*) and in plant stress responses (the PR10 *Sola l 4*) (see Chapter 2). The edited tissues (leaves and fruits) were characterized, compared to the WT counterparts, by the absence of the *Sola l 4* protein, and by a strong reduction in total SGAs (greater than 99%), with a simultaneous accumulation of steroidal saponins.

In the present study, we compared the bacterial communities in the rhizosphere of WT and GAME 4/Sola l 4 knock-out tomato lines, in order to assess if the alterations in the composition of molecules involved in the defense mechanisms (both SGAs and saponins) exuded from roots, as well as the absence of *Sola l 4* PR10-protein could also result in a different distribution at microbial composition and biodiversity levels.

3.2 Materials and methods

3.2.1 Plant materials, growth conditions, rhizosphere and roots sampling

Seeds of GAME 4 – Sola l 4 edited tomato lines, previously generated using Moneymaker as genetic background (see Chapter 2), and WT (as control), were surface-sterilized with 70% ethanol

for 30 seconds, rinsed with sterile distilled water and washed with 1% sodium hypochlorite for 10 min. The seeds were quickly rinsed five times with sterile distilled water and finally washed in sterile water for at least thirty minutes with gentle agitation. For each line, 20 sterilized seeds were germinated in Petri dishes on wet sterile paper, and placed in a growth chamber at 25 ± 1°C and 60 ± 5% of relative humidity (RH) at darkness. Upon roots emergence, plantlets were transferred to a polystyrene plateau with universal RADICOM soil substrate (Vigorplant Italia s.r.l., Fombio Lodi, Italy) and were grown in a in a containment greenhouse at 24 °C during the day (16 hr) and at 18 °C during night (8 hr) as previously described (Diretto et al., 2020). After 2 weeks, plants were placed in small pots (ARCAVasi, 10x10x12 cm) and finally, after 4 weeks, five plants for each line were transferred to bigger pots (ARCAVasi, 22x15x20 cm) disposed according a fully randomized scheme. Three replicates (250 mg) of rhizosphere soil from 5 individual plants were collected at the harvest stage using the following procedure: the entire root apparatus from the pot was taken out paying attention to maintain its integrity. Then, all the non-adhering soil from the plant roots was removed manually by vigorous shaking. Then, the rhizosphere soil intimately attached to roots was detached with a sterile brush (sterilized with 70% EtOH after each treatment/condition) and collected. Samples were stored at -80°C. Subsequently, the roots were washed twice in PBS buffer (pH 7.0) and sterile water, before being stored at -80 C prior to analysis.

3.2.2 Metabolite extraction and metabolomics analysis

A semi polar metabolomic analysis of tomato roots was performed. Roots were freeze-dried and ground with tungsten beads at 25 Hz for 2 min with the tissuelyser (mixermill MM 300, Retsch). Four replicates of 10 mg for each line were resuspended in 500µl of 75% (v/v) cold methanol with 0.1% Formic Acid (Sigma-Aldrich, Cat. 5630-50ML-F), spiked with 0.5 mg/mL formononetin (Sigma–Aldrich, Cat. No. 47752-25MG-F) and total metabolites were extracted at RT by orbital shaker (VDRL MOD. 711+ Asal S.r.l.) at 20 Hz for 20 min; subsequently, samples were centrifuged at 20,000g for 20 min and 400 µl of supernatant was collected and filtered with HPLC filter tubes (0.45 mm pore size, Whatmann, Cat. 514-8110). Liquid Chromatography coupled to High-Resolution Mass Spectrometry (LC-HRMS) analyses were carried out as previously reported (Noronha et al., 2022). Full scan MS with data-dependent MS/MS fragmentation in both positive and negative ionization mode was used for metabolite identification, through the comparison with authentic standards, when available, and on the basis of the accurate masses obtained from the Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) for native compounds or from the Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator

(http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/) for adducts. Data were quantified as fold internal standard.

3.2.3 DNA extraction and 16S rRNA amplicon sequencing

DNA was extracted from rhizosphere samples using a DNeasy PowerSoil Kit (QIAGEN Group, Hilden, Germany) and according to the manufacturer's instructions. Quality and quantity of extracted DNAs were assessed by a NanoDrop 2000 spectrophotometer (Thermo Scientific) and Qubit 2.0 fluorometer (Invitrogen, Life technologies) and then stored at −20 °C until further analysis. V3-V4 16S rRNA amplification, libraries construction and sequencing were performed by the Zymo Research Europe (zymorearch.eu) using custom designed primer set. (Quick-16S™ Primer Set V3-V4, Zymo Research, Irvine, CA, USA). The final libraries were sequenced on Illumina MiSeq platform in 2x300 paired ends.

3.2.4 Bioinformatics Analysis

Unique amplicon sequences variants were inferred from raw reads using the DADA2 pipeline (Callahan et al, 2016). Potential sequencing errors and chimeric sequences were also removed with the Dada2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database, a 16S database that is internally designed and curated, as reference. All data analyses were performed in R version 3.3.6 (R Core Team, 2020). To evaluate bacterial alpha diversity, we used the observed richness and the Shannon index applying the package 'vegan' version 2.5–4 (Oksanen et al., 2016). Wilcoxon rank-sum test was applied to test the difference in alpha diversity between WT and edited plants. Beta diversity was investigated by using weighted UniFrac distances. To represent the dissimilarity matrices, we used principal coordinate analysis (PCoA). Significant variation between the bacterial communities was performed by Permutational analysis of variance (PERMANOVA) (Anderson, 2001). Finally, to evidence the divergence among WT and edited plants in the bacterial taxa, we applied the DAtest package (version 2.7.11) (Russel et al., 2018). To evaluate potential differences among WT and edited samples, the best performing method was EdgeR exact - TMM (ere) normalization, whereas divergences between each comparison (WT vs 1.2-line, WT vs 13A line and 1.2 line vs 13A line) were calculated with EdgeR exact - TMM (ere), EdgeR quasi loglikelihood (qll) - TMM (erq) and EdgeR exact - TMM (ere) normalizations, respectively.

3.3 Results

3.3.1 Accumulation and secretion of SGAs and saponins in roots

Although SGAs and steroidal saponins are widely studied metabolic classes, their effect on shaping the microbial community in the rhizosphere of tomato plants has only been partially evaluated (Nakayasu, Ohno, et al., 2021; Nakayasu, Yamazaki, et al., 2021). We have previously obtained tomato plants edited for the genes *GAME 4* and *Sola l 4*, characterized by a strong reduction in the SGAs content of more than 99% in both leaves and fruit, at the mature green and red ripe stages (see Chapter 2). In order to verify that the same effects on the local secondary metabolism was also exerted at root level, a semi-polar analysis was performed using LC-HRMS (Table 1).

Table 1. LC-HRMS analysis of the SGAs and saponins composition in rhizosphere of tomato plants. Bold font indicates statistical significance (Student's t-test, $p < 0.01$).

Metabolite	Class	AVG ± SD		
		WT	1.2	13A
α-tomatine	SGA	3619.5 ± 98.7	**68.5 ± 14.6**	**2.1 ± 0.2**
Acetoxyhydroxytomatine	SGA	0.2 ± 0.03	n. f.	n. f.
Acetoxytomatine	SGA	27.1 ± 1.8	**0.3 ± 0.06**	n. f.
Dehydrotomatine	SGA	286.8 ± 9.5	**4.5 ± 0.9**	**0.2 ± 0.02**
Esculeoside A	SGA	19.6 ± 0.5	**0.2 ± 0.04**	n. f.
Esculeoside B	SGA	0.01 ± 0.005	n. f.	n. f.
Hydroxytomatine	SGA	416.3 ± 28.2	**5.4 ± 0.9**	**0.07 ± 0.01**
Tomatidine	SGA	497.3 ± 9.4	**2.1 ± 0.3**	**0.2 ± 0.02**
Tigogenin	Saponin	3.8 ± 0.6	**45.9 ± 4.0**	**112.7 ± 8.5**
Tigonin	Saponin	n. f.	671.7 ± 25.1	842.5 ± 52.1
Uttroside B	Saponin	0.1 ± 0.03	**141.7 ± 6.5**	**175.2 ± 11.3**

In WT roots, the most abundant SGA was α-tomatine (74.4%), followed by tomatidine (10.2%), hydroxy-tomatine (8.6%) and dehydrotomatine (5.9%). On the contrary, a reduction in total SGAs content of 98.3% and 99.9% was observed in 1.2 and 13A lines, respectively. Interestingly, the residual composition of the main SGAs in the roots of the edited plants also differed from WT: indeed, while α-tomatine was still the most abundant compound (83.4% and 84.6%, respectively), a drastic decrease in the content of tomatidine was observed, representing only the 2.6% in line 1.2 and 6.8% in line 13A. Considering the branching point where *GAME 4* is located (Itkin et al., 2013), the almost complete absence of SGAs resulted in a substantial accumulation of steroidal

saponins in the roots as well. In fact, a saponin increase of approximately 221-291 times was observed in the 1.2 and 13A lines, respectively. The most abundant member was tigonin, absent in the roots of WT plants, and ranging from 671.7-fold IS in line 1.2 and 842.5-fold IS in line 13A. Differently from WT leaves and fruits, in which uttroside B was absent (Sevi et al., 2023), traces of this compound were found in the roots. However, uttroside B was accumulated about 1,125.9-1,392.1 up-fold in edited roots.

3.3.2 Sequencing data

Rhizosphere samples were analyzed by V3-V4 16S rRNA amplicon sequencing. After the elimination of chimeras and singletons with the DADA2 pipeline, a total of 2,474,571 raw reads were obtained that assigned to 7,179 ASVs. Subsequently, the three replicates per plant were merged and an average of 171,599.8 ± 9,472.3; 163,417.8 ± 8,648.9 and 159,896.6 ± 9,152.5 raw reads were obtained for WT, line 1.2 and line 13A respectively.

ASVs belonging to unassigned Kingdom or Phylum (229 ASV representing 1.967% of total reads), mitochondria (5 ASV representing 0.128% of total reads) and chloroplasts (7 ASV representing 0.489% of total reads) were removed from the dataset using the phyloseq package (McMurdie & Holmes, 2013) of RStudio (RStudio, Boston, MA, United States) version R4.2.1. Thus, after cleaning, a total of 97.416% of the dataset was kept with a number of 6,938 ASVs. Among these, 25 ASVs with the 0.005% of reads belong to the Archaea Kingdom. The rarefaction curve (Supplementary Figure 1) revealed that the library size was adequate to cover microbial diversity. Altogether, a total of 27 phyla, 58 classes, 116 orders, 214 families and 535 genera were assigned to the bacterial ASVs. The microbial community structure was quite similar for the rhizosphere of WT and edited plants (Figure 1). In fact, Phyla *Actinobacteria*, *Acidobacteria*, *Bacteroidetes* and *Proteobacteria* represented more than 70% of the total microorganisms. In particular, the most abundant phylum in all rhizosphere soil was *Proteobacteria* with more than 40%. The two most abundant bacterial classes, *Alphaproteobacteria* and *Actinobacteria*, were also conserved in the rhizosphere of WT and edited plants (Supplementary Figure 2). More in detail, within the *Proteobacteria* Phylum, it was shown that the *Alphaproteobacteria* class represented about the 58-63%, followed by *Gammaproteobacteria* (~14-17.5%) in both WT and edited lines. Interestingly, while *Betaproteobacteria* was the third most abundant class in the rhizosphere of edited plants (12.7 % in line 1.2 and 14.8 % in line 13A), *Deltaproteobacteria* (13.7%) was the third more representative in the WT plants.

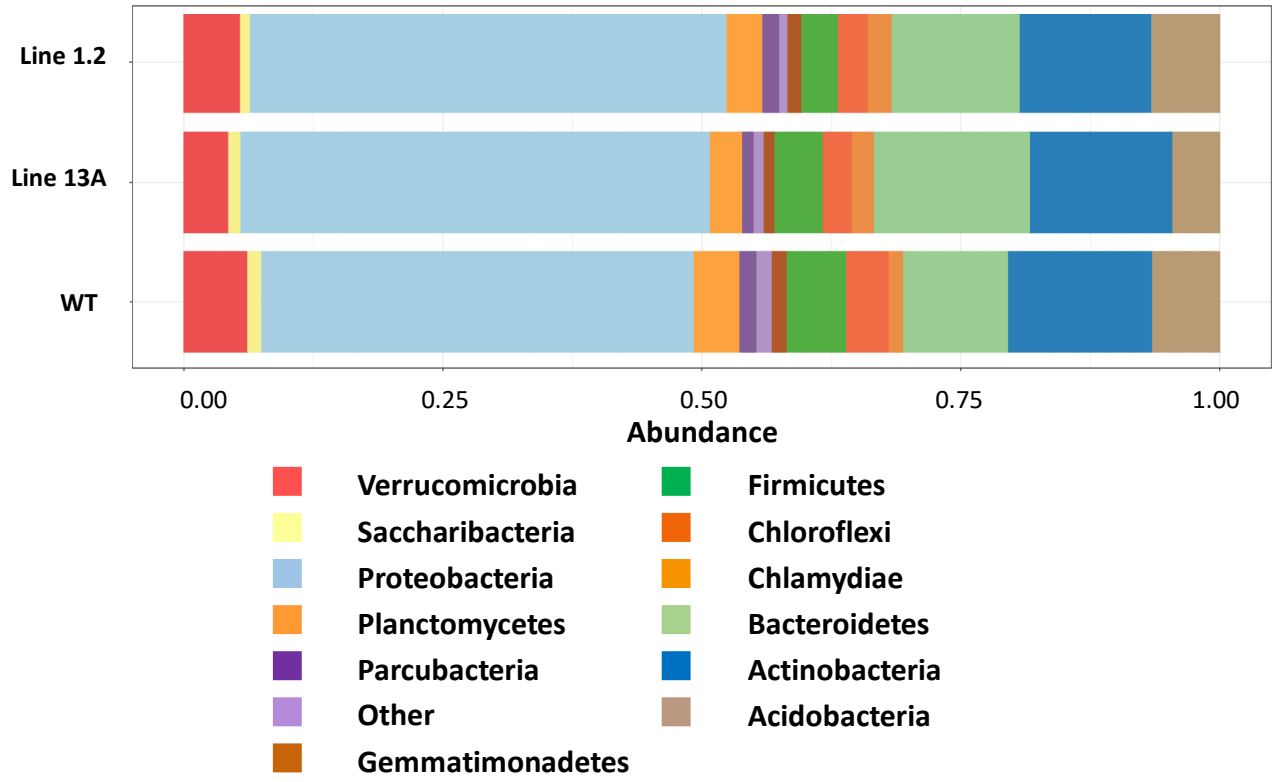


Figure 1. Relative abundances of taxa. Bar plot showing the relative abundance of the major phyla in the rhizosphere of WT plant, 1.2 and 13A edited lines. Bacterial phyla with low ASV abundance were classified as other.

3.3.3 Effects of metabolic alteration on bacterial community in the rhizosphere

To better understand the complexity of bacterial communities within the rhizosphere, the alpha diversity was evaluated by observed richness, which measures the number of observed ASVs, and Shannon diversity index, which measures the number of species and the evenness of species abundance (Figure 2). Although rhizosphere of WT plant showed a higher alpha diversity than those of edited plants, no significant differences were observed (Wilcoxon rank-sum test). More in detail, the mean ASV value was 1,601.6 ± 139.9 for WT, followed by line 13A (1,584.6 ± 196.2) and line 1.2 (1,524.6 ± 118.2) (Figure 2A). Similarly, as shown in Figure 2B, the highest Shannon Index value was found in WT rhizosphere (6.5 ± 0.162), followed by line 13A (6.43 ± 0.163) and line 1.2 showed the lowest (6.41 ± 0.09). To investigate the diversity between the microbial community, beta-diversity analysis by weighted UniFrac was performed. Visualization of beta-diversity by PCoA (Figure 3) showed a marked separation on the first axes between WT and edited lines that clustered together. Along the second axis, on the other hand, a separation between the two edited lines was observed; in particular, the line 13A showed a higher variance. According to PERMANOVA results, genotype variable had a greater influence on the composition of the bacterial community than line variable ($R2 = 0.27$, $p < 0.001$ and $R2 = 0.176$, $p < 0.01$, respectively). Subsequently, differential abundance analysis was performed to further investigate

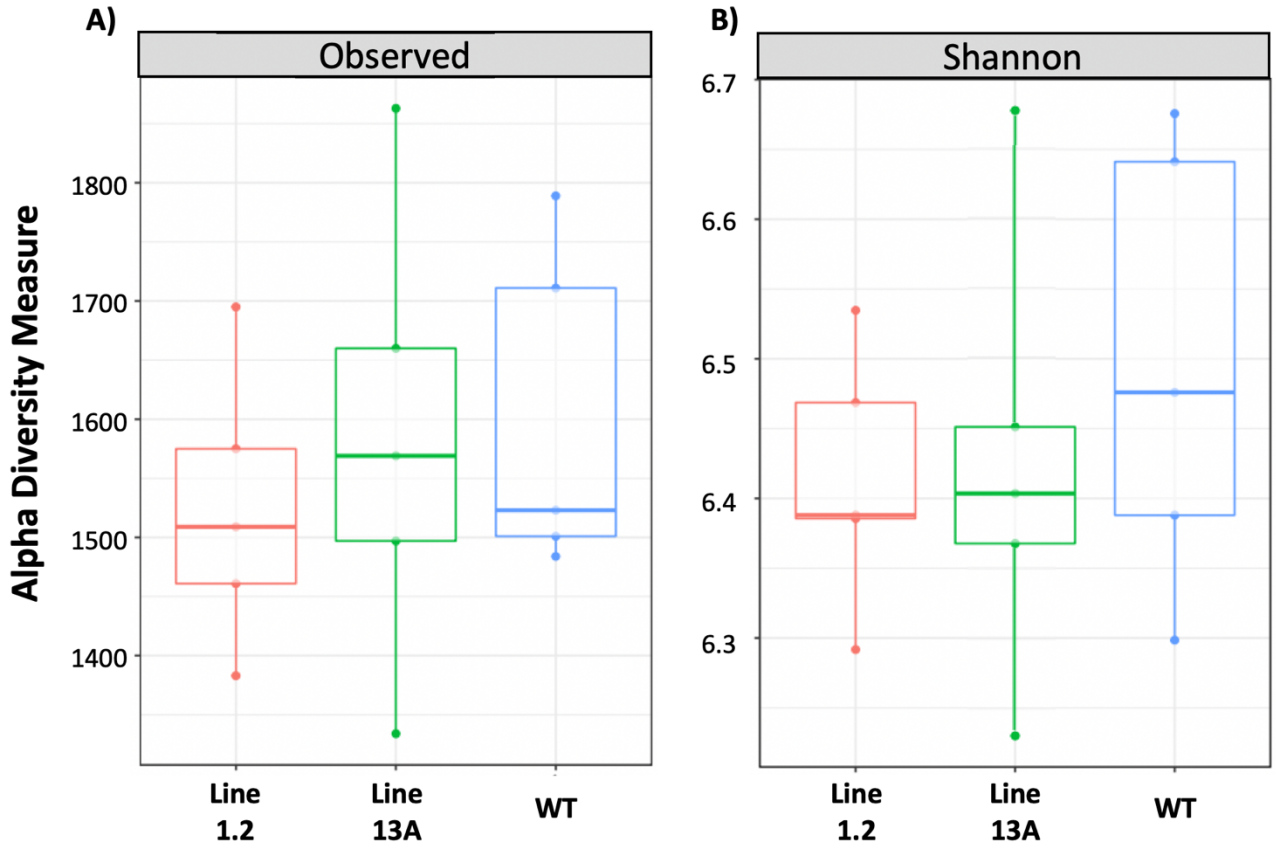


Figure 2. Alpha diversity measures. Box plots of observed ASVs (A) and Shannon diversity index (B) in the rhizosphere of edited and WT plants. Statistical analysis showed no difference for each condition (Wilcoxon rank-sum test, $p > 0.05$).

which bacterial genera were responsible for the divergence between the rhizosphere of WT and edited samples. This analysis demonstrated the existence of 23 genera enriched in the rhizosphere of edited plants compared to the WT [EdgeR exact - TMM (ere), p.adj < 0.005; Figure 4]. Among these, twelve belonged to the phylum *Proteobacteria* (*Aeromonas*, *Asticcacaulis*, *Dyella*, *Frigidibacter*, *Massilia*, *Methyloferula*, *Paraburkholderia*, *Peredibacter*, *Pseudomonas*, *Rhizobium*, *Sphingobium* and *Variovorax*) six to the phylum *Bacteroidetes* (*Chryseobacterium*, *Flavobacterium*, *Fluviicola*, *Hydrotalea*, *Taibaiella* and a genus belonging to the Order *Sphingobacteriales*) four to *Actinobacteria* (*Agromyces Microbacterium*, *Arthrobacter*, *Leifsonia* and *Nocardioides*) and one to the phylum *Chlamydiae*. On the other hand, only two genera were enriched in the WT rhizosphere: the genus *Micromonospora*, a member of family *Micromonosporaceae* and the genus *Hydrogenophaga*. The analysis of differential abundance at a higher taxonomic level allowed to identify, within the genera that showed significant differences, several species and ASVs that were prevalent in the rhizosphere of the edited lines (Table 2). More specifically, within the genus *Flavobacterium*, the species *F. cauense*, *F. hauense* and *F. caeni sp16948* were identified. The largest number of differential ASVs was found for the genus

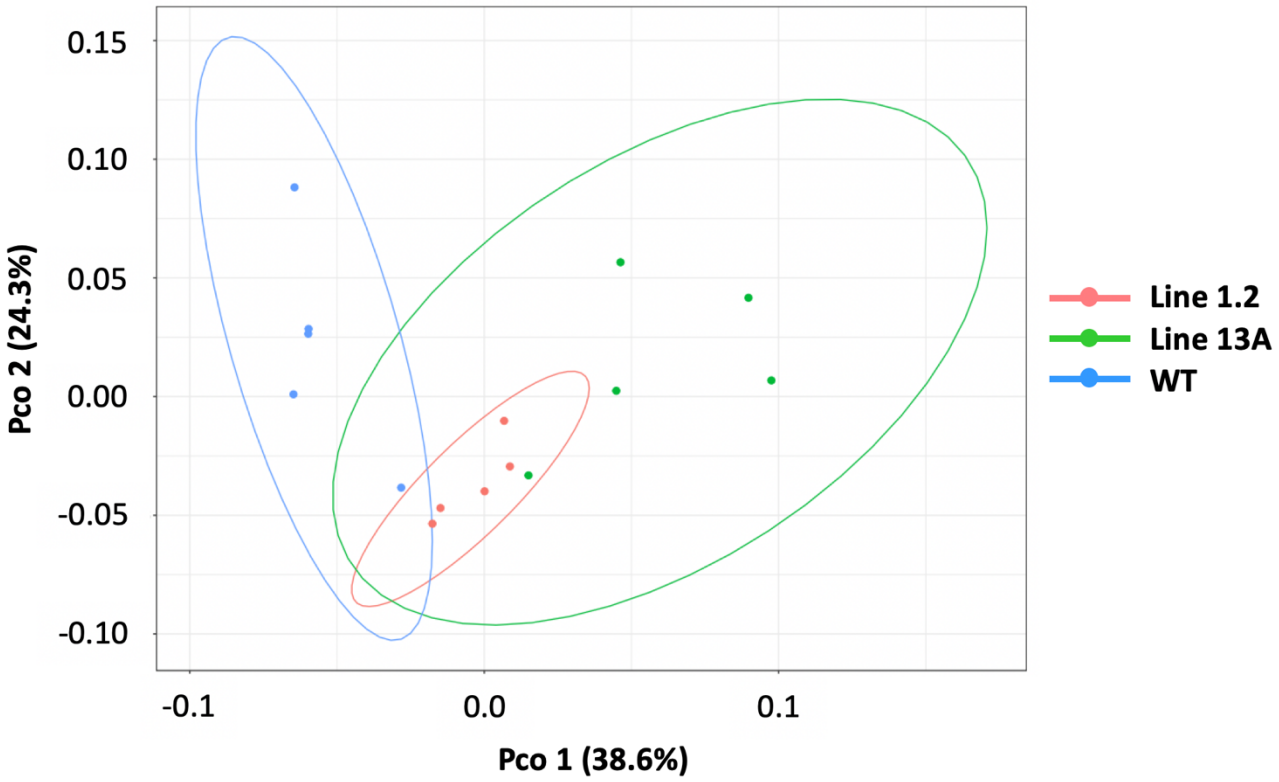


Figure 3. Microbial community composition of WT and edited rhizosphere plant. Principal Coordinate analysis (PCoA) of bacterial community from rhizosphere soil are based on weighted UniFrac metric.

Pseudomonas: the ASVs number 1, 3 and 4 were identified for the genus *Pseudomonas* and one for each species of *Pseudomonas putida* and *mediterranea*. Three different species were also found for the genus *Massilia*: *M. aerilata*, *M. cf timonae* and *M. alkalitolerans varians* for which two ASVs, the number 1 and 3, were identified. *Paraburkholderia udeis* and *phenazinium* were the two differently abundant species found mainly in the rhizosphere of the edited plants. A single differentially abundant ASV for all each other genera was revealed by the analysis.

Differential abundance analysis was carried out to assess potential differences between the edited lines as well. The differences between the WT-1.2 line were less evident than those shown between the WT-13A line (Supplementary Figure 3). Of interest, only 4 genera, *Fluviicola*, *Sphingobium*, *Metachamydia* and *Methyloferula*, were found to be enriched in the rhizosphere of line 1.2 compared to WT [EdgeR exact - TMM (ere), p.adj < 0.001; Supplementary Figure 3A]. However, 13 genera were most abundant in line 13A samples in comparison with WT. At the same time, the genus *Micromonospora* was prevalent in WT [EdgeR qll - TMM (erq), p.adj < 0.001; Supplementary Figure 3B]. Comparing the two edited lines showed that line 13A was the most divergent: in fact, 12 genera were differentially abundant, all belonging to the phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* [EdgeR exact - TMM (ere), p.adj < 0.001; Supplementary Figure 3C].

3.4 Discusssion

It is well known that root exudates have a strong influence in shaping the rhizosphere microbiome and that plant-associated indigenous microbes are primary for the host phenotype and plant protection against fungi, bacteria and consequently diseases (Köhl et al., 2019; J. Li et al., 2021). Indeed, the metabolic alterations of engineered plants could modify the soil microbiome on the basis of the remodulation of molecules produced from roots (R. P. Jacoby et al., 2021; Pascale et al., 2020). In particular, tomato represents a model to investigate plant-microbe interactions (Dong et al., 2019). Previous studies have shown that tomato roots exudate many steroidal glycoalkaloids (SGAs) such as α-tomatine, a molecule showing both antibiotic activity against many plant pathogens (Sandrock & VanEtten, 1998) and that is able to exert an effect on the microbial composition of the rhizosphere (Nakayasu et al., 2022).

In this work, we analyzed microbial rhizosphere composition of edited plant previously generated in our laboratory. These CRISPR/Cas9 tomato plants were edited in two target genes: *GAME4,* a key gene involved in the biosynthetic pathway of glycoalkaloids, and *Sola l 4*, one of the major allergens in tomato belonging to the PR-10 class. As a result of knock-out of these genes, a very strong reduction of total SGAs was achieved, greater than 99% in leaves and fruits (see Chapter 2). Interestingly, when we analyzed the root SGAs content, we observed a reduction in total SGAs content of 98.3% and 99.9% in 1.2 and 13A lines, respectively. In parallel, a great accumulation of steroidal saponins in the roots was achieved. In fact, saponin increase of approximately 250 times in the two lines. The most abundant, tigonin, increases from 600 to 800 times in both edited lines. Concerning the alfa- diversity, our findings showed that the rhizosphere microbial community structure of WT and edited plants was very similar (Figure 1, Supplementary Figure 2), and in particular that *Actinobacteria*, *Acidobacteria*, *Bacteroidetes* and *Proteobacteria* represented more than 70% of the total microorganisms. Likewise, no shift in the bacterial community was observed; in fact, even if WT plants showed a higher alpha diversity than edited plants, no significant changes were observed on the basis of Wilcoxon rank-sum test (Figure 2). Interestingly, when we performed a beta-diversity analysis by PCoA (Figure 3), a marked separation between WT and the two edited lines was found, and the two lines clustered together and separately from WT. Moreover, differential abundance analysis highlights several genera enriched in the rhizosphere of the two edited plants compared to the WT. More in detail, concerning the two edited lines, we noticed that line 13A was the most divergent: in fact, 12 genera resulted differentially abundant with respect to the only 4 genera of the line 1.2.

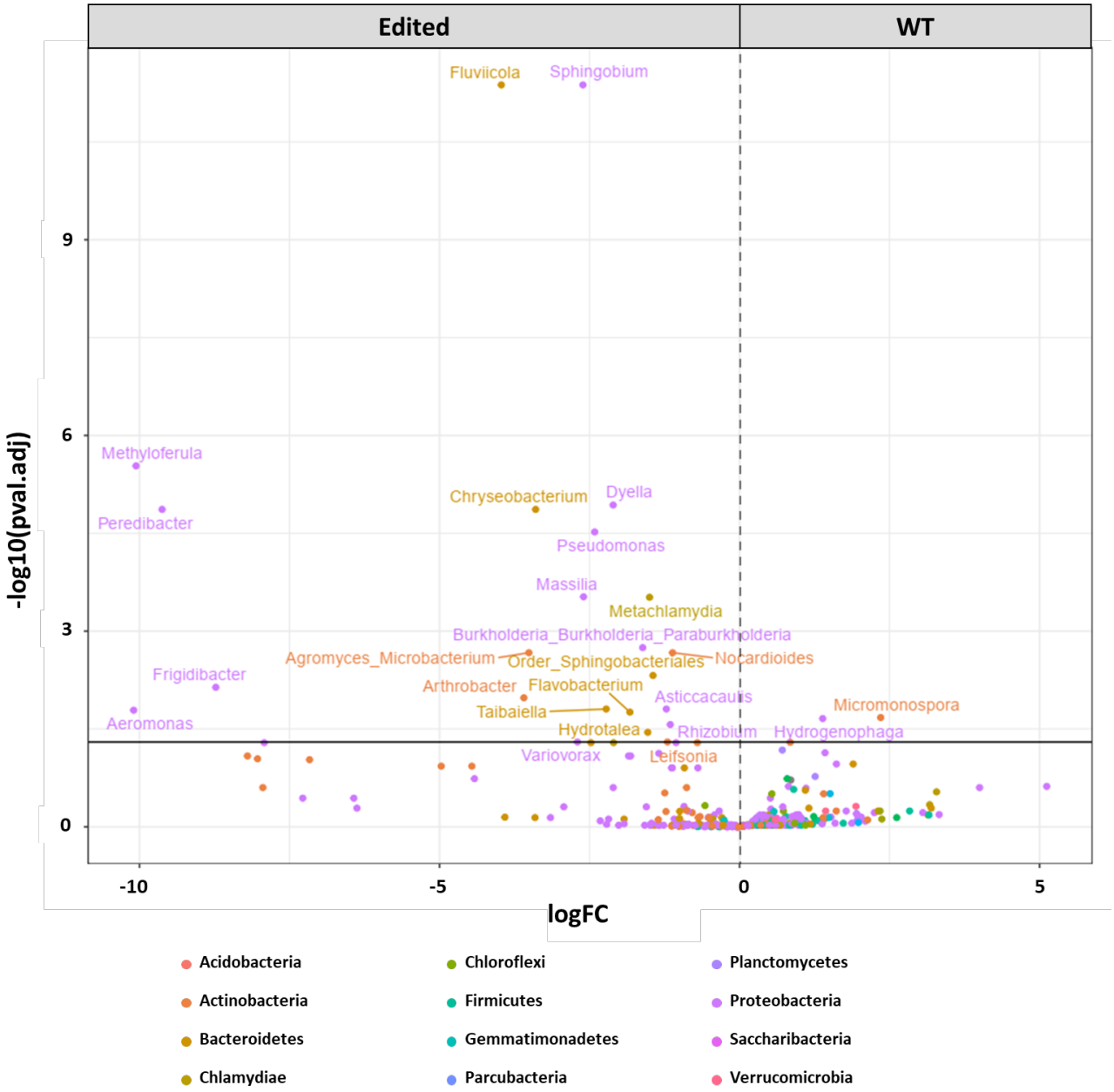


Figure 4. Volcano plot of differential abundance at genus level in the rhizosphere of tomato plants. The differences among WT and edited plants in bacterial taxa were tested with DAtest package and EdgeR exact - TMM (ere) was the best method. The dotted line marks the division between WT, on the right, and edited plants, on the left. The continuous line, represents the value of -log10 (pval.adj = 0.05) above which the taxa are differentially significant.

Among the genera differentially abundant in the rhizosphere of edited plants several have been already shown beneficial effects on plant development for their plant growth promotion (PGP) activity. Various species belonging to the PGP bacterial groups (*Rhizobium*, *Dyella*, *Aeromonas* and *Pseudomonas*), which are most abundant in the edited lines, are known as root nodulators and free-living nitrogen-fixing bacteria. These bacteria are well characterized for their ability to fix

Table 2. List of differentially abundant ASVs in the rhizosphere of the plants edited. Only ASVs with adjusted p-value <0.05 were shown.

Genus	Species	ASV	logFC
Flavobacterium	Flavobacterium cauense	Flavobacterium cauense	-6.75
Pseudomonas	Pseudomonas	Pseudomonas_3	-11.66
Fluviicola	Fluviicola hefeinensis	Fluviicola hefeinensis_1	-6.88
Flavobacterium	Flavobacterium hauense	Flavobacterium hauense_1	-11.43
Pseudomonas	Pseudomonas putida	Pseudomonas putida_1	-5.55
Sphingobium	Sphingobium fulginis quisquiliarum	Sphingobium fulginis quisquiliarum_1	-2.55
Pseudomonas	Pseudomonas mediterranea	Pseudomonas mediterranea	-11.03
Pseudomonas	Pseudomonas	Pseudomonas_4	-10.91
Leifsonia	Leifsonia lichenia	Leifsonia lichenia_1	-10.79
Massilia	Massilia alkalitolerans varians	Massilia alkalitolerans varians_1	-10.57
Metachlamydia	Metachlamydia Mesochlamydia	Metachlamydia Mesochlamydia_1	-1.54
Arthrobacter	Arthrobacter bambusae	Arthrobacter bambusae	-10.17
Paraburkholderia	Paraburkholderia udeis	Paraburkholderia udeis_1	-10.13
Asticcacaulis	Asticcacaulis taihuensis	Asticcacaulis taihuensis	-2.12
Massilia	Massilia cf timonae	Massilia cf timonae	-10.17
Variovorax	Variovorax paradoxus	Variovorax paradoxus_1	-9.98
Dyella	Dyella ginsengisoli	Dyella ginsengisoli_2	-9.99
Massilia	Massilia alkalitolerans varians	Massilia alkalitolerans varians_3	-9.89
Pseudomonas	Pseudomonas	Pseudomonas_1	-6.62
Peredibacter	Peredibacter sp50801	Peredibacter sp50801_1	-9.19
Massilia	Massilia aerilata	Massilia aerilata_1	-3.51
Paraburkholderia	Paraburkholderia phenazinium	Paraburkholderia phenazinium_1	-8.99
Frigidibacter	Frigidibacter albus	Frigidibacter albus	-8.67
Dyella	Dyella ginsengisoli	Dyella ginsengisoli_1	-2.39
Methyloferula	Methyloferula sp44639 stellata	Methyloferula sp44639 stellata_2	-8.55
Flavobacterium	Flavobacterium caeni sp16948	Flavobacterium caeni sp16948	-11.92

atmospheric nitrogen by increasing the nitrogen content in the soil available to plants, one of the most mineral nutrients necessary for their growth (Igiehon & Babalola, 2018). More in detail in the rhizosphere of line 1.2 and 13A we observed an increase of *Pseudomonas putida* and *Pseudomonas mediterranea* species, which are known to exert beneficial effects on plant health (Gu et al., 2020). *Pseudomonas putida*, in addition to the above-mentioned nitrogen-fixing activity, exerts other PGP activities such as producing plant hormone precursors, favoring the mobilization of nutrients and producing antibiotics that prevent the growth of pathogens (Molina et al., 2020). Among the PGP bacteria, also an increase of the genus *Arthrobacter*, belonging to the phylum *Actinobacteria*, was observed in the edited lines. It is well known that several strains of this genus are able to survive under different abiotic stress conditions. More specifically, a differential abundance of this genus was highlighted in the rhizosphere of heavy metal-contaminated soils and under drought and salt stress (Bian et al., 2023; Pereira et al., 2019; Upadhyay et al., 2012). Furthermore, Fan and colleagues observed that some *Arthrobacter* strains

are able to alleviate salt stress in tomato plants enhancing seed germination, seedling length and plant fresh and dry weight (Fan et al., 2016).

We observed other PGP bacteria more abundant in edited plants: for example, the species *Variovorax paradoxus*, commonly found in the rhizosphere as a symbiont of nitrogen-fixing microbes and plants, was identified as a specialist in sulfonate cycling in wheat rhizosphere (Schmalenberger et al., 2008). Moreover, a new strain belonging to this genus was recently isolated, and its potential use as a biocontrol agent and biostimulant to reduce the susceptibility of tomato plants to abiotic and biotic stresses has been highlighted (Kim et al., 2018). We also found an increase in the genus *Sphingobium* in both edited plants compared to wild type plants. Even if these results might seem to contradict what previously observed from Nakayasu findings (Nakayasu et al., 2021), we hypothesize that the absence of tomatine that normally stimulate the growth of *Sphingomonadaceae* in the rhizosphere, could be balanced by the simultaneous increase of additional secondary metabolites in the edited plants; indeed, saponins could exert a similar attractive role for *Sphingobium* played by tomatine. In addition, it would seem that strains belonging to the genus *Sphingobium* can promote crop resistance towards various pathogenic bacteria (Yuan et al., 2019). Interestingly, another genus increased in both edited plants compared to wild type plants, the *Fluviicola* genus. The recently identified species, *Fluviicola chungangensis*, which was isolated from soil sample of a rice field, belongs to this genus (Akter & Huq, 2020). Further insights on the enzymatic activities and metabolism of this species could clarify the reason for its increase in the two edited species.

Finally, an increase of microorganisms belonging to genera mainly involved in defense mechanisms, protecting plant from other pathogenic microorganisms, have also been highlighted in the rhizosphere of edited plants. In particular *Flavobacterium*, *Chryseobacterium* and *Peredibacter* have been already used as biological control of diseases; indeed, they could produce antimicrobial compounds and stimulate plant immune systems (Gu et al., 2022; Jaiswal et al., 2017; Nishioka et al., 2016). Recently, Kwak and colleagues identified a new *Flavobacterium* strain in rhizosphere of tomato variety Hawaii, more resistant to the soil-borne pathogen *Ralstonia solanacearum* than Moneymaker variety (Kwak et al., 2018).

Nowadays, it is well known that plant health is closely related to the microbial community in the rhizosphere. The plant is able to regulate its metabolism and produce a number of compounds released in the form of root exudates. Once we have identified which compounds might attract beneficial microorganisms to plant fitness, with the recent advent of genome editing technologies, it is possible to modify key genes involved in metabolic pathways and as a consequence to shape root exudates composition. Our results showed that both SGAs and saponins can potentially

contribute to the modulation of the rhizosphere microbiota trough a re-distribution of the most present species, thus increasing bacteria species involved in defense mechanisms against pathogens. Moreover, in this study we demonstrated how CRISPR/Cas9 technology can be used as an efficient tool to modulate metabolites distribution in the rhizosphere that could influence the microbial community of the tomato plants rhizosphere. In this way, by targeting plant secondary metabolites it is possible attract and or repel specific bacterial genera, which could provide plant health and productivity.

3.5 References

Akter, S., & Huq, Md. A. (2020). Fluviicola chungangensis sp. Nov., a bacterium isolated from rice field. *Archives of Microbiology*, *202*(2), 293–298. https://doi.org/10.1007/s00203-019-01746-5

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, *26*(1), 32–46. https://doi.org/10.1046/j.1442-9993.2001.01070.x

Berendsen, R. L., Pieterse, C. M. J., & Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Science*, *17*(8), 478–486. https://doi.org/10.1016/j.tplants.2012.04.001

Bian, F., Zhong, Z., Zhang, X., Li, Q., & Huang, Z. (2023). Bamboo-based agroforestry changes phytoremediation efficiency by affecting soil properties in rhizosphere and non-rhizosphere in heavy metal-polluted soil (Cd/Zn/Cu). Journal of Soils and Sediments, 23(1), 368–378. https://doi.org/10.1007/s11368-022-03303-y

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R. H., Uroz, S., & Martin, F. (2009). 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist*, *184*(2), 449–456. https://doi.org/10.1111/j.1469-8137.2009.03003.x

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E. V. L., & Schulze-Lefert, P. (2013). Structure and Functions of the Bacterial Microbiota of Plants. *Annual Review of Plant Biology*, *64*(1), 807–838. https://doi.org/10.1146/annurev-arplant-050312-120106

Colagiero, M., Rosso, L. C., Catalano, D., Schena, L., & Ciancio, A. (2020). Response of Tomato Rhizosphere Bacteria to Root-Knot Nematodes, Fenamiphos and Sampling Time Shows Differential Effects on Low Level Taxa. *Frontiers in Microbiology*, *11*, 390. https://doi.org/10.3389/fmicb.2020.00390

Compant, S., Samad, A., Faist, H., & Sessitsch, A. (2019). A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. *Journal of Advanced Research*, *19*, 29–37. https://doi.org/10.1016/j.jare.2019.03.004

Diretto, G., Frusciante, S., Fabbri, C., Schauer, N., Busta, L., Wang, Z., Matas, A. J., Fiore, A., K.C. Rose, J., Fernie, A. R., Jetter, R., Mattei, B., Giovannoni, J., & Giuliano, G. (2020). Manipulation of β-carotene levels in tomato fruits results in increased ABA content and extended shelf life. *Plant Biotechnology Journal*, *18*(5), 1185–1199. https://doi.org/10.1111/pbi.13283

Dong, C.-J., Wang, L.-L., Li, Q., & Shang, Q.-M. (2019). Bacterial communities in the rhizosphere, phyllosphere and endosphere of tomato plants. *PLOS ONE*, *14*(11), e0223847. https://doi.org/10.1371/journal.pone.0223847

Dono, G., Rambla, J. L., Frusciante, S., Fabene, E., Gómez-Cadenas, A., Granell, A., Diretto, G., & Mazzucato, A. (2022). Pigment-Related Mutations Greatly Affect Berry Metabolome in San Marzano Tomatoes. *Horticulturae*, *8*(2), 120. https://doi.org/10.3390/horticulturae8020120

Egamberdieva, D., Kamilova, F., Validov, S., Gafurova, L., Kucharova, Z., & Lugtenberg, B. (2007). High incidence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown on salinated soil in Uzbekistan. *Environmental Microbiology*, *0*(0), 070816220133002-??? https://doi.org/10.1111/j.1462-2920.2007.01424.x

Fan, P., Chen, D., He, Y., Zhou, Q., Tian, Y., & Gao, L. (2016). Alleviating salt stress in tomato seedlings using *Arthrobacter* and *Bacillus megaterium* isolated from the rhizosphere of wild plants grown on saline–alkaline lands. *International Journal of Phytoremediation*, *18*(11), 1113–1121. https://doi.org/10.1080/15226514.2016.1183583

Fitzpatrick, C. R., Salas-González, I., Conway, J. M., Finkel, O. M., Gilbert, S., Russ, D., Teixeira, P. J. P. L., & Dangl, J. L. (2020). The Plant Microbiome: From Ecology to Reductionism and

Beyond. *Annual Review of Microbiology*, *74*(1), 81–100. https://doi.org/10.1146/annurev-micro-022620-014327

French, E., Tran, T., & Iyer-Pascuzzi, A. S. (2020). Tomato Genotype Modulates Selection and Responses to Root Microbiota. *Phytobiomes Journal*, *4*(4), 314–326. https://doi.org/10.1094/PBIOMES-02-20-0020-R

Gu, Y., Banerjee, S., Dini-Andreote, F., Xu, Y., Shen, Q., Jousset, A., & Wei, Z. (2022). Small changes in rhizosphere microbiome composition predict disease outcomes earlier than pathogen density variations. *The ISME Journal*, *16*(10), 2448–2456. https://doi.org/10.1038/s41396-022-01290-z

Gu, Y., Wang, J., Xia, Z., & Wei, H.-L. (2020). Characterization of a Versatile Plant Growth-Promoting Rhizobacterium Pseudomonas mediterranea Strain S58. *Microorganisms*, *8*(3), 334. https://doi.org/10.3390/microorganisms8030334

Haney, C. H., Samuel, B. S., Bush, J., & Ausubel, F. M. (2015). Associations with rhizosphere bacteria can confer an adaptive advantage to plants. *Nature Plants*, *1*(6), 15051. https://doi.org/10.1038/nplants.2015.51

Igiehon, N., & Babalola, O. (2018). Rhizosphere Microbiome Modulators: Contributions of Nitrogen Fixing Bacteria towards Sustainable Agriculture. *International Journal of Environmental Research and Public Health*, *15*(4), 574. https://doi.org/10.3390/ijerph15040574

Itkin, M., Heinig, U., Tzfadia, O., Bhide, A. J., Shinde, B., Cardenas, P. D., Bocobza, S. E., Unger, T., Malitsky, S., Finkers, R., Tikunov, Y., Bovy, A., Chikate, Y., Singh, P., Rogachev, I., Beekwilder, J., Giri, A. P., & Aharoni, A. (2013). Biosynthesis of Antinutritional Alkaloids in Solanaceous Crops Is Mediated by Clustered Genes. *Science*, *341*(6142), 175–179. https://doi.org/10.1126/science.1240230

Jacoby, R. P., Koprivova, A., & Kopriva, S. (2021). Pinpointing secondary metabolites that shape the composition and function of the plant microbiome. *Journal of Experimental Botany*, *72*(1), 57–69. https://doi.org/10.1093/jxb/eraa424

Jacoby, R., Peukert, M., Succurro, A., Koprivova, A., & Kopriva, S. (2017). The Role of Soil Microorganisms in Plant Mineral Nutrition—Current Knowledge and Future Directions. *Frontiers in Plant Science*, *8*, 1617. https://doi.org/10.3389/fpls.2017.01617

Jaiswal, A. K., Elad, Y., Paudel, I., Graber, E. R., Cytryn, E., & Frenkel, O. (2017). Linking the Belowground Microbial Composition, Diversity and Activity to Soilborne Disease Suppression and Growth Promotion of Tomato Amended with Biochar. *Scientific Reports*, *7*(1), 44382. https://doi.org/10.1038/srep44382

Kim, H. S., Lee, S. A., Kim, Y., Sang, M. kyung, Song, J., Chae, J.-C., & Weon, H.-Y. (2018). Enhancement of Tomato Tolerance to Biotic and Abiotic Stresses by Variovorax sp. PMC12. *Research in Plant Disease*, *24*(3), 221–232. https://doi.org/10.5423/RPD.2018.24.3.221

Köhl, J., Kolnaar, R., & Ravensberg, W. J. (2019). Mode of Action of Microbial Biological Control Agents Against Plant Diseases: Relevance Beyond Efficacy. *Frontiers in Plant Science*, *10*, 845. https://doi.org/10.3389/fpls.2019.00845

Kwak, M.-J., Kong, H. G., Choi, K., Kwon, S.-K., Song, J. Y., Lee, J., Lee, P. A., Choi, S. Y., Seo, M., Lee, H. J., Jung, E. J., Park, H., Roy, N., Kim, H., Lee, M. M., Rubin, E. M., Lee, S.-W., & Kim, J. F. (2018). Rhizosphere microbiome structure alters to enable wilt resistance in tomato. *Nature Biotechnology*, *36*(11), 1100–1109. https://doi.org/10.1038/nbt.4232

Lakshmanan, V., Selvaraj, G., & Bais, H. P. (2014). Functional Soil Microbiome: Belowground Solutions to an Aboveground Problem. *Plant Physiology*, *166*(2), 689–700. https://doi.org/10.1104/pp.114.245811

Li, J., Wang, C., Liang, W., & Liu, S. (2021). Rhizosphere Microbiome: The Emerging Barrier in Plant-Pathogen Interactions. *Frontiers in Microbiology*, *12*, 772420. https://doi.org/10.3389/fmicb.2021.772420

Li, J.-G., Ren, G.-D., Jia, Z.-J., & Dong, Y.-H. (2014). Composition and activity of rhizosphere microbial communities associated with healthy and diseased greenhouse tomatoes. *Plant and Soil*, *380*(1–2), 337–347. https://doi.org/10.1007/s11104-014-2097-6

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H. M., Piceno, Y. M., DeSantis, T. Z., Andersen, G. L., Bakker, P. A. H. M., & Raaijmakers, J. M. (2011). Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria. *Science*, *332*(6033), 1097–1100. https://doi.org/10.1126/science.1203980

Molina, L., Segura, A., Duque, E., & Ramos, J.-L. (2020). The versatility of Pseudomonas putida in the rhizosphere environment. In *Advances in Applied Microbiology* (Vol. 110, pp. 149–180). Elsevier. https://doi.org/10.1016/bs.aambs.2019.12.002

Nakayasu, M., Ohno, K., Takamatsu, K., Aoki, Y., Yamazaki, S., Takase, H., Shoji, T., Yazaki, K., & Sugiyama, A. (2021). Tomato roots secrete tomatine to modulate the bacterial assemblage of the rhizosphere. *Plant Physiology*, *186*(1), 270–284. https://doi.org/10.1093/plphys/kiab069

Nakayasu, M., Takamatsu, K., Yazaki, K., & Sugiyama, A. (2022). Plant specialized metabolites in the rhizosphere of tomatoes: Secretion and effects on microorganisms. *Bioscience, Biotechnology, and Biochemistry*, *87*(1), 13–20. https://doi.org/10.1093/bbb/zbac181

Nakayasu, M., Yamazaki, S., Aoki, Y., Yazaki, K., & Sugiyama, A. (2021). Triterpenoid and Steroidal Saponins Differentially Influence Soil Bacterial Genera. *Plants*, *10*(10), 2189. https://doi.org/10.3390/plants10102189

Nguyen, C. (2003). Rhizodeposition of organic C by plants: Mechanisms and controls. *Agronomie*, *23*(5–6), 375–396. https://doi.org/10.1051/agro:2003011

Nishioka, T., Elsharkawy, M. M., Suga, H., Kageyama, K., Hyakumachi, M., & Shimizu, M. (2016). Development of Culture Medium for the Isolation of *Flavobacterium* and *Chryseobacterium* from Rhizosphere Soil. *Microbes and Environments*, *31*(2), 104–110. https://doi.org/10.1264/jsme2.ME15144

Noronha, H., Silva, A., Silva, T., Frusciante, S., Diretto, G., & Gerós, H. (2022). VviRafS5 Is a Raffinose Synthase Involved in Cold Acclimation in Grapevine Woody Tissues. *Frontiers in Plant Science*, *12*, 754537. https://doi.org/10.3389/fpls.2021.754537

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., … Weedon, J. (2016). *vegan: Community Ecology Package*.

Pascale, A., Proietti, S., Pantelides, I. S., & Stringlis, I. A. (2020). Modulation of the Root Microbiome by Plant Molecules: The Basis for Targeted Disease Suppression and Plant Growth Promotion. *Frontiers in Plant Science*, *10*, 1741. https://doi.org/10.3389/fpls.2019.01741

Patra, A., Sharma, V. K., Chattopadhyay, A., Mohapatra, K. K., & Rakshit, A. (2021). Rhizodeposition: An Unseen Teaser of Nature and Its Prospects in Nutrients Dynamics. In A. Rakshit, S. K. Singh, P. C. Abhilash, & A. Biswas (Eds.), *Soil Science: Fundamentals to Recent Advances* (pp. 85–126). Springer Singapore. https://doi.org/10.1007/978-981-16-0917-6_6

Pereira, L. B., Andrade, G. S., Meneghin, S. P., Vicentini, R., & Ottoboni, L. M. M. (2019). Prospecting Plant Growth-Promoting Bacteria Isolated from the Rhizosphere of Sugarcane Under

Drought Stress. *Current Microbiology*, *76*(11), 1345–1354. https://doi.org/10.1007/s00284-019-01749-x

Philippot, L., Raaijmakers, J. M., Lemanceau, P., & van der Putten, W. H. (2013). Going back to the roots: The microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, *11*(11), 789–799. https://doi.org/10.1038/nrmicro3109

R Core Team. (2020). *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.* http://www.r-project.org/index.html

Russel, J., Thorsen, J., Brejnrod, A. D., Bisgaard, H., Sørensen, S. J., & Burmølle, M. (2018). *DAtest: A framework for choosing differential abundance or expression method* [Preprint]. Bioinformatics. https://doi.org/10.1101/241802

Sandrock, R. W., & VanEtten, H. D. (1998). Fungal Sensitivity to and Enzymatic Degradation of the Phytoanticipin α-Tomatine. *Phytopathology®*, *88*(2), 137–143. https://doi.org/10.1094/PHYTO.1998.88.2.137

Schmalenberger, A., Hodge, S., Bryant, A., Hawkesford, M. J., Singh, B. K., & Kertesz, M. A. (2008). The role of Variovorax and other Comamonadaceae in sulfur transformations by microbial wheat rhizosphere communities exposed to different sulfur fertilization regimes: Variovorax in wheat rhizosphere. *Environmental Microbiology*, *10*(6), 1486–1500. https://doi.org/10.1111/j.1462-2920.2007.01564.x

Torsvik, V., Øvreås, L., & Thingstad, T. F. (2002). Prokaryotic Diversity—Magnitude, Dynamics, and Controlling Factors. *Science*, *296*(5570), 1064–1066. https://doi.org/10.1126/science.1071698

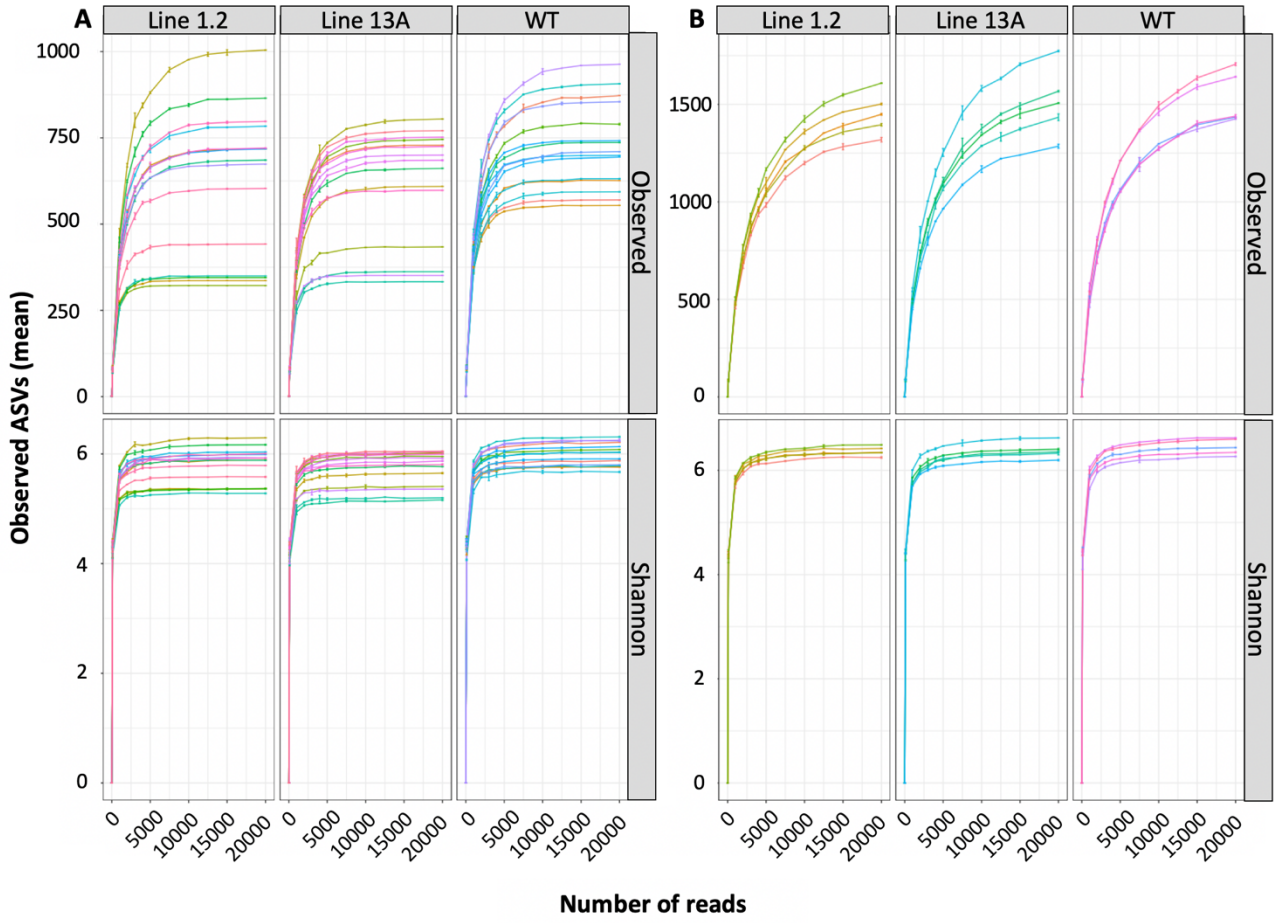
Upadhyay, S. K., Singh, J. S., Saxena, A. K., & Singh, D. P. (2012). Impact of PGPR inoculation on growth and antioxidant status of wheat under saline conditions: Effect of rhizobacteria on wheat in saline conditions. *Plant Biology*, *14*(4), 605–611. https://doi.org/10.1111/j.1438-8677.2011.00533.x

Yin, C., Casa Vargas, J. M., Schlatter, D. C., Hagerty, C. H., Hulbert, S. H., & Paulitz, T. C. (2021). Rhizosphere community selection reveals bacteria associated with reduced root disease. *Microbiome*, *9*(1), 86. https://doi.org/10.1186/s40168-020-00997-5

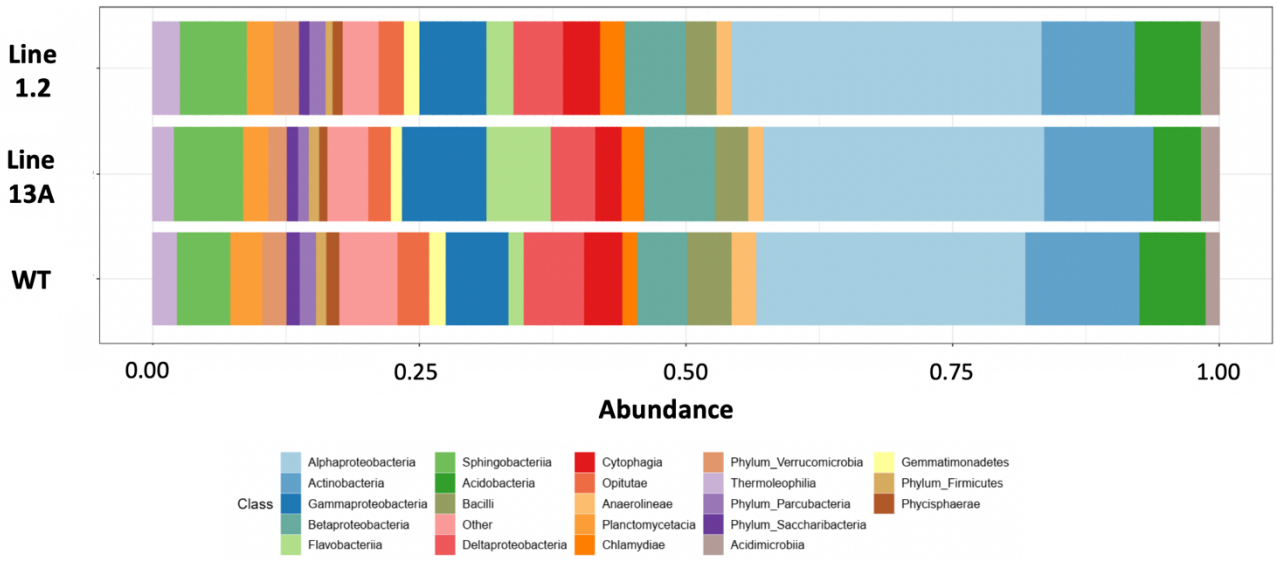
Yuan, X. F., Song, T. J., Yang, J. S., Huang, X. G., & Shi, J. Y. (2019). Changes of microbial community in the rhizosphere soil of Atractylodes macrocephala when encountering replant disease. *South African Journal of Botany*, *127*, 129–135. https://doi.org/10.1016/j.sajb.2019.08.046

Zhalnina, K., Louie, K. B., Hao, Z., Mansoori, N., da Rocha, U. N., Shi, S., Cho, H., Karaoz, U., Loqué, D., Bowen, B. P., Firestone, M. K., Northen, T. R., & Brodie, E. L. (2018). Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nature Microbiology*, *3*(4), 470–480. https://doi.org/10.1038/s41564-018-0129-3

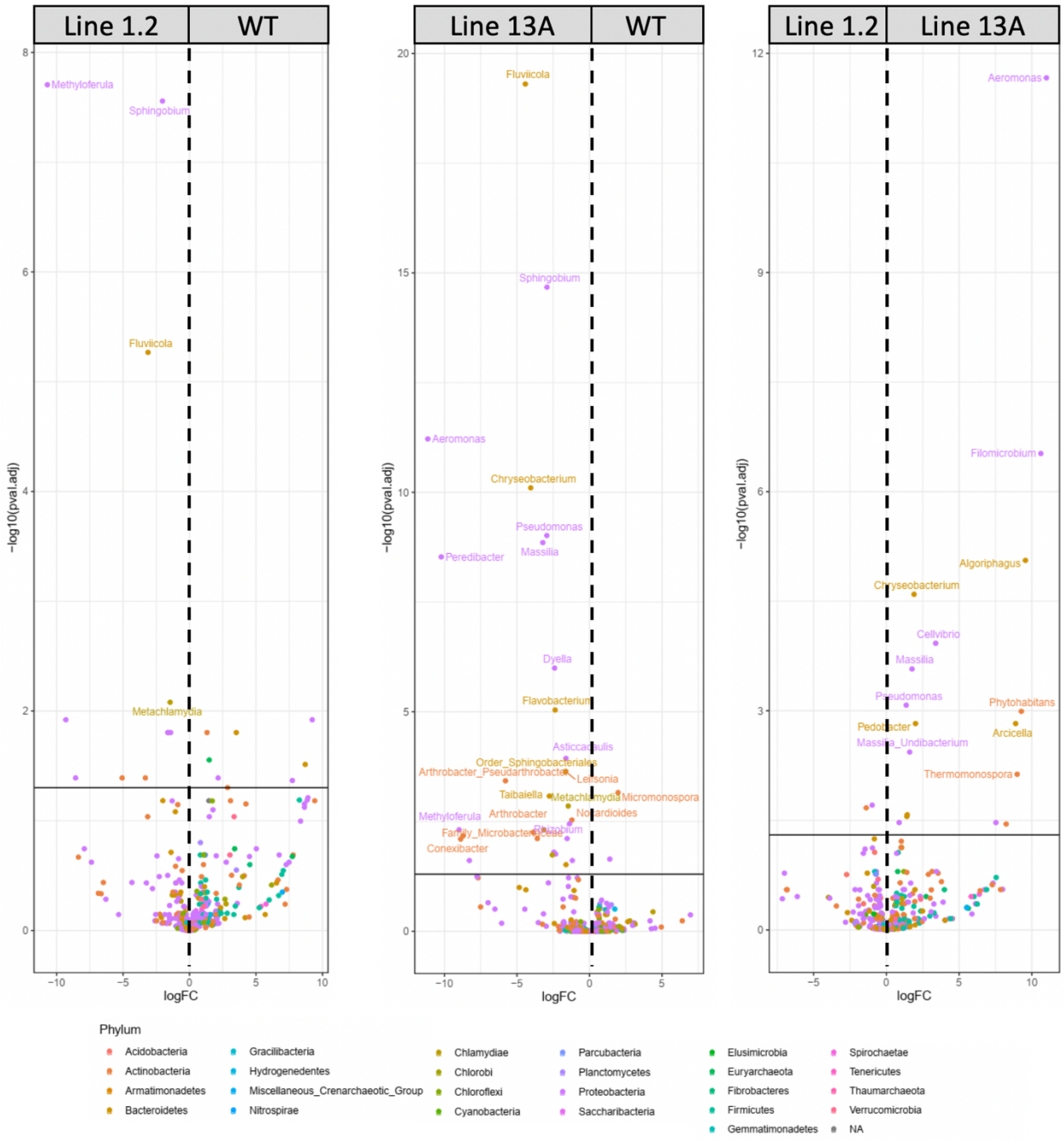
3.6 Supplemental figures



Supplementary Figure 1. Rarefaction curves for line 1.2, line 13A and WT. Observed ASV richness plotted before (A) and after (B) merging the three technical replicates of each line. Each curve is a sample.



Supplementary Figure 2. Bar plot showing the relative abundance of the major classes in the rhizosphere of WT plants, 1.2 and 13A edited lines. Bacterial classes with low ASV abundance were classified as other.



Supplementary Figure 3. Volcano plot of differential abundance at genus level in the rhizosphere of tomato plants. The differences among WT-line 1.2 and line 1.2-line 13A in bacterial taxa were analyzed with EdgeR exact - TMM (ere) method. Divergence between WT-line 13A was calculated with EdgeR qll – TMM (erq) method. The continuous line represents the value of -log10 (pval.adj = 0.05) above which the taxa are differentially significant.

Chapter 4. Metabolic profiling of two tomato edited lines in the *GREENFLESH* locus obtained by CRISPR/Cas9 Editing

Note: The experiments included in this chapter were published in the journal Frontiers in Plant Science.

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In-Depth Characterization of *greenflesh* Tomato Mutants Obtained by CRISPR/Cas9 Editing: A Case Study With Implications for Breeding and Regulation

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Gene editing has already proved itself as an invaluable tool for the generation of mutants for crop breeding, yet its ultimate impact on agriculture will depend on how crops generated by gene editing technologies are regulated, and on our ability to characterize the impact of mutations on plant phenotype. A starting operational strategy for evaluating gene editing-based approaches to plant breeding might consist of assessing the effect of the induced mutations in a crop- and locus-specific manner: this involves the analysis of editing efficiency in different cultivars of a crop, the assessment of potential off-target mutations, and a phenotypic evaluation of edited lines carrying different mutated alleles. Here, we targeted the *GREENFLESH* (*GF*) locus in two tomato cultivars ('MoneyMaker' and 'San Marzano') and evaluated the efficiency, specificity and mutation patterns associated with CRISPR/Cas9 activity for this gene. The *GF* locus encodes a Mg-dechelatase responsible for initiating chlorophyll degradation; in *gf* mutants, ripe fruits accumulate both carotenoids and chlorophylls. Phenotypic evaluations were conducted on two transgene-free T_2 'MoneyMaker' *gf* lines with different mutant alleles (a small insertion of 1 nucleotide and a larger deletion of 123 bp). Both lines, in addition to reduced chlorophyll degradation, showed a notable increase in carotenoid and tocopherol levels during fruit ripening. Infection of *gf* leaves and fruits with *Botrytis cinerea* resulted in a significant reduction of infected area and pathogen proliferation compared to the wild type (WT). Our data indicates that the CRISPR/Cas9-mediated mutation of the *GF* locus in tomato is efficient, specific and reproducible and that the resulting phenotype is robust and consistent with previously characterized

4.1 Introduction

CRISPR-mediated gene editing has opened exciting perspectives for the development of sustainable, innovative crops with improved agronomic, nutritional quality and industrial processing traits (Maximiano et al., 2021; Zhang et al., 2020; H. Zhu et al., 2020).

In collaboration with the laboratory of Prof. Antonio Granell (IBMCP), CRISPR/Cas9 technology was used to knock out the *GF* (*GREENFLESH*/*STAYGREEN*) locus, which encodes a Mg dechelatase required for chlorophyll catabolism (Shimoda et al., 2016), in tomato cultivars 'MoneyMaker'. The *GF* locus was chosen for both its biological significance and its genetic characteristics: it belongs, indeed, to the group of so-called 'staygreen' mutants, which comprises a class of phenotypes with impaired or delayed chlorophyll catabolism. In some of these phenotypes, called functional staygreen, senescence and ripening are halted or delayed, while in others, called cosmetic staygreen, senescence and ripening mostly proceed as in wild types, but vegetative and reproductive organs display a characteristic phenotype caused by chlorophyll retention (Thomas & Ougham, 2014). In the case of *gf* tomatoes, which belong to the latter group, not only senescent leaves have extremely delayed or no yellowing, but most distinctively ripe fruits also develop a characteristic color due to the simultaneous presence of chloroplasts and chromoplasts/plastoglobules (Barry et al., 2008). A number of such tomato varieties, referred to as brown, black or "chocolate," are commercially available and are highly appreciated by consumers; they carry different mutations at the same locus (deletions ranging in size from 2 to 1,163 bp, single-base substitutions and single-base insertions) and all result in comparable phenotypes (Barry & Pandey, 2009). Despite not dramatically altering senescence and ripening, the gf mutation does result in some potentially useful functional changes mainly related to carotenoid accumulation and plastid morphology (Grassl et al., 2012; Luo et al., 2013; Roca et al., 2006; K. Zhu et al., 2021).

In the present study, two *Cas9* free tomato plants (line 2B19 and 12A41) homozygous for the gf locus, were selected. In this chapter, metabolomic analyses of the edited berries from two independent lines were carried out to elucidate how the knock out of the GF gene could affect the accumulation of some isoprenoid compounds such as carotenoids, chlorophylls, tocochromanols and quinones.

4.2 Materials and methods

4.2.1 Extraction and analysis of isoprenoids

Extractions of isoprenoids in fruits at six stages of ripening were performed as described previously (Diretto et al., 2020). Briefly, for each sample 5 mg of freeze-dried powder were

extracted with chloroform (spiked with 50 mg/l DL-α-tocopherol acetate as internal standard) and methanol (2:1 by volume); 1 volume of 50 mM Tris buffer (pH 7.5, containing 1 M NaCl) was then added and the samples were kept for 20 min on ice before a centrifugation step at 15,000 g for 10 min at 4◦C. The hypophase was collected and the aqueous phase was re-extracted with the same amount of spiked chloroform; the two organic phases were merged and dried by speedvac and the resulting pellets were resuspended in 50 ml of ethyl acetate. For each sample, at least two independent extractions were performed. LC-DAD analyses were carried out using an Accela U HPLC system (Thermo Fisher Scientific, Waltham, MA, United States). LC separations were performed using a C30 reverse-phase column (100 x 3.0 mm) from YMC (YMC Europe GmbH, Schermbeck, Germany) with mobile phases composed by methanol (A), water–methanol (20:80 by volume) containing 0.2% ammonium acetate (B) and tert–methyl butyl ether (C). The gradient was 95% A : 5% B for 1.3 min, followed by 80% A : 5% B : 15% C for 2.0 min and by a linear gradient to 30% A : 5% B : 65% C over 9.2 min. UV–visible detection was performed continuously from 220 to 700 nm with an online Accela Surveyor photodiode array detector (PDA; Thermo Fisher Scientific). Mass ionization was performed with an atmosphericpressure chemical ionization (APCI) probe, operating in both + and – voltage conditions. Nitrogen was utilized at 20 and 10 units as sheath and auxiliary gas, respectively. The vaporizer and capillary temperature were set at 300 and 250°C, respectively. The discharge current was 5.5 μA, while S-lens RF level was set at 50. A mass range of 110/1,600 *m/z* was used both in positive and in negative voltage with the following parameters: resolution set at 70,000; microscan, AGC target and maximum injection time equal to, respectively, 1, 1x106 and 50. All solvents used were LC-MS grade quality (CHROMASOLVR from Sigma-Aldrich, Saint Louis, MO, United States). Different isoprenoid classes (carotenoids, chlorophylls, tocochromanols, and quinones) were identified based on the accurate masses and by comparison with authentic standards, when available, and quantified based on the internal standard (IS) amounts (thus named Fold IS); finally, *gf* data were normalized and expressed relatively to WT data (thus reported as Fold WT).

4.3 Results

4.3.1 Metabolic profiling of two isogenic mutant lines of ‘MoneyMaker’ at the GF locus

The levels of a total of 29 isoprenoid compounds (chlorophylls, carotenoids, tocochromanols, and quinones) were monitored and significant differences were found between gf mutants and WT fruits throughout the ripening process for all classes of compounds (Figure 1).

Chlorophylls

As expected, the most dramatic changes were associated with chlorophyll metabolism. More in detail, in *gf* mutants the levels of chlorophyll *a* and *b* and of their catabolites were comparable to the WT at the MG stage. After breaker, chlorophylls decreased with a similar trend in WT and mutant fruits, but the latter were characterized by significantly higher values until Br+8 (Figure 1). Concurrently, chlorophyll catabolites (chlorophyllide *b*, pheophytin *a* and *b*, and pheophorbide *a* and *b*) were markedly less abundant at all time points, starting from breaker. Both trends are consistent with the role of the GF protein as the Mg dechelatase which converts chlorophyll a to pheophytin *a* initiating chlorophyll catabolism (Shimoda et al., 2016).

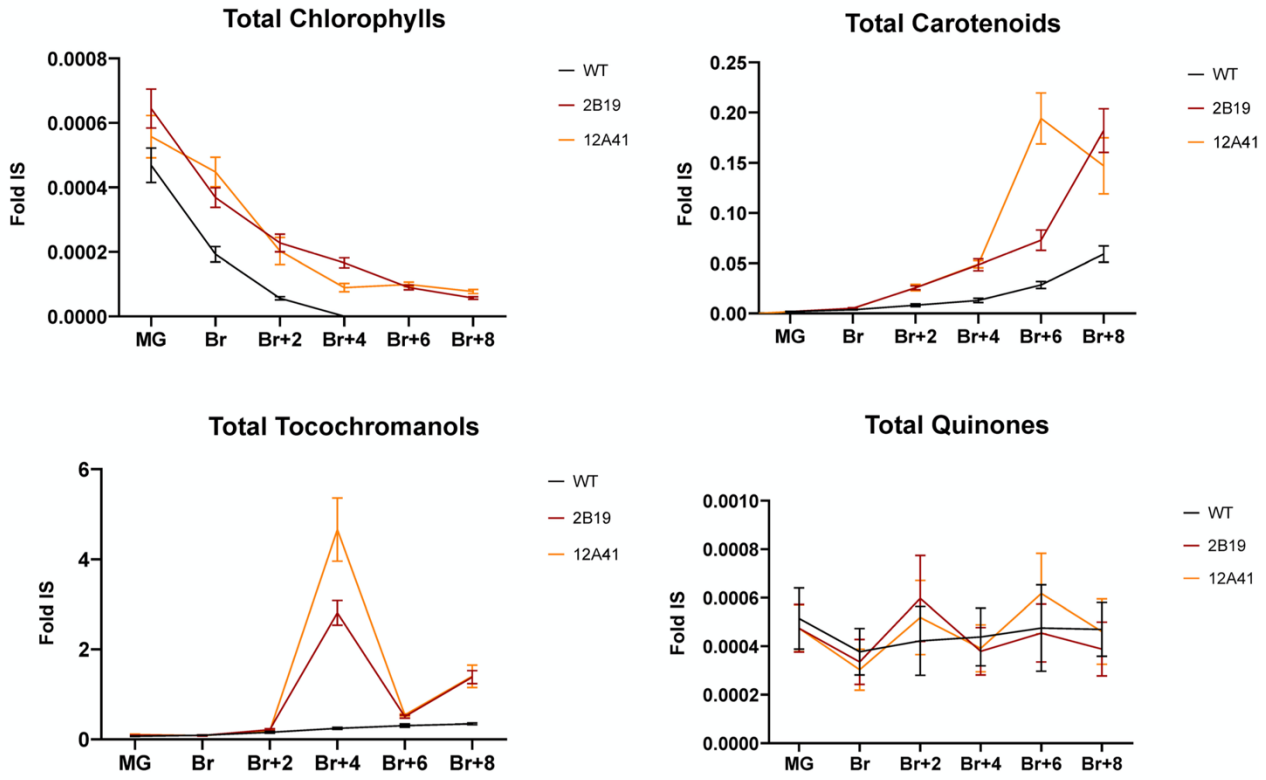

Figure 1. LC-HRMS analysis of the isoprenoid composition of two isogenic gf 'MoneyMaker' lines. Abundance of different isoprenoid classes during ripening. The accumulation trend of four classes of isoprenoid compounds (chlorophylls, carotenoids, tocochromanols, and quinones) was monitored across six ripening stages (MG to Br+8). The abundance of each compound is expressed relative to the internal standard (Fold IS).

Carotenoids

In addition to chlorophylls, total carotenoid content increased in mutant fruits after breaker: indeed, both *gf* lines displayed a 3.7-fold increase in total carotenoid content with respect to WT at Br+4, and line 12A41 further showed a 6.85-fold increase at Br+6. In both edited and WT fruits, carotenoid levels continued to increase until Br+8, when 2B19 and 12A41 gf lines still displayed

3- and 2.5-fold increase over the WT, respectively (Figure 1). At the MG stage, the difference in composition between *gf* and WT fruits lied mostly in lycopene content (Figure 2). Notably, while

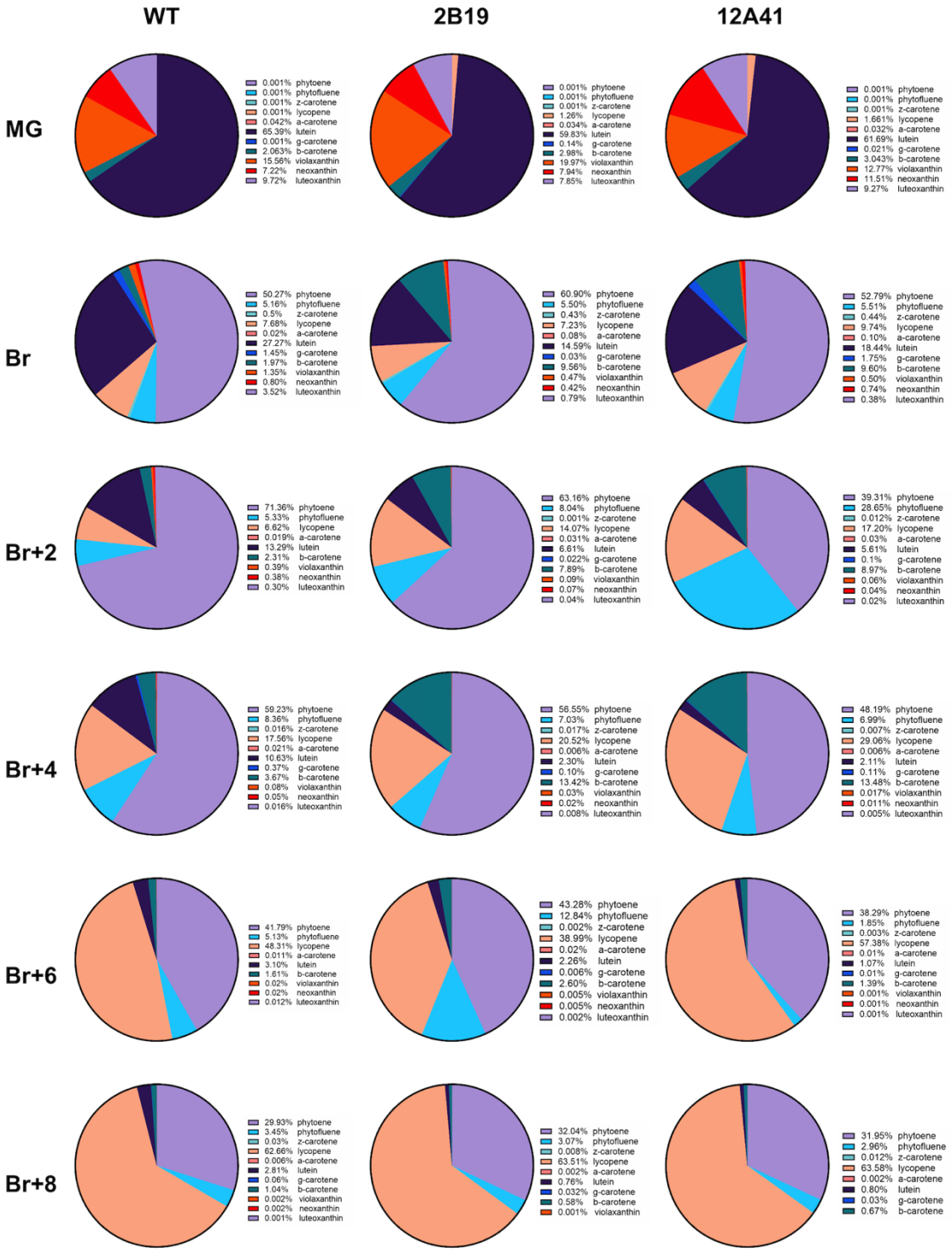

Figure 2. Carotenoid composition across tomato fruit ripening. Relative abundance of carotenoids in the fruits of WT MoneyMaker and 2B19 and 12A41 gf mutants from MG to eight days after breaker (Br+8).

carotenoids produced in the early steps of the biosynthetic pathway were enriched in *gf* fruits at all ripening stages, xanthophyll levels did not vary with the same amplitude. In particular, lutein levels slightly increased at Br+2 and Br+6, but had an opposite tendency at Br, Br+4 and Br+8, and violaxanthin, luteoxanthin, and neoxanthin resulted unaltered or were even less abundant in *gf* compared to WT fruits at all ripening stages. At Br+6 and Br+8 carotenoid composition converged in edited and WT lines, while total carotenoid content remained higher in *gf* fruits.

Tocochromanols and Quinones

A series of additional changes were observed in other isoprenoid classes: for instance, tocochromanol content was generally enriched in *gf* fruits with an approximate 1.47-fold increase over the WT; however, it peaked at Br+4, with a 11-fold increase in line 2B19 and a 19-fold increase in line 12A41, and again less prominently at Br+8, with a 4-fold over-accumulation in both lines. Finally, additional alterations were found in the quinone group, with a series of positive (ubiquinone-9 and ubiquinone-10) and negative (α-tocopherol quinone and plastoquinone) changes, although they are statistically significant only in line 2B19 (Figure 1).

The greatest alteration in fruit metabolite composition and abundance was revealed between breaker and Br+4, with enrichments mostly in phytoene, β-carotene and lycopene. At Br+4 (Figure 3A), carotenoids from phytoene to α and β-carotene were consistently up-regulated (2- to 8-fold, approximately), with a similar trend to tocochromanols. Chlorophylls remained high, while all their catabolites were depleted. The consistency of the phenotype observed in the two edited *gf* lines was reinforced by a principal component analysis (PCA) of the isoprenoid data at Br+4 (Figure 3B) which showed the clear separation between the WT and *gf* genotypes, which consistently clustered in a similar way. In summary, the metabolic profiling of *gf* and WT 'MoneyMaker' plants pointed to a significant shift in isoprenoid metabolism and enrichment in valuable secondary compounds in *gf* fruits.

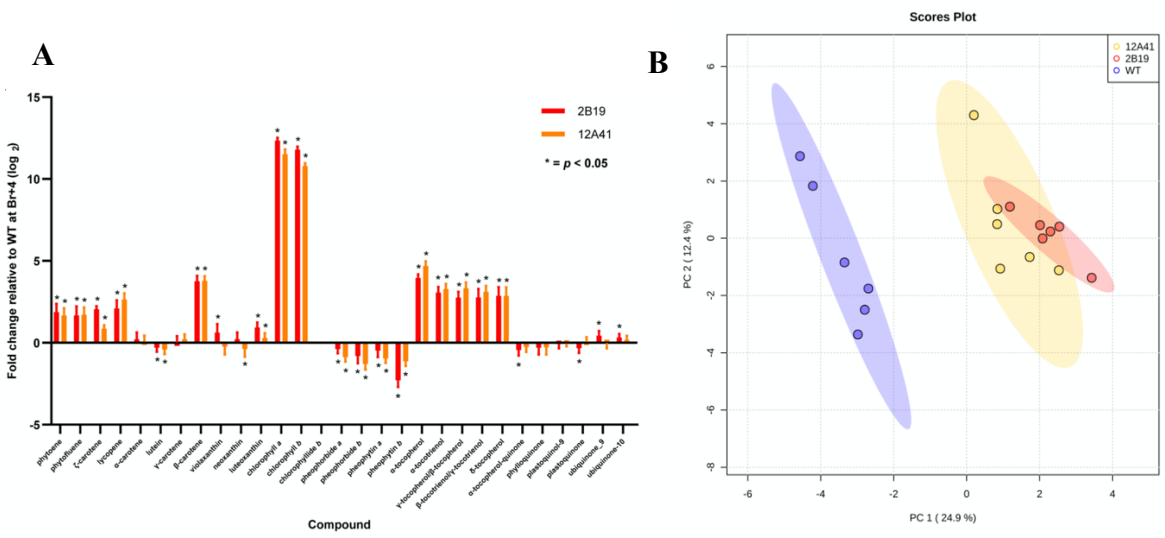

Figure 3. A) Metabolite composition at Br+4. Relative abundance of a total of 29 isoprenoid compounds in edited gf fruits compared to the wild-type, 4 days after breaker. Asterisks signal significant differences between mutant and WT (Student's t-test, p < 0.5). B) Principal Component Analysis (PCA) of the metabolite composition of WT and gf fruits at Br+4.

4.4 Discussion

The correlation between *gf* and increased carotenoid levels in tomato was previously described (Li et al., 2018; Luo et al., 2013; Ma et al., 2022). In the model proposed by Luo et al. (2013), phytoene synthase (PSY1), the enzyme responsible for fruit carotenoid biosynthesis, is negatively regulated by native GF, either post-transcriptionally by direct interaction, or indirectly through modulation of PIF1. Mutations in the *GF* locus lift this negative regulation, increasing phytoene synthase activity to levels comparable to those of a constitutively overexpressed PSY1: *gf* mutants accumulate higher levels of carotenoids, especially phytoene, lycopene and β-carotene. The metabolic profile of our edited fruits fits this model well, showing an increase of metabolites of the early steps of the carotenoid pathway. Comparable levels of lycopene and α- and β-carotene were also observed by Li et al. (2018) in *gf* tomato fruits at Br+7. On the contrary, xanthophyll levels resulted less affected by the mutation: this could be explained by the fact that in *gf* plants the retention of chlorophyll does not correspond to increased or continued photosynthetic activity, making it unnecessary for cells to accumulate greater quantities of antenna pigments.

Chlorophyll retention was the most apparent metabolic change in our edited fruits. More in detail, chlorophyll *a* and *b* concentrations greatly increased in mutants at all stages of ripening compared to the WT, but still decreased by the end of fruit ripening. However, alternative mechanisms for chlorophyll degradation might be in place and, especially, two other loci in tomato might be implicated in starting chlorophyll breakdown. In addition to the described *GF* locus (Solyc08g080090), tomato also encodes a staygreen-like (SGRL) protein (Solyc04g063240).

SGRL proteins are known to intervene in chlorophyll degradation in leaves prior to senescence and under stress conditions (Rong et al., 2013; Sakuraba et al., 2014; Wu et al., 2016); SlSGRL is known to take part in chlorophyll breakdown in an ABA-dependent manner (Yang et al., 2020), albeit its specific role in tomato has not been elucidated yet. Alignment of GF against the tomato proteome also identifies another locus (Solyc12g056480) which encodes a putative staygreen protein with a 72.69% similarity with GF (Matsuda et al., 2016). Unfortunately, it was not possible to make a direct comparison between our *gf* mutants and those of Luo et al. (2013) and Li et al. (2018) for chlorophyll retention. This finding might be due to the fact that the first did not detect differences in chlorophyll accumulation between mutants and WT fruits, something which might be attributed to an incomplete disruption of GF activity by RNAi, while the second did not investigate this feature. However, the visual phenotype reported by Li et al. (2018) is indicative of chlorophyll retention during ripening.

Unexpectedly, we found that *gf* fruits accumulate considerably higher levels of vitamin E, especially at Br+4 and Br+8. High chlorophyll levels should in principle reduce vitamin E biosynthesis during fruit ripening, inhibiting the recycling of the chlorophyll-derived phytol towards tocochromanol biosynthesis. Previous studies have reported simultaneous increases in carotenoids and tocochromanols, as in the case of tomato fruits overexpressing PSY1 (Fraser et al., 2007), a biochemical phenotype occurring together with chlorophyll degradation. However, it is possible that the activation of the carotenoid pathway provides an abundance of precursors which are additionally channeled toward tocochromanol biosynthesis. An overview of the general isoprenoid pathways can be seen in Figure 4. Overall, these data suggest the existence of a complex and not yet fully understood equilibrium between chlorophyll synthesis/degradation, phytyl salvage pathway and synthesis and accumulation of other isoprenoid classes. However, it is worth noticing that these changes mainly affect plastidial (carotenoids and tocochromanols) rather than cytosolic (quinones) isoprenoids, thus indicating a reduced capacity of *gf* to affect carbon exchanges between MVA and MEP pathways.

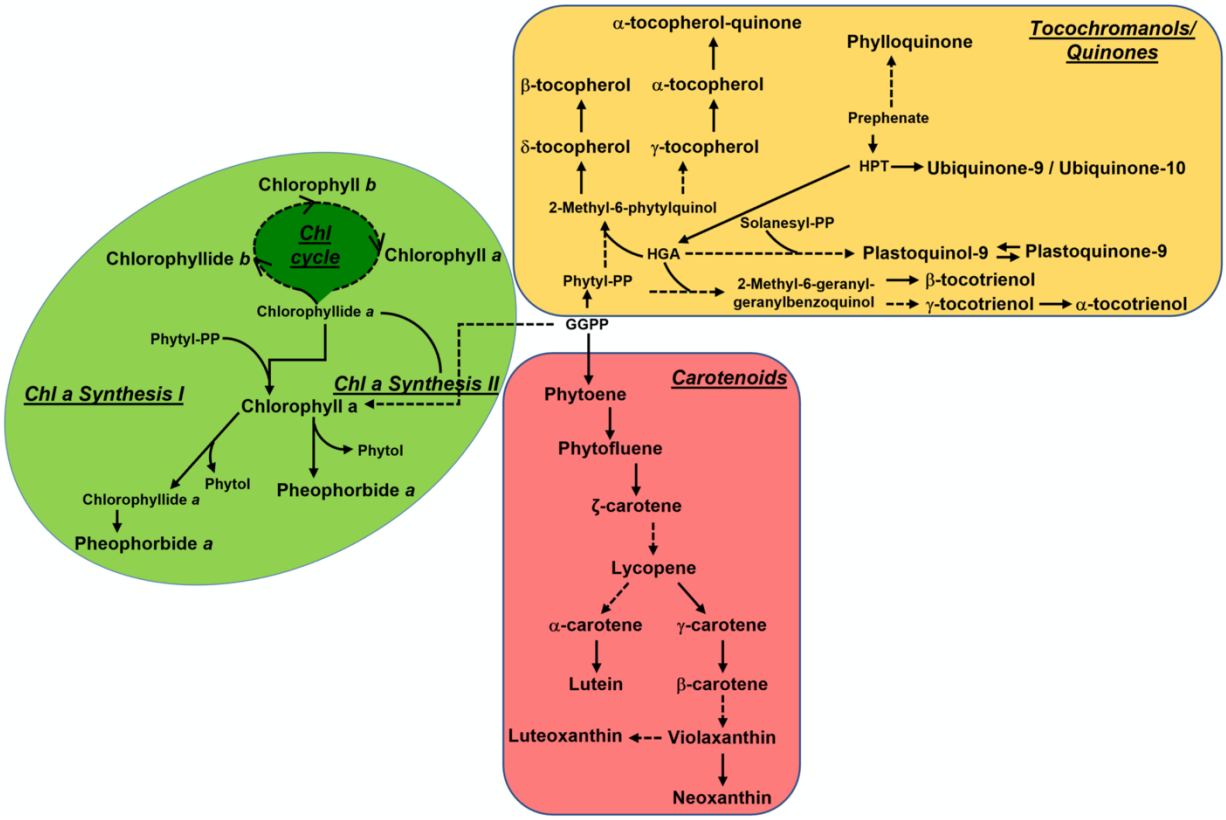

Figure 4. Overview of the main isoprenoid biosynthetic pathways. Metabolic relationships between the main isoprenoid classes (chlorophylls, carotenoids, and tocochromanols/quinones) referred to in this study. The metabolism of carotenoids, chlorophylls, and tocochromanols occurs in the plastid, while quinones are cytosolic.

Notably, the acceptable daily intake (ADI) for vitamin E is 12 mg/die, and its concentration in tomato is on average 1 mg/100 g fw (https://fdc.nal.usda.gov/). The increase of vitamin E (especially α-tocopherol) in ripe fruits to up to around 10-fold represents a significant enrichment of this class of compounds with respect to WT fruits, meaning that just 120 g of gf fruits would be needed to meet the ADI.

In summary, our findings highlight how the *gf* mutation does not solely lead to a visible alteration of tomato fruit coloration, but also induces functional shifts which promote the accumulation of valuable health-promoting secondary compounds. While an increase in carotenoid levels had been already reported by other authors (Li et al., 2018; Luo et al., 2013), overaccumulation of vitamin E is a novel trait associated with these mutants.

4.5 References

Barry, C. S., McQuinn, R. P., Chung, M.-Y., Besuden, A., & Giovannoni, J. J. (2008). Amino Acid Substitutions in Homologs of the STAY-GREEN Protein Are Responsible for the *green-flesh* and *chlorophyll retainer* Mutations of Tomato and Pepper. *Plant Physiology*, *147*(1), 179–187. https://doi.org/10.1104/pp.108.118430

Barry, C. S., & Pandey, P. (2009). A survey of cultivated heirloom tomato varieties identifies four new mutant alleles at the green-flesh locus. *Molecular Breeding*, *24*(3), 269–276. https://doi.org/10.1007/s11032-009-9289-4

Diretto, G., Frusciante, S., Fabbri, C., Schauer, N., Busta, L., Wang, Z., Matas, A. J., Fiore, A., K.C. Rose, J., Fernie, A. R., Jetter, R., Mattei, B., Giovannoni, J., & Giuliano, G. (2020). Manipulation of β-carotene levels in tomato fruits results in increased ABA content and extended shelf life. *Plant Biotechnology Journal*, *18*(5), 1185–1199. https://doi.org/10.1111/pbi.13283

Fraser, P. D., Enfissi, E. M. A., Halket, J. M., Truesdale, M. R., Yu, D., Gerrish, C., & Bramley, P. M. (2007). Manipulation of Phytoene Levels in Tomato Fruit: Effects on Isoprenoids, Plastids, and Intermediary Metabolism. *The Plant Cell*, *19*(10), 3194–3211. https://doi.org/10.1105/tpc.106.049817

Grassl, J., Pružinská, A., Hörtensteiner, S., Taylor, N. L., & Millar, A. H. (2012). Early Events in Plastid Protein Degradation in *stay-green Arabidopsis* Reveal Differential Regulation beyond the Retention of LHCII and Chlorophyll. *Journal of Proteome Research*, *11*(11), 5443–5452. https://doi.org/10.1021/pr300691k

Li, X., Wang, Y., Chen, S., Tian, H., Fu, D., Zhu, B., Luo, Y., & Zhu, H. (2018). Lycopene Is Enriched in Tomato Fruit by CRISPR/Cas9-Mediated Multiplex Genome Editing. *Frontiers in Plant Science*, *9*, 559. https://doi.org/10.3389/fpls.2018.00559

Luo, Z., Zhang, J., Li, J., Yang, C., Wang, T., Ouyang, B., Li, H., Giovannoni, J., & Ye, Z. (2013). A STAY-GREEN protein S l SGR 1 regulates lycopene and β-carotene accumulation by interacting directly with S l PSY 1 during ripening processes in tomato. *New Phytologist*, *198*(2), 442–452. https://doi.org/10.1111/nph.12175

Ma, L., Zeng, N., Cheng, K., Li, J., Wang, K., Zhang, C., & Zhu, H. (2022). Changes in fruit pigment accumulation, chloroplast development, and transcriptome analysis in the CRISPR/Cas9-mediated knockout of *Stay-green 1* (*slsgr1*) mutant. *Food Quality and Safety*, *6*, fyab029. https://doi.org/10.1093/fqsafe/fyab029

Matsuda, K., Shimoda, Y., Tanaka, A., & Ito, H. (2016). Chlorophyll a is a favorable substrate for Chlamydomonas Mg-dechelatase encoded by STAY-GREEN. *Plant Physiology and Biochemistry*, *109*, 365–373. https://doi.org/10.1016/j.plaphy.2016.10.020

Maximiano, M. R., Távora, F. T. P. K., Prado, G. S., Dias, S. C., Mehta, A., & Franco, O. L. (2021). CRISPR Genome Editing Technology: A Powerful Tool Applied to Developing Agribusiness. *Journal of Agricultural and Food Chemistry*, *69*(23), 6379–6395. https://doi.org/10.1021/acs.jafc.1c01062

Roca, M., Hornero-Méndez, D., Gandul-Rojas, B., & Mínguez-Mosquera, M. I. (2006). Stay-Green Phenotype Slows the Carotenogenic Process in *Capsicum annuum* (L.) Fruits. *Journal of Agricultural and Food Chemistry*, *54*(23), 8782–8787. https://doi.org/10.1021/jf062007r

Rong, H., Tang, Y., Zhang, H., Wu, P., Chen, Y., Li, M., Wu, G., & Jiang, H. (2013). The Stay-Green Rice like (SGRL) gene regulates chlorophyll degradation in rice. *Journal of Plant Physiology*, *170*(15), 1367–1373. https://doi.org/10.1016/j.jplph.2013.05.016

Sakuraba, Y., Kim, D., Kim, Y.-S., Hörtensteiner, S., & Paek, N.-C. (2014). *Arabidopsis* STAYGREEN-LIKE (SGRL) promotes abiotic stress-induced leaf yellowing during vegetative growth. *FEBS Letters*, *588*(21), 3830–3837. https://doi.org/10.1016/j.febslet.2014.09.018

Shimoda, Y., Ito, H., & Tanaka, A. (2016). Arabidopsis *STAY-GREEN*, Mendel's Green Cotyledon Gene, Encodes Magnesium-Dechelatase. *The Plant Cell*, *28*(9), 2147–2160. https://doi.org/10.1105/tpc.16.00428

Thomas, H., & Ougham, H. (2014). The stay-green trait. *Journal of Experimental Botany*, *65*(14), 3889–3900. https://doi.org/10.1093/jxb/eru037

Wu, S., Li, Z., Yang, L., Xie, Z., Chen, J., Zhang, W., Liu, T., Gao, S., Gao, J., Zhu, Y., Xin, J., Ren, G., & Kuai, B. (2016). NON-YELLOWING2 (NYE2), a Close Paralog of NYE1, Plays a Positive Role in Chlorophyll Degradation in Arabidopsis. *Molecular Plant*, *9*(4), 624–627. https://doi.org/10.1016/j.molp.2015.12.016

Yang, M., Zhu, S., Jiao, B., Duan, M., Meng, Q., Ma, N., & Lv, W. (2020). SlSGRL, a tomato SGR-like protein, promotes chlorophyll degradation downstream of the ABA signaling pathway. *Plant Physiology and Biochemistry*, *157*, 316–327. https://doi.org/10.1016/j.plaphy.2020.10.028

Zhang, Y., Pribil, M., Palmgren, M., & Gao, C. (2020). A CRISPR way for accelerating improvement of food crops. *Nature Food*, *1*(4), 200–205. https://doi.org/10.1038/s43016-020-0051-8

Zhu, H., Li, C., & Gao, C. (2020). Applications of CRISPR–Cas in agriculture and plant biotechnology. *Nature Reviews Molecular Cell Biology*, *21*(11), 661–677. https://doi.org/10.1038/s41580-020-00288-9

Zhu, K., Zheng, X., Ye, J., Huang, Y., Chen, H., Mei, X., Xie, Z., Cao, L., Zeng, Y., Larkin, R. M., Xu, Q., Perez-Roman, E., Talón, M., Zumajo-Cardona, C., Wurtzel, E. T., & Deng, X. (2021). Regulation of carotenoid and chlorophyll pools in hesperidia, anatomically unique fruits found only in *Citrus*. *Plant Physiology*, *187*(2), 829–845. https://doi.org/10.1093/plphys/kiab291

Chapter 5. General conclusions

Human and animal diet and, thus, food quality rely on the presence and abundance of a series of pro- and anti-nutritional compounds accumulating in all edible crops. However, and overall, the extent of investigations and efforts to improve the nutritional status of a plant-based food is strongly unbalanced in favor of the former, rather than the latter. In particular tomato, even if is source of many nutritional molecules, such as the carotenoids, flavonoids, phenolic and ascorbic acid, also accumulates numerous anti-nutritional factors (such as phytates, tannins, SGAs, etc.) and allergens (**Chapter 1**). For this reason, a limited number of studies, aimed to reduce or get rid of them have been carried out, using classic GMO-based strategies (e.g., RNAi). In this context, the goal of the present thesis has been to improve the overall nutritional quality of tomato berries by deleting, simultaneously, antinutritional and allergenic compounds through the CRISPR/Cas9 technology with two different aims: on one side, to elucidate, by multi-omics approach, the molecular and biochemical remodeling of plant metabolism as a consequence of the knocking-out of the two target genes for, respectively, glycoalkaloid and allergen synthesis; on the other side, to clarify how the perturbation in secondary metabolism can influence or modify the microbial community of the rhizosphere.

In the first part of this study (**Chapter 2**), we isolated and characterized two edited lines in the following target genes: the first, *GAME 4,* involved in glycoalkaloids accumulation and located at a crucial point in the biosynthesis of SGAs, and the second, *Sola l 4,* one of the most characterized in tomatoes, belonging to PR-10 proteins and homologous to the major allergen in *Betula verrucose.* Selected T3 lines 1.2 and 13A, further analyzed by PCR and sequencing to confirm the editing in both genes, were used in this research. Concerning *Sola l 4* gene, we confirmed that the adopted editing strategy led to the formation of a non-functional truncated protein in both edited lines, keeping 1/3 of the original sequence and totally misfolded, suggesting the loss of its allergenic properties. Subsequently, and to investigate the effects of *GAME 4* and *Sola l 4* editing at the metabolic level, a semi-polar and non-polar targeted characterization was carried out. As expected, a very strong reduction of total SGAs was achieved, greater than 99% in leaves and fruits. In parallel, a marked increase of some saponins, for example, uttroside B and tigonin, compounds reported to exert strong anticancer activities and conferring protection against plant pathogens like insects, bacteria, viruses, and fungi. Moreover, a series of additional metabolic changes were found, although some of them were genotype-specific, thus suggesting the occurrence of stochastic events, while, in most of the consensus cases, the magnitude of the alterations was within |1.5| fold, indicating a significant but limited extent of metabolic changes following GAME 4-Sola l 4 knock-out. More in detail, some metabolic perturbations could be

associated to the sensorial properties of tomato: for instance, a significant increase of the D-raffinose and hexose contents in edited ripe fruits were observed; this finding together with the depletion in SGA, which are known to confer a bitter taste, and of some polyamines (e.g., spermine, spermidine), often associated to unpleasant smell, might suggest the presence of a distinct sensorial attitude. Additional alterations were also found in compounds with nutritional-related properties: notable examples were represented by a series of flavonoids such as luteolin, morin, and naringenin and myricetin, as well as vitamins as nicotinamide and thiamine, which slightly decreased in fruits at the red ripe stage; however, these potentially negative traits were counterbalanced by the over-accumulation of metabolites with pro-nutritional effects such as the amino acids phenylalanine and tyrosine. Similarly, either positive or relatively negative changes at metabolic level were also found in leaves and MG fruits: this aspect is of particular interest since these results, together with the achieved removal of toxic SGAs might pave the way for the exploitation of generally unused tomato organs (leaves and green fruits) for generating novel edible products for human and/or animal diet. Similarly, the analyses of the volatile profile in the edited tissues highlighted the presence of metabolic changes of note: more specifically, for instance, ripe fruits emitted higher levels of branched chain amino acid-derived compounds (3-methylbutanol, 3-methylbutanal, 3-methylbutanoic acid), often associated to positive aromatic notes in tomato; as well as lower accumulation of terpenes, as guaicol, which is considered an off-flavor molecule. In addition, lipid-derived volatiles, either with up- (1-hexanol) and down-accumulation ((E, E)-2,4-hexadienal) in edited vs WT lines were observed. Since all these compounds can strongly impact on sensory traits, as well on the capacity to efficiently face biotic stressors, an interesting hypothesis to further investigate will imply the evaluation of taste and aroma-related attitude of the edited fruits, as well as the determination of the whole GAME 4-Sola l 4 to respond less or more efficiently to the attack of biotic agents.

Some of these perturbations could find an explanation in the analyses of the global proteome: for example, the increase in the acid beta-fructofuranosidase in MG and RR edited fruits, which results in a higher sucrose degradation with a consequent increase in free hexoses (glucose, fructose). Interestingly, the analyses of leaf proteomic data highlighted the presence, among the up-regulated proteins, of numerous hits involved in defense mechanisms against biotic stresses, such as several peroxidases (peroxidase (EC 1.11.1.7), Peroxidase 4 domain-containing protein), chitinases (chitinase (EC 3.2.1.14), chitin-binding type-1 domain-containing protein) and PR-proteins (pathogenesis-related protein P2, Protein NP24 (Pathogenesis-related protein PR P23; salt induced ptotein) suggesting the occurrence of altered stress responses in the GAME 4-Sola l 4 lines.

In particular, these analyses allowed to observe how the plant metabolism tends to remodel itself and compensate the absence of some molecules with others; in our situation, we hypothesize that plant counterpoise the absence of both glycoalkaloids and one PR-protein with different compounds like saponins and other protein involved in the same functions missing due to the knock-out of the two target genes.

It has been reported that deep metabolic and protein changes occur along fruit ripening; thus, taking into consideration the aforementioned perturbations, we decided to evaluate the fitness of the edited fruits, particularly at ripening level. To this aim, a series of phenotypic parameters including firmness, water loss and Brix° index were measured; overall, we could not identify any significant perturbation, thus proving that the metabolic shift in GAME 4-Sola l 4 fruits is not a consequence of perturbation in their ripening attitude.

Thus, our first conclusions were that, as consequence of genome editing engineering in SGAs and allergens content, a remodeling of plant metabolism, deeper for secondary compounds, and more limited for primary metabolites, was observed in tomato edited plants. More in detail, these changes often regarded molecules associated to sensorial traits and in responses to biotic stresses. In the agreement with the latter, a simultaneous accumulation of proteins playing a fundamental role in stress tolerance was shown, with chitinase (EC 3.2.1.14), Protein NP24 (Pathogenesis-related protein PR P23) etc. as notable members.

Main conclusions can be summarized:

1: Efficient genome editing for *GAME 4* and *Sola l 4* target genes was achieved and two Cas9-free homozygous lines were subjected to a deep omics multi-level characterization;

2: Metabolomic analysis showed a drastic reduction in the SGA content of more than 99.9 % in either leaves and fruits. Simultaneously, a large accumulation of saponins content was obtained, thus indicating a redirection in the local metabolic flux;

3: The allergen Sola l 4 was completely removed from edited plants;

4: GAME 4-Sola l 4 editing triggered a series of changes in non-volatile and volatile metabolomes, and either in primary and secondary compounds. Notable examples were represented by phenylalanine, tyrosine, 3-methylbutanol, 3-methylbutanal, 3-methylbutanoic acid (over-accumulated) and polyamines, flavonoids, guaicol (down- represented). All these compounds have been reported to exert not dispensable roles in the generation of fruit taste and aroma and/or are part of the plant machinery to react against biotic stressors. Thus, these findings may suggest an altered capacity of the GAME4-Sola l 4 plants in relation to these aspects;

5: Proteomic analysis showed up-regulation of proteins there involved in defense mechanisms against biotic stresses in leaves chitinase (EC 3.2.1.14), Protein NP24 (Pathogenesis-related protein PR P23) and up-regulation of several proteins involved in carbohydrate metabolism (acid beta-fructofuranosidase) in tomato fruits;
6: At fruit level, all these alterations were not due to a change in the ripening attitude.

In the second part of this thesis (**Chapter 3**), in order to elucidate if the alterations in the accumulation of secondary molecules, exuded from roots and involved in the defense mechanisms (e.g., SGAs and saponins), could result in a different distribution at microbial composition, we analyzed the bacterial communities in the rhizosphere of WT and GAME 4/Sola l 4 edited lines. As previously discussed, plants live in close association with soil microbes, which could be beneficial, harmful or neutral. Soil pathogens, in particular, affect crop health and production whereas, on the other hand, beneficial microbes can play a role in the stimulation of plant growth, by producing phytohormones, improving tolerance to various stresses and enhancing nutrients availability.
In addition, more recently, it has been discovered that the SGAs α-tomatine and tomatidine can have an effect on the microbial composition of the rhizosphere of tomato plants; thus, we investigated if microbial rhizosphere composition of edited plants were affected form the metabolic-biochemical changes occurred in these plants. Interestingly, by performing a beta-diversity analysis by PCoA, we observed a marked separation between WT and the two edited lines. In particular, these lines clustered together and separately from WT and, moreover, differential abundance analysis highlights that several genera were enriched in their rhizosphere compared to the WT. In addition, a great increase in the genus *Spingobium* was found in both edited plants with respect to WT plants; even if this finding might seem to contradict previous results (Nakayasu et al., 2021), reporting an increase of the abundance of the *Sphingomonadaceae* family in tomato plant rhizosphere, we hypothesize that the absence of α-tomatine, normally stimulating the growth of *Sphingomonadaceae* in the rhizosphere, could be balanced by the simultaneous increase of other secondary compounds such as saponins, accumulated at high amounts in the edited plants; consequently, saponins could exert a similar attractive role for *Spingobium* as usually performed by α-tomatine. Interestingly, other genera like *Cryseobacterium*, previously reported to reduce disease severity in pathogen infected tomato plants, was also enriched in both edited vs WT lines.
In conclusion, our results unraveled that both SGAs and saponins can contribute to the modulation of the rhizosphere microbiota trough a re-distribution in the abundance of the most present species

in the rhizosphere; most notably, this effect potentially regarded bacteria species involved in plant defense mechanisms against pathogens, which increased in both edited lines.

Main conclusions can be summarized:

1: As a consequence of a remodeling of the secondary metabolic pool in edited plants, a different distribution in microbial community of rhizosphere was found;

2: Beta-diversity analysis showed a marked separation between WT and the two edited lines which, in fact, clustered together and separately from WT;

3: A great increase in the genus *Spingobium* was observed in both edited plants with respect to WT plants;

4: A significant increase in the genera *Cryseobacterium,* known to be involved in the reduction of the disease severity in pathogen tomato infections, was also shown in both edited lines.

In the **Chapter 4** of this thesis, in order to confirm the potential of CRISPR-based systems to introduce target mutations at selected loci, and in order to verify that this technique is accurate in generating mutants already available by classical methods, a metabolomic characterization of *greenflesh* tomato mutants generated in collaboration with the "Istituto de Biologia Molecular y Celular de Plantas" (IBMCP, laboratory of Professor Antonio Granell) was carried out. The obtained two lines, despite structural differences in mutations from those deriving from the five spontaneous mutations described by Barry and Pandey (2009), show remarkable consistency in their phenotypes.

Specifically, chlorophyll retention is the most apparent metabolic change in our edited fruits with a great increase of chlorophyll *a* and *b* concentrations at all stages of ripening compared to the WT. Furthermore, an accumulation of other valuable health-promoting secondary compounds was observed, in particular an increase in carotenoid levels and overaccumulation of vitamin E, that represents a novel trait associated with these mutants.

Main conclusions can be summarized:

1: *gf* mutation does not solely lead to a visible alteration of tomato fruit coloration, but also induces functional shifts which promote the accumulation of valuable health-promoting secondary compounds.