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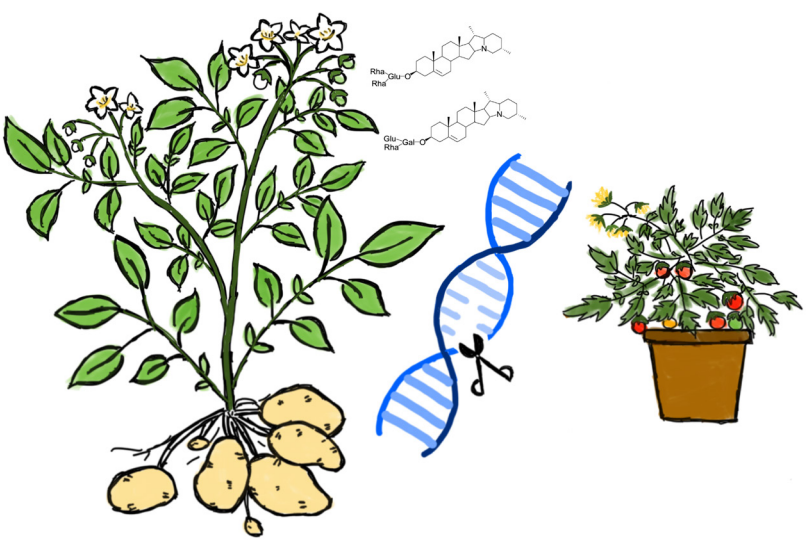
Modulation of the glycoalkaloid biosynthesis pathway in potato (*Solanum tuberosum* L.) and development of CRISPR/Cas9 methodology for tomato (*Solanum lycopersicum* L.)

Ying Liu



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Cover: The gene edited potato plant with reduced glycoalkaloid content, and the gene edited tomato plant with determinate phenotype, respectively (Illustration by Ying Liu)

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Modulation of the glycoalkaloid biosynthesis pathway in potato (*Solanum tuberosum* L.) and development of CRISPR/Cas9 methodology for tomato (*Solanum lycopersicum* L.)

Abstract

Steroidal glycoalkaloids (SGAs) are toxic defence substances present in *Solanum* species, including the tomato and potato. Concerns about SGA toxicity have long been raised regarding table potato cultivars, and more recently regarding starch potato cultivars, where SGAs negatively affect the use of proteins and fibre from starch by-products. Aiming at reducing the SGA hazard in potato, the present investigation has characterized SGA metabolism in starch and table potato cultivars. Further, using CRISPR/Cas9 technology, a number of gene-edited potato mutants with reduced SGA levels were generated, targeting the transcription factor *StGAME9*, as well as six key genes acting in SGA biosynthesis.

Transcriptomic and metabolomic profiling revealed both differences and similarities regarding the regulation of SGA levels in starch compared to table potato cultivars. Starch potato cultivars exhibited higher basal SGA levels and dry matter content but lower protein levels compared to table potato cultivars. In all seven types of key gene mutants, a significant SGA reduction was observed in leaves, tubers at harvest, and in tubers subjected to two SGA-inducing conditions: wounding and light exposure. The regulatory role of *StGAME9* in SGA biosynthesis was investigated, and key SGA-related genes regulated by *StGAME9* were identified. The reduction of SGA levels in mutants was stronger when genes in the post-cholesterol pathway of SGA biosynthesis were mutated, than for the pre-cholesterol ones. The results also showed that the mutation of each of these seven genes blocked both the basal and induced SGA synthesis. Notably, several mutants displayed close to SGA-free tubers and a relatively normal phenotype and tuber yield. A DNA-free genome editing method with a high mutation rate was established for the cultivated tomato, providing opportunities for extending the insights of SGA biosynthesis in potato to tomato.

The generation of SGA-free starch potato cultivars now offers an interesting opportunity for industrial applications, e.g. by transforming starch by-products into economically valuable compounds.

Keywords: glycoalkaloids, *Solanum tuberosum*, CRISPR/Cas9, starch potato cultivars, metabolomics, transcriptomics, *Solanum lycopersicum*

Modulation of the glycoalkaloid biosynthesis pathway in potato (*Solanum tuberosum* L.) and development of CRISPR/Cas9 methodology for tomato (*Solanum lycopersicum* L.)

Abstract

De steroida glykoalkaloiderna (SGA) är giftiga försvarsämnen som finns i *Solanum*-arter, inklusive tomat och potatis. SGA-toxicitet har länge varit en fråga när det gäller matpotatissorter, men på senare tid även gällande stärkelsepotatissorter, där SGA negativt påverkar användningen av proteiner och fibre från stärkelsebiprodukter. I syfte att minska SGA-risken i potatis har den föreliggande undersökningen karakteriserat SGA-metabolismen i stärkelse- och matpotatissorter. Vidare, med användning av CRISPR/Cas9-teknologi, genererades ett antal genredigerade potatismutanter med reducerade SGA-nivåer riktade mot transkriptionsfaktorn *StGAME9*, såväl som sex nyckelgener som verkar i SGA-biosyntes.

Transkriptom och metabolom karaktärisering avslöjade både skillnader och likheter när det gäller regleringen av SGA-nivåer i stärkelse jämfört med matpotatissorter. Stärkelsepotatissorter uppvisade högre basala SGA-nivåer och torrsubstanshalt men lägre proteinnivåer jämfört med matpotatissorter. Alla sju typer av nyckelgenmutanter uppvisade en signifikant SGA-reduktion i observerades i blad, knölar vid skada och i knölar som utsatts för två SGA-inducerande förhållanden: skada- och ljusexponering. Den reglerande rollen för *StGAME9* i SGA-biosyntes undersöktes och nyckel-SGA-relaterade gener reglerade av *StGAME9* identifierades. Minskningen av SGA-nivåer i mutanter var starkare när gener i post-kolesterolvägen för SGA-biosyntes muterades än för pre-kolesterol. Resultaten visade också att mutation av var och en av dessa sju gener blockerade både den basala och inducerade SGA-syntesen. Noterbart uppvisade flera mutanter nära SGA-fria knölar och en relativt normal fenotyp och knölutbyte. En DNA-fri genomredigeringsmetod med hög mutationshastighet etablerades för odlad tomat, vilket ger möjligheter att utöka förståelsen om SGA-biosyntes från potatis till tomat.

Genereringen av SGA-fria stärkelsepotatissorter erbjuder nu en intressant möjlighet för industriella tillämpningar, t.ex. genom att omvandla stärkelsebiprodukter till ekonomiskt värdefulla produkter.

Keywords: glykoalkaloider, *Solanum tuberosum*, CRISPR/Cas9, stärkelsepotatissorter, metabolomics, transcriptomics, *Solanum lycopersicum*

Dedication

To my parents for their endless love, support and encouragement.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Merino, I., Liu, Y., Johansson, A., Olarte Guasca, A., Andersson, M., Hofvander, P., & Sitbon, F. Transcriptomic and metabolomic profiling of starch vs. table potato cultivars subjected to two glycoalkaloid-inducing treatments. (manuscript)

II. Liu, Y., Merino, I., Potgieter, L., Andersson, M., Sitbon, F., & Hofvander, P. CRISPR/Cas9-mediated genome editing of the AP2/ERF transcription factor GAME9 alters gene expression and glycoalkaloid levels in leaves and tubers of potato. (manuscript)

III. Liu, Y., Merino, I., Andersson, M., Hofvander, P., & Sitbon, F. CRISPR/Cas9 -mediated generation of glycoalkaloid-free potato plants by mutation of key genes in the glycoalkaloid biosynthesis pathway. (manuscript)

IV. Liu, Y., Andersson, M., Granell, A., Cardi, T., Hofvander, P., & Nicolia, A. (2022). Establishment of a DNA-free genome editing and protoplast regeneration method in cultivated tomato (*Solanum lycopersicum*). *Plant Cell Rep* 41, 1843–1852. https://doi.org/10.1007/s00299-022-02893-8

Paper IV is reproduced with the permission of the publishers.

The contribution of Ying Liu to the papers included in this thesis was as follows:

I. Performed protein and dry matter determinations and prepared samples for metabolomics analyses. Contributed to the manuscript writing.

II. Generated and characterized the mutated potato plants, conducted transcriptomic analyses, performed data collection and all data analysis. Wrote the final version of the manuscript with input from co-authors.

III. Generated and characterized the mutated potato plants, performed data collection and all data analysis. Wrote the final version of the manuscript with input from co-authors.

IV. Designed the study with co-authors. Performed all the experimental work with assistance in tissue culture. Wrote the final version of the manuscript with input from co-authors.

Abbreviations

16DOX	16α-hydroxylase
2-ODD	2-oxoglutarate-dependent dioxygenase
AP2/ERF	APETALA 2/Ethylene-Responsive Element Binding Factor
CAS	Cycloartenol synthase
CYP	Cytochrome P450 monooxygenase
DEG	Differentially expressed gene
DPS	Dioxygenase for Potato Solanidane synthesis
DWF1-L	Sterol Δ24-reductase-like
DWF5-L	Δ7 sterol reductase-like
DWF7-L	Δ7-C5 sterol desaturase-like
FW	Fresh weight
GAME	GLYCOALKALOID METABOLISM
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
LAS	Lanosterol synthase
LC-MS	Liquid chromatography-mass spectrometry
PEG	Polyethylene glycol
PFJ	Potato fruit juice
RNP	Ribonucleoprotein
SA	Steroidal alkaloid
SGA	Steroidal glycoalkaloid
SMO1-L	Sterol methyl-oxidase 1-like
SMT	Sterol C-24 methyltransferase
SQE	Squalene epoxidase
SQS	Squalene synthase
STS	Steroidal saponin
TGA	Total glycoalkaloids

1. Introduction

1.1 Solanum species

1.1.1 The potato

Importance of the potato

The cultivated potato (*Solanum tuberosum* L.) is a dicotyledonous plant belonging to the *Solanum*, the largest genus of the Solanaceae family (Friedman *et al.* 1997). Other examples of important food crop plants in the *Solanum* species are the tomato (*Solanum lycopersicum* L.) and eggplant (*Solanum melongena* L.). Potato is the fourth most important food crop in the world after maize (*Zea mays* L.), wheat (*Triticum aestivum*), and rice (*Oryza sativa*). Potato is native to the Andean region of South America, where is has been cultivated for thousands of years by indigenous populations. Potato was first introduced to the Canary Islands during the Spanish conquest of the Americas in the 16th century, after which it was spread throughout Europe (Bradshaw and Ramsay 2009). Potato was first introduced to Sweden as a medical curiosity in the botanical garden in Uppsala in 1658, it took a long time and a great deal of effort until it was cultivated as a staple food and was widespread in Sweden during the 18th century (Eriksson *et al.* 2016).

Potato is not only a staple food but also a versatile raw material for various industrial applications such as starch-related products, biofuel products, animal feed and bioplastic products. It is cultivated in more than 100 countries and the production and consumption increases every year due to high demand. According to FAOSTAT data, the world production of potato

in the year 2021 was 376 million tonnes, with China being the leading potato-producing country, followed by India and Ukraine (FAOSTAT 2023).

Potato nutrients

Potato tubers are storage organs, rich in various essential nutrients, including carbohydrates, vitamins, minerals, dietary fibre, proteins and micronutrients. Starch, constituting the predominant carbohydrate in potato tubers, exhibits a low lipid content, typically ranging from 10-30% of fresh weight (Camire *et al.* 2009). Raw potato starch manifests a crystalline structure, imparting resistance to digestion in the small intestine. The process of cooking and subsequent cooling serves to enhance the retrogradation of starch, thereby augmenting its resistance to digestion (McGill *et al.* 2013). Potato tubers contain ca. 2% protein per unit of fresh weight (FW), marked by a high lysine content compared to the cereal proteins (Storey 2007; Waglay *et al.* 2014). Potato proteins have the potential to substitute other plant- and animal-based proteins in food production, duo to their properties of low allergenicity and high nutritional value (Koppelman *et al.* 2002; McGill *et al.* 2013; Waglay *et al.* 2014; Hussain *et al.* 2021).

Potato cultivars

Currently, there are over 4000 cultivated potato varieties for commercial use across the world. The abundance of potato varieties with various traits and their production globally reflects the adaptability and versatility of the potato, which allows for customized trait improvement for different regions and market demands (Bradshaw and Ramsay 2009). In Sweden, the first market potato cultivars can be traced back to 1910, such as “Bintje” and “King Edward”, which are still actively cultivated for food production. Potato cultivars can be classified into two categories based on their intended applications: table cultivars and starch cultivars. Table cultivars are selected for culinary purposes, these include the King Edward, Rocket, and Bintje. Starch cultivars are suitable for industrial processing into starch-based products due to their high starch content or quality, e.g. Kuras, Saprodi, and Avarna.

Potato breeding

With the increased cultivation of potatoes used for both food and industrial applications, more effort has been put into potato breeding. The focus on potato breeding encompasses several key aspects, such as plant resistance to

disease and pests, yield and productivity of tubers, quality and nutritional values, stress tolerance and environmental sustainability (Bradshaw 2007; Eriksson *et al.* 2016). Most cultivated potatoes are auto-tetraploid (2n = 4x = 48) with high heterozygosity, which is a significant barrier to potato improvement. Conventional potato breeding involves several steps, including selecting parental lines with desirable traits followed by sexual crosses and evaluating the performance of the hybrid offsprings with multiple selection cycles, which is time and labour consuming (Bradshaw and Bonierbale 2010). Modern potato breeding combines traditional methods with advanced technologies, such as marker-assisted selection to expedite the breeding process. The complete genome sequence of potato was elucidated by the Potato Genome Sequencing Consortium, which facilitates the advanced genomic study and breeding efforts of potatoes (2011). The development of genome editing technologies, such as TALEN- and CRIPSR-based methods, has a great potential for potato breeding, with several reports highlighting successfully improved traits such as increased resistance, reduced glycoalkaloid content, and improved tuber and starch quality (Sawai *et al.* 2014; Butler *et al.* 2016; Clasen *et al.* 2015; Andersson *et al.* 2017; Kieu *et al.* 2021).

1.1.2 The tomato

Importance of the tomato

The tomato (*Solanum lycopersicum* L.) is an important vegetable crop, which is extensively cultivated in diverse regions across the world. Tomato originates from Peru, South America and was first domesticated in Mexico due to the abundance of cultivated and wild tomato relatives in the region (Jenkins 1948). Tomato is regarded as protective food, considering the antioxidants it contains, such as lycopene, vitamin C, vitamin E, phenolics, and flavonoids (George *et al.* 2004). The production of tomato has increased over the decades, with Asia emerging as the dominated region, accounting for 63% of the global tomato yield (FAOSTAT 2023). It is one of the most popular vegetables suitable for cultivation throughout the tropics and subtropics, from private gardens, greenhouses, to open fields. Additionally, tomato have served as a model organism for genetic and molecular research due to its simple diploid genetics (2n = 2x = 24) and short life cycle,

contributing to advancements in plant biology and crop improvement (Ito *et al.* 2015).

Tomato breeding

Tomato is self-pollinating and can be easily hybridized within the species or crossed with wild relatives, such as *Solanum pennellii*, *Solanum pimpinellifolium*, and *Solanum peruvlanum*. Tomato breeding has been based around various standard methods, including hybridization followed by pedigree selection and introgression by backcrossing for desired traits. Breeding targets for tomatoes depend on market demands and consumer preferences, such as yield and quality of tomato fruits, resistance to biotic and abiotic stress, and resistance to diseases and pests. Like the potato, the availability of the tomato's genomic sequence (Tomato Genome Consortium 2012) facilitates the development of modern tomato breeding methods, including maker-assisted selection and genome editing technology (Foolad & Panthee R 2012; Brooks *et al.* 2014).

"Determinate" and "indeterminate"

Due to the different growth habits, tomatoes can be classified into two categories: "determinate" and "indeterminate" cultivars (Pnueli *et al.* 1998; Vicente *et al.* 2015). The "determinate" cultivars exhibit a compact growth habit, forming small bushes, and are characterized by synchronous fruit ripening; while "indeterminate" cultivars display a vining growth, continuing to produce the fruits throughout the growing season. It has been reported that the mutations in either or both of the flowering repressor gene *SELF PRUNING (SP)* and its paralog, *SELF PRUNING 5G (SP5G)*, can convert "indeterminate" cultivars into "determinate" cultivars (Carmel-Goren *et al.* 2003; Soyk *et al.* 2017; Kwon *et al.* 2020).

1.2 Glycoalkaloids

1.2.1 Glycoalkaloids - secondary metabolites

Secondary metabolites in Solanaceae plants

The production of biologically active metabolites is pervasive throughout the plant kingdom, serving critical roles in various aspects of plant life cycles. Primary metabolites are essential compounds involved in the plant's basic

life processes, while secondary metabolites are crucial for plant adaptation to the environment (Bourgaud *et al*. 2001). Alkaloids, one of the most important classes of secondary metabolites, are nitrogen-containing compounds and are found in 20% plant species, some examples from the Solanaceae family are steroidal alkaloids, tropane alkaloids, pyridine alkaloids and piperidine alkaloids (Cordell *et al.* 2001; Matsuura & Fett-Neto 2015; Chowański *et al.* 2016). Steroidal alkaloids (SAs) and their glycosylated forms, known as steroidal glycoalkaloids (SGAs), are present in over 350 plant species, with significant accumulations observed in the Solanaceae and Liliaceae families (Roddick 1996； Friedman *et al.* 1997). The investigation of the biological activities and biosynthesis of Solanaceae glycoalkaloids has garnered increasing attention, driven by their important role in plant defence as well as their high value in pharmaceutical applications.

Toxicity of glycoalkaloids

Glycoalkaloids, particularly those found in the Solanaceae family, are natural toxins that can cause adverse effects on human health. Acute toxicity may lead to symptoms such as vomiting, gastrointestinal disturbances, abdominal pain, or even more severe neurological symptoms, such as headaches, dizziness and confusion. Studies have estimated that a toxic dose of potato glycoalkaloids can be from 1mg/kg body weight (BW), with a potentially lethal dose being 3-6 mg/kg BW (Morris & Lee 1984; Friedman *et al.* 1997). For reasons of food safety, an upper limit of 200 SGA/kg (FW) in raw, unpeeled potato tubers is widely recommended. Some countries, such as The Netherlands and Denmark, have established stricter guidelines of a maximum of 100 mg TGA/kg (FW) glycoalkaloids, specifically for new potato cultivars (Schrenk *et al.* 2020). Concerns about glycoalkaloid toxicity in tomato fruits are comparatively lesser than for potato tubers, since the major glycoalkaloid in tomatoes, α-tomatine, is less toxic and has a much lower concentration in ripe fruits (ca. 5 mg/kg FW), as it is largely converted into a non-toxic glycoalkaloid (esculeoside A) during fruit ripening (Friedman 2002).

Role in plant resistance

The diverse and chemical properties of glycoalkaloids contribute to the plant defense systems, protecting against herbivores, pathogens, insects, fungi and bacteria, ensuring the survival and adaption of plants in various natural

environments. The biological activity of glycoalkaloids is based on their membrane-destabilizing properties against various organisms (Roddick *et al.* 1990; Keukens *et al.* 1995). The foliar total glycoalkaloids (TGA) level in potatoes is highly associated with resistance against pests, such as the Colorado potato beetle (*Leptinotarsa decemlineata*) and the potato leafhopper (*Empoasca fabae*) (Sinden *et al.* 1980; Tingey 1984; Sanford *et al.* 1992; Friedman *et al.* 1997). It has also been shown that α-chaconine can inhibit the growth of several fungi and nematodes (Fewell & Roddick 1993; Li *et al.* 2022).

Structure of glycoalkaloids

To date, more than 100 structurally different SGAs have been identified within the *Solanum* species, including both wild and cultivated potatoes, tomatoes, eggplants, and other nightshades (Milner *et al.* 2011; Zhao *et al.* 2021). The structure of glycoalkaloids consists of a cholesterol-derived hydrophobic C_{27} nitrogen-containing skeleton (aglycone), and a sugar moiety attached to the side chain (Friedman *et al.* 1997). The constitution of the sugar moieties attached to the side chain of glycoalkaloids plays a pivotal role in delineating their biological activities, even when sharing an identical aglycone, and vice versa.

The typical aglycones of Solanum glycoalkaloids identified so far are solanidane and spirosolane types, also referred to as alkamines (Friedman *et al.* 1997; Milner *et al.* 2011). For example, the major glycoalkaloids in the potato, α-chaconine and α-solanine, are of the solanidane type (solanidine aglycone), whereas the major glycoalkaloid in the tomato, α-tomatine, has the spirosolane aglycone (tomatidine aglycone). Another important part of the glycoalkaloid structure, glycoside residues, are attached at the 3-OH position on the A ring of the aglycone, usually in the form of tri- or tetrasaccharide with different combinations of various sugar molecules (Friedman *et al.* 1997). For example, both α-solanine and α-chaconine have a trisaccharide side chain, (solatriose and chacotriose, respectively), while α-tomatine possess a tetrasaccharide (lycotetraose) (Friedman *et al.* 1997; Milner *et al.* 2011; Iijima *et al.* 2013; Sonawane *et al.* 2020; Zhao *et al.* 2021).

1.2.2 SGAs in the potato

SGAs in potato plants

In cultivated potatoes, the predominant SGAs, α-chaconine and α-solanine, comprise up to 95% of TGA, with a ratio of α-chaconine/α-solanine varying from 2:1 to 1:1. Wild potato species exhibit a broader SGA composition, including minor SGAs, such as solamarine, solamargine, solasonine, demissine and leptine (Friedman & Dao 1992; Shakya & Navarre 2008; Kozukue *et al.* 2008; Koleva *et al.* 2012). SGAs are found in the entire potato plant, with the highest levels in flowers and berries, followed by sprouts and leaves (Friedman *et al*. 1997). The SGA levels in the tuber flesh are generally low, and SGAs are mainly formed in the areas of high metabolic activity, such as the peels and the eyes of tubers (Maga & Fitzpatrick 1980).

Factors influencing SGA levels in potato tubers

Genetic determinants play a fundamental role in setting the baseline levels of SGAs, as different species and cultivars exhibit distinct SGA profiles (Sinden *et al.* 1984; Friedman *et al*. 1997). However, the level of SGAs in potato tubers can be significantly increased upon various abiotic stresses during growth and storage, such as drought, heat, wounding and light exposure (Sinden *et al.* 1984; Friedman *et al*. 1997; Friedman & McDonald 1999; Knuthsen *et al*. 2009). SGAs are resistant to degradation during standard cooking processes, such as boiling, baking and frying. Therefore, it is important to ensure that the SGA level in tubers used as food is within safety limits (Bushway & Ponnampalam 1981).

SGAs in by-products of industrial potato starch production

During potato starch production, substantial by-products, notably potato fruit juice (PFJ), are generated, necessitating either disposal or transformation into value-added side stream products. PFJ is enriched with economically valuable compounds, such as proteins (ca. 2%) (Schoenbeck *et al.* 2013). However, the presence of SGAs poses a significant challenge, due to their concentration in PFJ, complicating the extraction of proteins while preserving their functional properties. Several techniques, including acidic precipitation and thermal coagulation, have been explored to extract proteins from PFJ (Zwijnenberg *et al.* 2002; Cheng *et al.* 2010; Miedzianka *et al.* 2012; Schoenbeck *et al.* 2013; Waglay *et al.* 2014). The functional integrity of proteins isolated via thermal treatment may be compromised, limiting

their application as animal feed (Schoenbeck *et al.* 2013; Hussain *et al*. 2021).

1.2.3 SGAs in other *Solanum* species

The major SGA in the tomato, tomatine, is a mixture of α-tomatine and dehydrotomatine in a 10:1 ratio, distributed throughout various parts of tomato plants (Friedman *et al*. 1994; Friedman 2002). High SGA concentration in tomato plants is often observed in immature parts of the plant, such as green fruits (Friedman 2002). Other SGAs identified in tomato include tomatoside A, esculeoside A and B, and dehydrotomatoside. In eggplants, the predominant SGAs are α-solamargine and α-solasonine, which are also synthesized in over 100 other plant species (Milner *et al.* 2011; Friedman 2015). The cultivated eggplant generally exhibits lower levels of TGA (< 20 mg/kg FW) compared to other *Solanum* plants, with the highest concentration usually found in the peels, especially the mesocarp with seeds (Sánchez-Mata *et al.* 2010; Milner *et al.* 2011). Similar to tomato SGAs, eggplant SGAs are relatively non-toxic; however, their embryotoxicity and teratogenic properties necessitate careful monitoring of SGA levels in eggplant (Milner *et al.* 2011).

1.3 The biosynthesis of SGAs

Solanum plants, including the potato, tomato, and eggplant, share a common SGA biosynthesis pathway. This pathway starts with the formation of cycloartenol from acetyl CoA, followed by cholesterol biosynthesis, ultimately resulting in the diverse structural forms of SGAs (Friedman *et al.* 1997; Cárdenas *et al.* 2015; Sonawane *et al.* 2020; Zhao *et al.* 2021). The intricate process involves a large number of genes across multiple steps, with cholesterol as the primary precursor for SGA (Ohyama *et al.* 2013; Petersson *et al.* 2013). Despite multiple steps and many key enzymes having been identified in recent years, the complete pathway has not yet been elucidated. The biosynthesis of SGAs can be broadly divided into two parts: pre-cholesterol pathway and post-cholesterol pathway. This PhD project is primarily focused on the study of SGA biosynthesis in potato plants, and a model detailing the SGA biosynthesis pathway in potato plants is illustrated in Fig 1.

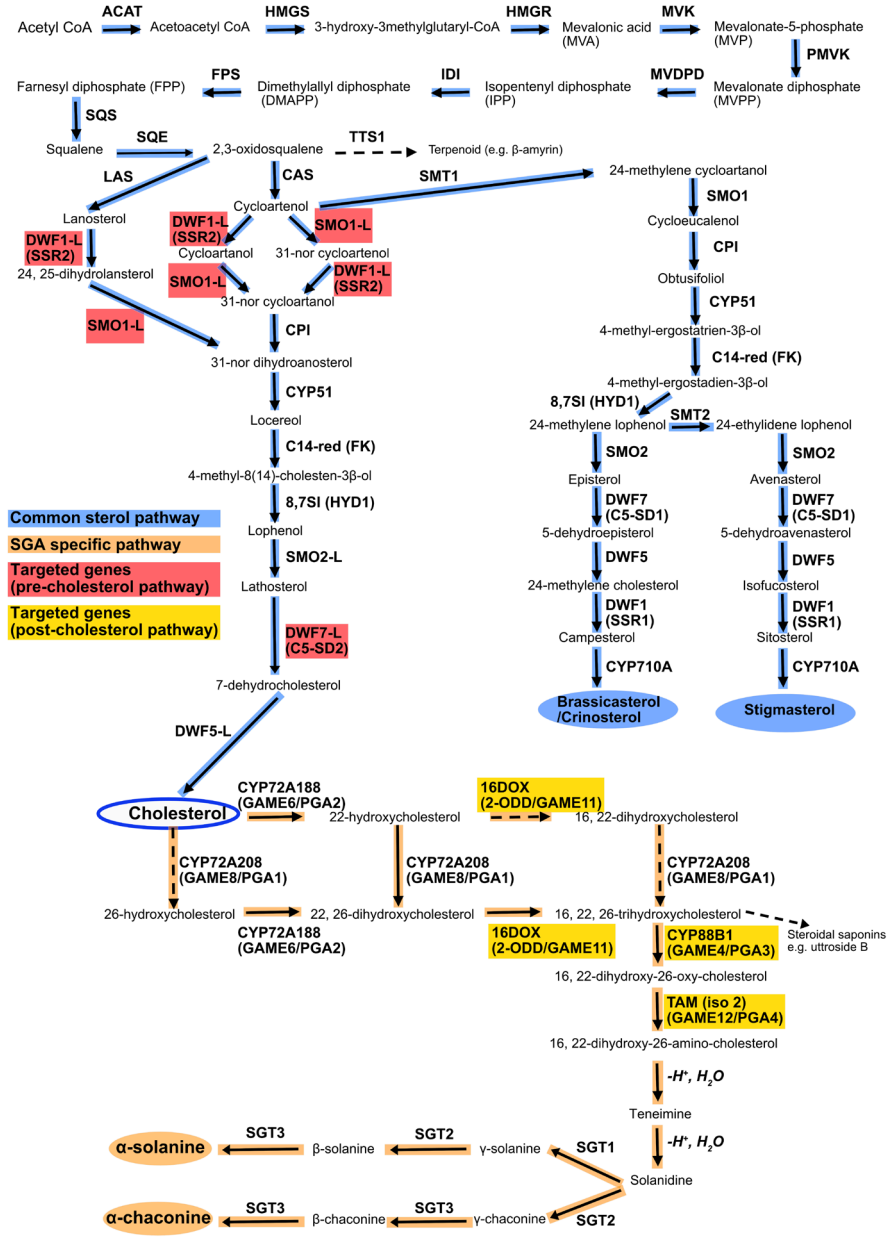

Figure 1. Pathway models of sterol and SGA biosynthesis in the potato. L denotes duplicated '-like' genes, encoding proteins with a special role in cholesterol synthesis.

1.3.1 Pre-cholesterol pathway: from acetyl-CoA to cholesterol

The pre-cholesterol pathway is initiated by the mevalonate (MVA) pathway from acetyl-CoA, catalysed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) (Stermer *et al.* 1994; Krits *et al*. 2007; Ginzberg *et al.* 2012). Further downstream, the early enzymes such as squalene synthase (SQS), squalene epoxidase (SQE), cycloartenol synthase (CAS), lanosterol synthase (LAS), not only participate in cholesterol biosynthesis, but also contribute to the synthesis of phytosterols and terpenoids at crucial branching points (Ginzberg *et al.* 2009; Sonawane *et al*. 2016). The sterol side chain reductase2 (SSR2, also denoted as DWF1-L) is a key enzyme responsible for converting cycloartenol to cycloartanol, controlling the metabolic flux towards cholesterol and C-24 alkyl phytosterols at this branch point (Sawai *et al.* 2014). A separation between cholesterol synthesis for either housekeeping or for the production of SGAs is mediated through the duplication of a small set of differentially regulated sterol-biosynthetic genes (Sonawane *et al*. 2016). This results in two parallel routes to cholesterol, with examples of duplicated genes such as *DWF1-L* and *DWF1*, *STEROL METHYL-OXIDASE1-LIKE* (*SMO1-L*) and *SMO1*, Δ*7-C5 STEROL DESATURASE-LIKE* (*DWF7-L*) *and DWF7*, and Δ*7 STEROL REDUCTASE-LIKE* (*DWF5-L*) *and DWF5* (Sawai *et al.* 2014; Sonawane *et al*. 2016; Nahar *et al*. 2017). Each of these genes is implicated in either SGA (the former) or sterol (the latter) biosynthesis.

1.3.2 Post-cholesterol pathway: from cholesterol to specific SGAs

Cholesterol subsequently undergoes a series of SGA-specific reactions, including hydroxylation, oxidation, transamination and glycosylation, leading to the formation of various SGAs (Sonawane et al. 2020). A cluster of *GLYCOALKALOID METABOLISM* (*GAME*) genes were reported, being involved in the post-cholesterol pathway which converts cholesterol into aglycones (Itkin *et al*. 2013). Several cytochrome P450 monooxygenases (CYPs), such as CYP72A186/GAME7 and CYP72A188/PGA2/GAME6 in the tomato and potato, respectively, hydroxylate cholesterol at the C-22 position, followed by re-hydroxylation at the C-26 position by CYP72A208/PGA1/GAME8 (Itkin *et al*. 2013; Umemoto *et al.* 2016). The first 2-oxoglutarate-dependent dioxygenase (2-ODD) identified in SGA biosynthesis, 16α-hydroxylase (16DOX/GAME11), is responsible for hydroxylation at the C-16 position (Nakayasu *et al.* 2017). CYP88B1, also

denoted as GAME4/PGA3, plays a key role in a later oxidation step before nitrogen incorporation, contributing to the synthesis of nitrogenous SGAs and non-nitrogenous steroidal saponins (STSs) at the branching point (Itkin *et al*. 2013; Paudel *et al.* 2017). Further along in the pathway, a nitrogen atom is introduced into the cholesterol backbone at the C-26 position catalysed by an aminotransferase, gamma amino butyric acid (GABA) transaminase (TAM/GAME12/PGA4) (Itkin *et al*. 2013; Sonawane *et al.* 2020; Nakayasu *et al.* 2021). Several UDP-glycosyltransferases (UGTs), encoding *STEROIDAL ALKALOID GLYCOSYLTRANSFERASE 1* (*SGT1*), *SGT2* and *SGT3* in potato, and *GAME1*, *GAME17*, *GAME18*, and *GAME2* in tomato, are involved in several glycosylation reactions, adding various sugar moieties to the aglycone and contributing to the formation of distinct SGAs (Moehs *et al.* 1997; McCue *et al.* 2005, 2006, 2007). Another DOX enzyme, encoding *DIOXYGENASE FOR POTATO SOLANIDANE SYNTHESIS* (*DPS*), catalyzes the ring arrangement from spirosolanes to solanidanes, contributing to the structural diversity of SGAs in Solanaceae species (Akiyama *et al.* 2021).

1.3.3 Regulatory genes involved in the SGA biosynthesis pathway

The APETALA 2/Ethylene-Responsive Element Binding Factor (AP2/ERF) family constitutes a group of transcription factors, playing key roles in regulating the biosynthesis of specialized metabolites in plants (Sakuma *et al.* 2002; Feng *et al.* 2020). GAME9, also known as Jasmonate-Responsive Ethylene Response Factor 4 (JRE4), belongs to the JRE subset family of ERFs. It has been identified as a transcriptional regulator for a series of metabolic genes involved in cholesterol and SGA biosynthesis in both tomato and potato (Cárdenas *et al*. 2016; Thagun *et al.* 2016; Nakayasu *et al.* 2018b). GAME9 functions by recognizing and binding to GCC box-like elements in the promoters of its target genes, either directly or in conjunction with MYC2 as an interacting factor (Cárdenas *et al.* 2016; Thagun *et al.* 2016). Recently, two tomato jasmonate-related clade IIIe bHLH transcription factors, MYC1 and MYC2, have been found to play a similar role in regulating constitutive SGA biosynthesis by controlling the basal expression of SGA-related genes (Panda *et al.* 2022; Swinnen *et al.* 2022).

1.3.4 Previous studies in SGA biosynthesis targeting key genes in *Solanum* species

Previous studies have highlighted the efficacy of silencing key genes in both pre- and post-cholesterol pathways of SGA biosynthesis, leading to a significant reduction in SGA content in the potato and tomato. These targeted key genes include *DWF1-L*, three *CYP* genes (*CYP72A208*, *CYP72A188*, and *CYP88B1*), *16-DOX*, *TAM* isoform 2, and *DPS* (Itkin. *et al*. 2013; Sawai *et al.* 2014; Umemoto *et al.* 2016; Nahar *et al.* 2017; Paudel *et al.* 2017; Nakayasu *et al.* 2017, 2021; Akiyama *et al.* 2021). Moreover, the silencing of *GAME9* resulted in suppressed SGA levels by altering the expression of key metabolic genes in the SGA biosynthesis pathway (Cárdenas *et al*. 2016; Thagun *et al.* 2016; Nakayasu *et al.* 2018b). Recent advancements in modern breeding technologies, such as TALEN and CRIPSR/Cas9, expedite the generation of SGA-reduced or even SGA-free Solanum plants by targeting all alleles of key SGA metabolic genes, including *DWF1-L*, *16DOX*, and *CYP88B1* (Akiyama *et al.* 2017; Nakayasu *et al*. 2018a; Yasumoto *et al.* 2019, 2020; Zheng *et al.* 2021). However, there has been no investigation into the comparative SGA reduction in mutants targeting different key genes in the pre- or post-cholesterol pathway of SGA biosynthesis.

1.4 Sterols

1.4.1 Sterols in plants

Sterols, vital isoprenoid-derived molecules for eukaryotic life, are either synthesized *de novo* or acquired from the environment. While cholesterol predominates in vertebrates, plants contain a mixture of various sterols, typically featuring lower cholesterol levels than mammals, such as 1-2% found in Arabidopsis (Benveniste 2004). However, certain plant species, like Solanaceous plants (e.g. potato and tobacco), exhibit higher levels of cholesterol, constituting 15-20 % of total sterols (Arnqvist *et al.* 2003). Plant sterols, also called phytosterols (C-24 alkylsterols), serve as essential structural elements in the membrane, regulating fluidity and permeability (Hartmann 1998; Schaller 2003). They exist in various forms, such as free sterols, sterol esters (coupled with fatty acids), sterol glycosides, and acylsterol glycosides, contributing to the sterols diversity and performing a wide range of functions in plants (Valitova *et al.* 2016). Beyond their

structural role in the membrane, phytosterols act as precursors to brassinosteroids, which are a group of steroidal growth hormones involved in plant growth and development (Clause & Sasse 1998; Benveniste 2004). Additionally, phytosterols are also substrates for the synthesis of a wide range of secondary metabolites, including SGAs, cardenolides, withanolids, pregnane derivatives, and saponins (Hartmann 1998; Piironen *et al.* 2000).

Phytosterols can be categorized into three groups based on the number of methyl substituents at the C-4 position: 4-desmethyl sterols, 4α-monomethyl sterols, and 4,4-dimethyl sterols (Moreau *et al.* 2018). Among these, campesterol, stigmasterol and sitosterol, which fall into the 4-desmethyl sterols, are the most common phytosterols (Hartmann & Benveniste 1987; Moreau *et al.* 2018). The other two groups constitute minor components in most plants. Structurally akin to cholesterol, phytosterols differ in the side chain (Hartmann 1998; Piironen *et al.* 2000). For instance, sitosterol and campesterol deviate from the cholesterol structure by an ethyl and a methyl group, respectively. Stigmasterol, in comparison to sitosterol, features an additional double bond at the C-22 position of the side chain.

1.4.2 Phytosterol biosynthesis in *Solanum* species

The phytosterol biosynthesis pathway intersects with cholesterol biosynthesis, as indicated in Fig 1, involving specific genes related to SGA or sterol, as well as shared genes in both pathways (Nahar *et al*. 2017; Sonawane *et al.* 2020). The formation of phytosterols shares the same mevalonate/isoprenoid pathway as cholesterol biosynthesis, leading to the production of the sterol precursor, cycloartenol (Hartmann & Benveniste 1987; Piironen *et al.* 2000; Valitova *et al.* 2016; Sonawane *et al.* 2020). Then cycloartenol is catalyzed by Sterol C-24 methyltransferases (SMTs), diverting the biosynthesis towards phytosterols (Sonawane *et al.* 2020). Two distinct types of SMTs, SMT1 and STM2, participate in the synthesis of 24-methl and 24-ethyl sterols, respectively. SMT1 initiates the primary methylation of cycloartenol to 24-methylene cycloartanol (Bouvier-Navé *et al.* 1998; Arnqvist *et al.* 2003). SMT2 contributes to the second methylation step at the branching point, transforming 24-methylene lophenol into 24-ethylidene lophenol, leading to the formation of 24-ethylsterols, such as β-sitosterol and stigmasterol (Bouvier-Navé *et al*. 1998). DWF1 operates in reactions downstream of both SMT1 and SMT2, catalysing the formation of

campesterol and sitosterol, respectively, which are further desaturated by CYP710A to yield the final products, brassicasterol and stigmasterol, respectively (Nomura *et al.* 1998; Morikawa *et al.* 2006, 2009; Arnqvist *et al.* 2008; Nahar *et al*. 2017).

1.5 DNA-free genome editing method by CRISPR/Cas9

Advancements in traditional plant breeding have historically been time- and labour-intensive. However, plant biology and breeding have the potential of being advanced with the rapid development of genome editing and other new genomic techniques (NGTs). Among these, CRISPR/Cas9 based technology has emerged as a prominent method, utilizing the improvement of various traits in a wide range of plant species. The widespread CRISPR/Cas9 application is attributed to its versatility and high editing efficiency, surpassing earlier genome editing tools like zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Lowder *et al.* 2015). The success of CRISPR/Cas9 has been reported not only in model plants, e.g. *Arabidopsis thaliana* and *Nicotiana benthamiana*, but also in commercially significant crops such as wheat, rice, potato, and maize (Li *et al*. 2013; Nekrasov *et al.* 2013; Upadhyay *et al.* 2013; Jiang *et al.* 2013; Liang *et al.* 2014; Brooks *et al.* 2014; Zhou *et al.* 2014; Ito *et al.* 2015; Wang *et al.* 2015; Zhang *et al.* 2016; Andersson *et al.* 2017).

The current development of genome editing has sparked discussion on the regulation of NGTs and their applications in agriculture in many regions, including Europe (Purnhagen *et al*. 2023). The European Commission proposed new regulations for plants modified by NGTs in July 2023. The aim is to establish regulatory frameworks that harness the innovative potential of NGTs in plant breeding while upholding high safety standards. Therefore, the safety of NGT applications in plant breeding, e.g. the absence of integrated foreign DNA into plant cells, becomes paramount; this is achievable through a DNA-free genome editing method.

1.5.1 Delivery reagents of the CRISPR/Cas9 system

Efficient and precise genome editing via CRISPR/Cas necessitates a reliable delivery system into plant cells. Plasmid vectors delivered using agrobacterium or particle bombardment have dominated genome editing

reagent delivery across many plant species, an approach subjected to regulatory approval (Ghogare *et al.* 2021). An alternative method involves the use of ribonucleoprotein complexes (RNPs), consisting of Cas9 protein and single guide RNA (sgRNA), enabling the DNA-free delivery of the CRISPR/Cas9 system. RNPs offer advantages such as lower off-target frequency and faster degradation compared to the plasmid-based systems (Chen *et al.* 2019). Delivery of RNPs into plant cells has been achieved through particle bombardment or polyethylene glycol (PEG)-mediated transfection (Woo *et al.* 2015; Svitashev *et al.* 2016; Liang *et al.* 2017). Woo et al. (2015) pioneered the delivery of RNP complexes into plant protoplasts in *Arabidopsis*, tobacco, lettuce and rice. Subsequent studies have extended the application of RNPs for genome editing to other crops, including maize, wheat, potato, and canola (Svitashev *et al.* 2016; Liang *et al.* 2017; Andersson *et al.* 2018; Sidorov *et al.* 2021).

1.5.2 DNA-free genome editing in the potato and tomato

In the potato, the implementation of protocols for shoot regeneration from transfected protoplasts and the utilization of RNPs have facilitated a DNA-free genome editing system (Nicolia *et al.* 2015, 2021b; Andersson *et al.* 2017, 2018). This system is efficient from the transfection in regenerated plants, enabling modifications in all four alleles.

The application of CRISPR/Cas9 technology in the tomato for trait enhancement, including improved disease resistance and domestication of the wild tomato, has been reported previously (Ito *et al*. 2015; Pan *et al*. 2016; Li *et al.* 2018). CRISPR/Cas9 technology applied in tomato primarily utilized stable *Agrobacterium tumefaciens*-mediated transformation, often resulting in a low homozygous mutation rate and necessitating segregation in the next generation to eliminate foreign DNA. A recent study introduced the use of RNPs to deliver CRISPR/Cas9 into tomato protoplasts, achieving a high editing efficiency in regenerated calli (Nicolia *et al.* 2021a). However, the bottleneck remains in shoot regeneration from tomato RNP-transfected protoplasts. Limited reports detail successful shoot regeneration from unedited protoplasts in the cultivated tomato, and very recently (Morgan & Cocking 1982; Sakata *et al.* 1987; Tan *et al.* 1987), Lin et al. (2021) reported success in the wild tomato (*Solanum peruvianum*) with CRISPR/Cas9 mutations. Therefore, the establishment of a protocol with high editing

efficiency and regeneration rates in the cultivated tomato would be beneficial for both genetic studies and breeding purposes.

2. Aims and objectives of the thesis

Considering the importance of SGAs for potato quality and food safety, the overall aim of this thesis has been to investigate the regulation of SGA metabolism and biosynthesis in the potato, and to generate SGA-reduced mutated potato plants with a focus on starch potato cultivars. Part of the work was dedicated to developing a DNA-free CRISPR/Cas9 methodology for cultivated tomato.

The specific aims were to:

- compare the SGA metabolism in starch and table potato cultivars.
- generate SGA-reduced mutated potato plants by targeting different key SGA genes through CRISPR/Cas9 technology
- establish a protocol of DNA-free genome editing through CRISPR/Cas9 technology in cultivated tomato

3. Results and discussion

3.1 Metabolomic analysis of starch vs. table potato cultivars

To investigate the glycoalkaloid metabolism in less-explored starch potato cultivars, a three-year study was designed investigating a number of starch potato cultivars along with table potato cultivars. A set of 13 commonly used starch potato cultivars in Sweden and ten prevalent table potato cultivars were selected for this study.

SGA quantification using heavy isotope-labelled endogenous standards showed that the average tuber TGA level was significantly lower in the tubers of table potato cultivars (43 ± 18 mg/kg FW) than in starch potato cultivars (113 ± 62 mg/kg FW) (Paper I, Figure 1). However, there were no significant differences between the groups when subjected to two SGA-inducing conditions (wounding and light exposure). These results indicate a stronger selection for table potato cultivars at harvest, but also that the impact of accumulated SGAs under TGA-inducing conditions has not been considered for either group.

Metabolomic analyses revealed significant differences in the metabolite profiles between starch and table potato cultivars (Paper I, Figure 3). Particularly, α-solanine was identified with the highest basal levels in the tubers of starch potato cultivars than in the table potato cultivars (Paper I, Figure 4). In contrast, a lower level of a great number of amino acids was observed in the tubers of starch potato cultivars compared to those of table potato cultivars. A correlation analysis between α-solanine and target

metabolites confirmed that, as expected, glycoalkaloids (e.g. solanidine, α-chaconine, and solasonine) were the most positively correlated metabolites (Paper I, Figure 5). Conversely, a broad range of compounds exhibited a negative correlation, including sugars, purines, and fatty acids. Protein and dry matter determination showed a significant difference between starch and table cultivars: protein content was higher in table potato cultivars, while dry matter showed the opposite trend, implying distinct breeding priorities between these two groups (Paper I, Figure 6). Starch potato cultivars are selectively bred for high starch content, which is linked to high dry matter content, a trait that is not attractive in table potato cultivers. Transcriptomic profiling showed differing gene expression patterns in starch and table potato cultivars (Paper I, Figure 7). Among over 500 identified differentially expressed genes (DEGs), the expression of a few transporters (e.g. amino acid transporter) and biotic defence response-related genes was significantly higher in table and starch potato cultivars, respectively.

3.2 CRISPR/Cas9-mediated generation of SGA-free potato plants

To investigate the role of *GAME9*, the AP2/ERF transcript factor that regulates key genes in SGA biosynthesis, we generated *StGAME9* mutated events using DNA-free CRISPR/Cas9 technology (Paper II, Figure 1). Three full knockout mutants were selected for detailed studies, including SGA quantification, observation of plant appearance and growth under greenhouse conditions, sterol profiling, transcriptomic analyses, and the SGA response of tubers under two SGA-inducing conditions. LC-MS quantification of SGA levels revealed a significant reduction in TGA levels in leaves, accompanied by an almost complete absence of SGAs (<0.2 mg/kg FW) in the tubers in all three events compared to the wild-type plants (Paper II, Figure 2). Despite a slight reduction in plant height, all three events displayed a normal growth and developmental pattern compared to the wild-type plants. A normal phenotype was also observed for *SlGAME9* EMS-induced mutated tomato plants (Nakayasu *et al.* 2018b).

Transcriptome profiling unveiled a wide range of DEGs in leaves and tubers from two selected *StGAME9* events, including genes encoding UDP-glycosyltransferases, a Cellulose synthase-like protein, and genes involved

in the SGA biosynthesis pathway (Paper II, Table 1, 2). Examination of SGA- and sterol-related gene expression indicated that *StGAME9* regulates genes in both SGA and sterol biosynthesis pathways (Paper II, Figure 4), which compared well with results from transgenic tomato plants with down-regulated *SlGAME9* expression from RNAi (Cárdenas *et al*. 2016; Thagun *et al.* 2016; Nakayasu *et al.* 2018b). The significant down-regulation of key SGA genes, was observed for both pre- (e.g. *SMO1-L* and *DWF7-L*) and post-cholesterol (e.g. *16DOX* and *SGT1*) pathways of SGA biosynthesis. The altered sterol profile in the *StGAME9* events (Paper II, Figure 3), i.e. decreased phytosterols levels at varying degrees, was the final consequence of altered SGA/sterol-related gene expression within the metabolic network. Investigation of the responses of *StGAME9* mutated tubers under SGA-inducing conditions demonstrated consistently low TGA levels, in contrast to the significant SGA accumulation in wild-type tubers (Paper II, Figure 2). Moreover, the expression of inducible SGA-related genes identified in the wild-type remained down-regulated under SGA-inducing conditions in *StGAME9* mutants.

To investigate alternative methods of achieving SGA reductions in potato mutants, we also generated a number of mutated potato events via DNA-free CRISPR/Cas9 technology, by targeting different key genes in either the pre- or post-cholesterol pathway of SGA biosynthesis. These targeted genes included *DWF1-L*, *SMO1-L*, and *DWF7-L* acting in the pre-cholesterol pathway, and *TAM* isoform 2, *16DOX*, and *CYP88B1* acting in the post-cholesterol pathway of SGA biosynthesis (Paper III, Figure 1). While a considerable SGA variation both within and between different gene mutants was observed, the majority of mutated events that carried mutations in all four alleles showed reduced SGA levels in both leaves and tubers (Paper III, Figure 3, Supp. Fig 3, Supp. Table 3). Additionally, mutated events in the post-cholesterol pathway exhibited significantly lower SGA levels in both leaves and tubers, with tubers displaying a more pronounced reduction (Paper III, Figure 3). This finding might be explained through a greater SGA-specific action from the genes in the post-cholesterol pathway. The strategies for targeting genes positioning in either the pre- or post-cholesterol pathway of SGA biosynthesis both resulted in several full knockout events displaying close to SGA-free tubers (SGA levels < 0.1 mg/kg FW). Similar to the *StGAME9* mutated tubers, the SGA levels remained consistently low under

two SGA-inducing conditions (Paper III, Figure 3, Supp. Fig 3), suggesting that mutations in the different key genes have a capacity to block both basal and induced SGA synthesis. The morphology of the majority of mutated plants and tubers remained similar to that of the wild-type under greenhouse conditions (Paper III, Figure 2), and no correlations were observed between reduced or even the absence of SGA and the plant height or tuber yield, aligning with previous gene downregulation studies (Umemoto *et al.* 2016; Nakayasu *et al.* 2017, 2021).

The significantly reduced SGA levels in both leaves and tubers in mutated events targeting *DWF1-L*, *TAM* (iso2), *16DOX*, and *CYP88B1* in this study (Paper III, Figure 3) corroborated findings from previous studies of RNAi or knockout lines in potato, confirming the key roles of these genes in the SGA biosynthesis pathway (Itkin. *et al*. 2013; Sawai *et al.* 2014; Nahar *et al.* 2017; Paudel *et al.* 2017; Nakayasu *et al.* 2017, 2018a, 2021; Akiyama *et al.* 2017, 2021; Yasumoto *et al.* 2019, 2020; Zheng *et al.* 2021). The *SMO1-L* and *DWF7-L* mutated events, reported for the first time in our study, also exhibit a significant decrease in SGA levels, supporting their involvement in the SGA biosynthesis pathway. The significant accumulation of sterols in the *SMO1-L* and *DWF7-L* events further validate their positions in our pathway model (Paper III, Figure 1, Figure 4). Double mutants for *SMO1-L* and *DWF1-L* displayed a higher degree of reduction compared to single mutants of either *SMO1-L* or *DWF1-L*. The availability of SGA-free starch potatoes presents a valuable opportunity for potential industrial applications.

3.3 Development of a DNA-free CRISPR/Cas9 method in cultivated tomato

To establish a DNA-free genome editing and protoplast regeneration protocol for cultivated tomato, we enhanced the protoplast isolation method and focused on optimizing shoot regeneration on various solid media (Paper IV, Figure 1), building on previous research (Nicolia *et al*. 2021a, 2021b). Four tomato cultivars, Red Setter, Ailsa Craig, M82 and Moneymaker, were employed, and sgRNAs were designed to target two genes simultaneously, *SELF PRUNING* (*SP*) and *SELF PRUNING 5G* (*SP5G*), in all four cultivars. The targeted genes were selected to facilitate the visual observation of effects.

Several modifications to the protoplast isolation process were made to enhance yield, viability, and regenerability. The results revealed that older seedlings (21 days), a higher temperature (25 °C), and longer durations during enzyme treatment (16 h) improved protoplast yield across all four cultivars, ranging from 1.0 to 2.2 × 10^6 per gram of leaf materials (Paper IV, Figure 2). Conversely, preconditioning of donor plants before protoplast isolation had a negative impact on yield.

Under optimized protoplast isolation conditions, freshly isolated protoplasts were subjected to PEG transfection using a vector carrying GFP. Transfection efficiency, estimated at 30-50%, was observed through GFP expression. RNP complexes targeting both genes were then transfected into protoplasts for further shoot regeneration investigation. Ten different solid shoot regeneration media were designed, exploring the effect of different combinations or concentrations of plant hormones, gelling agents, and carbon sources (Paper IV, Supp. Table 1). The highest shoot regeneration rate was noted in Medium TSR-a (31.4%), followed by Medium TSR-b (21.3%), with no statistically significant difference (Paper IV, Table 1). The major difference between these two media was the different plant hormones and concentrations. Morphological variations in regenerated shoots were observed, with the healthiest shoots on TSR-b, using a plant hormone combination of 0.1 mg/L IAA and 0.75 mg/L zeatin. Genotype differences were observed among the four tested cultivars, with Red Setter exhibiting superiority. Such genotype differences in other cultivars were also observed in the previous reports (Morgan & Cocking 1982; Niedz *et al.* 1985; Tan *et al.* 1987).

Among the 110 regenerated M_0 plants, 35 (31.8%) harbored mutations in both *SP* and *SP5G* genes, achieving an editing efficiency of up to 60% in at least one allele in either *SP* or *SP5G* genes (Paper IV, Table 2). Mutations in M_0 plants included a mix of bi-allelic, mono-allelic and chimeric variants. These results suggest that the RNP-based CRISPR/Cas9 system is highly efficient in generating the desired mutations in cultivated tomato, comparable to the current predominated *Agrobacterium tumefaciens*-mediated transformation (Ito *et al*. 2015; Ueta *et al.* 2017; Dahan-Meir *et al.*

2018), with the added advantage of there being no stable integration of foreign DNA.

4. Conclusions and future perspectives

The work presented in this thesis has broadened the knowledge and understanding of the SGA metabolism and biosynthesis in the potato, and provided a DNA-free CRISPR/Cas9 genome editing method for cultivated tomato. The key conclusions are:

- Starch potato cultivars were thoroughly characterized for their SGA levels and metabolism, compared to table potato cultivars. Metabolic differences between these two groups were observed, e.g. the levels of basal SGA, amino acid, and total protein, along with distinct gene expression patterns. The significant accumulation of SGAs in both starch and table potato cultivars under inducing conditions underscores the importance of considering both stress response and environmentally regulated SGA responses in breeding programs.

- Detailed investigations into *StGAME9* mutants provided a comprehensive understanding of the regulatory role of StGAME9 in potato SGA biosynthesis. The identification of the core SGA-related genes regulated by *StGAME9* offers valuable insights for future strategies aimed at reducing SGA levels in the potato to enhance their nutritional value and to facilitate the use of side streams.

- Comparative analysis of SGA reduction in potato mutants targeting different key genes revealed that knocking out of genes in the post-cholesterol pathway of SGA biosynthesis resulted in more substantial reductions in SGA levels compared to those in the pre-

cholesterol pathway. The availability of SGA-free starch potatoes presents a promising opportunity for industrial applications.

- The challenge of shoot regeneration from transfected protoplasts of cultivated tomato has been overcome, leading to the successful establishment of a DNA-free genome editing method with a high regeneration rate and mutation rate. However, further adaptation may be necessary when applying this protocol to other cultivars.

The work presented in this thesis serves as an initial step, paving the way for potential future investigations. Future work could be directed towards the utilization of SGA-free starch potatoes for industrial applications, as well as delving into SGA metabolism and biosynthesis in other *Solanum* species, e.g. tomato. Key targets for future work may include:

- The analysis of metabolomic differences between mutated potato plants and wild-type plants to identify the mutated events with the lowest SGA levels and minimal impact on overall metabolism.

- The evaluation of the performance of mutated potato plants in the field, e.g. plant phenotype, resistance to insects and pathogens, and yield.

- The investigation of the feasibility of employing SGA-free starch potatoes for industrial applications, including assessing starch content and quality, SGA levels in by-products (e.g. potato fruit juice and pulp), and the quality and quantity of proteins and fibre extracted from by-products.

- The characterization of the role of other core SGA-related genes found regulated by *StGAME9*, to enhance comprehension of the SGA biosynthesis.

- The extension of the insights gained in SGA biosynthesis in potato from this work to tomato, by studying the corresponding homologous genes.

References

Akiyama, R., Nakayasu, M., Umemoto, N., Muranaka, T. & Mizutani, M. (2017). Molecular breeding of SGA-free potatoes accumulating pharmaceutically useful saponins. *Regulation of Plant Growth & Development*, vol. 52 (2), pp. 92–98. https://doi.org/10.18978/jscrp.52.2_92

Akiyama, R., Watanabe, B., Nakayasu, M., Lee, H.J., Kato, J., Umemoto, N., Muranaka, T., Saito, K., Sugimoto, Y. & Mizutani, M. (2021). The biosynthetic pathway of potato solanidanes diverged from that of spirosolanes due to evolution of a dioxygenase. *Nature Communications*, vol. 12 (1), pp. 6–15 Springer US. https://doi.org/10.1038/s41467-021-21546-0

Andersson, M., Turesson, H., Nicolia, A., Fält, A.S., Samuelsson, M. & Hofvander, P. (2017). Efficient targeted multiallelic mutagenesis in tetraploid potato (Solanum tuberosum) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Reports*, vol. 36 (1), pp. 117–128. https://doi.org/10.1007/s00299-016-2062-3

Andersson, M., Turesson, H., Olsson, N., Fält, A.S., Ohlsson, P., Gonzalez, M.N., Samuelsson, M. & Hofvander, P. (2018). Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiologia Plantarum*, vol. 164 (4), pp. 378–384. https://doi.org/10.1111/ppl.12731

Arnqvist, L., Dutta, P.C., Jonsson, L. & Sitbon, F. (2003). Reduction of cholesterol and glycoalkaloid levels in transgenic potato plants by overexpression of a type 1 sterol methyltransferase cDNA. *Plant Physiology*, vol. 131 (4), pp. 1792–1799. https://doi.org/10.1104/pp.102.018788

Arnqvist, L., Persson, M., Jonsson, L., Dutta, P.C. & Sitbon, F. (2008). Overexpression of CYP710A1 and CYP710A4 in transgenic Arabidopsis plants increases the level of stigmasterol at the expense of sitosterol. *Planta*, vol. 227 (2), pp. 309–317. https://doi.org/10.1007/s00425-007-0618-8

Benveniste, P. (2004). Biosynthesis and accumulation of sterols. *Annual Review of Plant Biology*, vol. 55, pp. 429–457. https://doi.org/10.1146/annurev.arplant.55.031903.141616

Bourgaud, F., Gravot, A., Milesi, S. & Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant Science*, vol. 161 (5), pp. 839–851. https://doi.org/https://doi.org/10.1016/S0168-9452(01)00490-3

Bouvier-Navé, P., Husselstein, T. & Benveniste, P. (1998). Two families of sterol methyltransferases are involved in the first and the second methylation steps of plant sterol biosynthesis. *European Journal of Biochemistry*, vol. 256 (1), pp. 88–96. DOI: 10.1046/j.1432-1327.1998.2560088.x

Bradshaw, E.J., Ramsay, G. (2009). Potato origin and production. (Singh, J., Kaur, L., ed.) *Advances in Potato Chemistry and Technology*, pp. 1–26. DOI:10.1016/B978-0-12-374349-7.00001-5

Bradshaw, J.E. (2007). Chapter 8 - Potato-Breeding Strategy. In: Vreugdenhil, D., Bradshaw, J., Gebhardt, C., Govers, F., Mackerron, D.K.L., Taylor, M.A., & Ross, H.A.B.T.-P.B. and B. (eds.) Amsterdam: Elsevier Science B.V., pp. 157–177. https://doi.org/10.1016/B978-044451018-1/50050-6

Bradshaw, J.E. & Bonierbale, M. (2010). Potatoes BT - Root and Tuber Crops. In: Bradshaw, J.E. (ed.) New York, NY: Springer New York, pp. 1–52. https://doi.org/10.1007/978-0-387-92765-7_1

Brooks, C., Nekrasov, V., Lipppman, 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*, vol. 166 (3), pp. 1292–1297. DOI: 10.1104/pp.114.247577

Bushway, R.J. & Ponnampalam, R. (1981). .alpha.-Chaconine and .alpha.-solanine content of potato products and their stability during several modes of cooking. *Journal of Agricultural and Food Chemistry*, vol. 29 (4), pp. 814–817 American Chemical Society. DOI: https://doi.org/10.1021/jf00106a033

Butler, N.M., Baltes, N.J., Voytas, D.F., Douches, D.S., Schiemann, J.H. & Federal, J.K. (2016). Geminivirus-Mediated Genome Editing in Potato (Solanum tuberosum L .) Using Sequence-Specific Nucleases. vol. 7 (July), pp. 1–13. https://doi.org/10.3389/fpls.2016.01045

Camire, M.E., Kubow, S. & Donnelly, D.J. (2009). Potatoes and Human Health. *Critical Reviews in Food Science and Nutrition*, vol. 49 (10), pp. 823–840 Taylor & Francis. DOI: https://doi.org/10.1080/10408390903041996

Cárdenas, P.D., Sonawane, P.D., Heinig, U., Bocobza, S.E., Burdman, S. & Aharoni, A. (2015). The bitter side of the nightshades: Genomics drives discovery in Solanaceae steroidal alkaloid metabolism. *Phytochemistry*, vol. 113, pp. 24–32. DOI: 10.1016/j.phytochem.2014.12.010

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*, vol. 7. https://doi.org/10.1038/ncomms10654

Carmel-Goren, L., Liu, Y.S., Lifschitz, E. & Zamir, D. (2003). The self-pruning gene family in tomato. *Plant Molecular Biology*, vol. 52 (6), pp. 1215–1222. DOI: 10.1023/b:plan.0000004333.96451.11

Chen, K., Wang, Y., Zhang, R., Zhang, H. & Gao, C. (2019). CRISPR/Cas Genome Editing and Precision Plant Breeding in Agriculture. *Annual Review of Plant Biology*, vol. 70, pp. 667–697. DOI: 10.1146/annurev-arplant-050718-100049

Cheng, Y., Xiong, Y.L. & Chen, J. (2010). Antioxidant and emulsifying properties of potato protein hydrolysate in soybean oil-in-water emulsions. *Food Chemistry*, vol. 120 (1), pp. 101–108 Elsevier Ltd. DOI: https://doi.org/10.1016/j.foodchem.2009.09.077

Chowański, S., Adamski, Z., Marciniak, P., Rosiński, G., Büyükgüzel, E., Büyükgüzel, K., Falabella, P., Scrano, L., Ventrella, E., Lelario, F. & Bufo, S.A. (2016). A review of bioinsecticidal activity of Solanaceae alkaloids. *Toxins*, vol. 8 (3), pp. 1–28. DOI: 10.3390/toxins8030060

Clasen, B.M., Stoddard, T.J., Li, J., Mathis, L., Voytas, D.F., Coffman, A., Yabandith, A., Demorest, Z.L., Cedrone, F., Luo, S., Clasen, B.M., Tibebu, R., Retterath, A., Ray, E.E., Zhang, F., Haun, W., Baltes, N.J. & Davison, S. (2015). Improving cold storage and processing traits in potato through targeted gene knockout. *Plant Biotechnology Journal*, vol. 14 (1), pp. 169–176. DOI: 10.1111/pbi.12370

Clause, S.D. & Sasse, J.M. (1998). Brassinosteroids: Essential Regulators of Plant Growth and Development. *Annual Review of Plant Biology*, vol. 49, pp. 427–451. https://doi.org/10.1146/annurev.arplant.49.1.427

Cordell, G.A., Quinn-Beattie, M. Lou & Farnsworth, N.R. (2001). The potential of alkaloids in drug discovery. *Phytotherapy Research*, vol. 15 (3), pp. 183–205. DOI: 10.1002/ptr.890

Dahan-Meir, T., Filler-Hayut, S., Melamed-Bessudo, C., Bocobza, S., Czosnek, H., Aharoni, A. & Levy, A.A. (2018). Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. *Plant Journal*, vol. 95 (1), pp. 5–16. DOI: 10.1111/tpj.13932

Eriksson, D., Carlson-Nilsson, U., Ortíz, R. & Andreasson, E. (2016). Overview and Breeding Strategies of Table Potato Production in Sweden and the Fennoscandian Region. *Potato Research*, vol. 59 (3), pp. 279–294 Potato Research. DOI: https://doi.org/10.1007/s11540-016-9328-6

FAOSTAT (2023). *FAOSTAT Statistical Database*. Available at: https://www.fao.org/faostat/en/#data/QCL

Feng, K., Hou, X.-L., Xing, G.-M., Liu, J.-X., Duan, A.-Q., Xu, Z.-S., Li, M.-Y., Zhuang, J. & Xiong, A.-S. (2020). Advances in AP2/ERF super-family transcription factors in plant. *Critical Reviews in Biotechnology*, vol. 40 (6), pp. 750–776 Taylor & Francis. DOI: https://doi.org/10.1080/07388551.2020.1768509

Fewell, A.M. & Roddick, J.G. (1993). Interactive antifungal activity of the glycoalkaloids α-solanine and α-chaconine. *Phytochemistry*, vol. 33 (2), pp. 323–328. https://doi.org/10.1016/0031-9422(93)85511-O

Foolad, M.R. & Panthee R, D. (2012). Marker-Assisted Selection in Tomato Breeding. *Critical Reviews in Plant Sciences*, vol. 31 (2), pp. 93–123 Taylor & Francis. DOI: https://doi.org/10.1080/07352689.2011.616057

Friedman, M. (2002). Tomato glycoalkaloids: Role in the plant and in the diet. *Journal of Agricultural and Food Chemistry*, vol. 50 (21), pp. 5751–5780. DOI: 10.1021/jf020560c

Friedman, M. (2015). Chemistry and Anticarcinogenic Mechanisms of Glycoalkaloids Produced by Eggplants, Potatoes, and Tomatoes. *Journal of Agricultural and Food Chemistry*, vol. 63 (13), pp. 3323–3337. DOI: 10.1021/acs.jafc.5b00818

Friedman, M. & Dao, L. (1992). Distribution of Glycoalkaloids in Potato Plants and Commercial Potato Products. *Journal of Agricultural and Food Chemistry*, vol. 40 (3), pp. 419–423. DOI: https://doi.org/10.1021/jf00015a011

Friedman, M., Levin, C.E. & McDonald, G.M. (1994). α-Tomatine Determination in Tomatoes by HPLC Using Pulsed Amperometric Detection. *Journal of Agricultural and Food Chemistry*, vol. 42 (9), pp. 1959–1964.

https://doi.org/10.1021/jf00045a024
Friedman, M., McDonald, G.M. & Filadelfi-Keszi, P.M. (1997). Potato Glycoalkaloids: Chemistry, Analysis, Safety, and Plant Physiology. *Critical Reviews in Plant Sciences*, vol. 16 (1), pp. 55–132. https://doi.org/10.1080/07352689709701946
George, B., Kaur, C., Khurdiya, D.S. & Kapoor, H.C. (2004). Antioxidants in tomato (Lycopersium esculentum) as a function of genotype. *Food Chemistry*, vol. 84, pp. 45–51. DOI: https://doi.org/10.1016/S0308-8146(03)00165-1
Ghogare, R., Ludwig, Y., Bueno, G.M., Slamet-Loedin, I.H. & Dhingra, A. (2021). Genome editing reagent delivery in plants. *Transgenic Research*, vol. 30 (4), pp. 321–335 Springer International Publishing. DOI: https://doi.org/10.1007/s11248-021-00239-w
Ginzberg, I., Thippeswamy, M., Fogelman, E., Demirel, U., Mweetwa, A.M., Tokuhisa, J. & Veilleux, R.E. (2012). Induction of potato steroidal glycoalkaloid biosynthetic pathway by overexpression of cDNA encoding primary metabolism HMG-CoA reductase and squalene synthase. *Planta*, vol. 235 (6), pp. 1341–1353. DOI: https://doi.org/10.1007/s00425-011-1578-6
Ginzberg, I., Tokuhisa, J.G. & Veilleux, R.E. (2009). Potato steroidal glycoalkaloids: Biosynthesis and genetic manipulation. *Potato Research*, vol. 52 (1), pp. 1–15. DOI:10.1007/s11540-008-9103-4
Hartmann, M.-A. & Benveniste, P.B.T.-M. in E. (1987). [58] Plant membrane sterols: Isolation, identification, and biosynthesis. *Plant Cell Membranes*. Academic Press, pp. 632–650.
Hartmann, M.A. (1998). Plant sterols and the membrane environment. *Trends in Plant Science*, vol. 3 (5), pp. 170–175. https://doi.org/10.1016/S1360-1385(98)01233-3
Hussain, M., Qayum, A., Xiuxiu, Z., Liu, L., Hussain, K., Yue, P., Yue, S., Y.F Koko, M., Hussain, A. & Li, X. (2021). Potato protein: An emerging source of high quality and allergy free protein, and its possible future based products. *Food Research International*, vol. 148 (600), p. 110583 Elsevier Ltd. DOI: https://doi.org/10.1016/j.foodres.2021.110583
Iijima, Y., Watanabe, B., Sasaki, R., Takenaka, M., Ono, H., Sakurai, N., Umemoto, N., Suzuki, H., Shibata, D. & Aoki, K. (2013). Steroidal glycoalkaloid profiling and structures of glycoalkaloids in wild tomato fruit. *Phytochemistry*, vol. 95, pp. 145–157 Elsevier Ltd. DOI: https://doi.org/10.1016/j.phytochem.2013.07.016
Itkin, M., Heinig, U., Tzfadia, O. & Bhide, A. (2013). Biosynthesis of Antinutritional Alkaloids in Solanaceous Crops Is Mediated by Clustered Genes Biosynthesis of Antinutritional Alkaloids in Solanaceous Crops Is Mediated by Clustered Genes. *Nature*, (June), pp. 1–7. DOI: 10.1126/science.1240230
Ito, Y., Nishizawa-Yokoi, A., Endo, M., Mikami, M. & Toki, S. (2015). CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. *Biochemical and Biophysical Research Communications*, vol. 467 (1), pp. 76–82 Elsevier Ltd. DOI: https://doi.org/10.1016/j.bbrc.2015.09.117
Jenkins, J.A. (1948). The origin of the cultivated tomato. *Economic Botany*, vol. 2

(4), pp. 379–392. DOI: https://doi.org/10.1007/BF02859492
Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B. & Weeks, D.P. (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Research*, vol. 41 (20), pp. 1–12. DOI: 10.1093/nar/gkt780
Keukens, E.A.J., de Vrije, T., van den Boom, C., de Waard, P., Plasman, H.H., Thiel, F., Chupin, V., Jongen, W.M.F. & de Kruijff, B. (1995). Molecular basis of glycoalkaloid induced membrane disruption. *BBA - Biomembranes*, vol. 1240 (2), pp. 216–228. DOI: https://doi.org/10.1016/0005-2736(95)00186-7
Kieu, N.P., Lenman, M., Wang, E.S., Petersen, B.L. & Andreasson, E. (2021). Mutations introduced in susceptibility genes through CRISPR / Cas9 genome editing confer increased late blight resistance in potatoes. *Scientific Reports*, (0123456789), pp. 1–12 Nature Publishing Group UK. DOI: https://doi.org/10.1038/s41598-021-83972-w
Koleva, I.I., van Beek, T.A., Soffers, A.E.M.F., Dusemund, B. & Rietjens, I.M.C.M. (2012). Alkaloids in the human food chain - natural occurrence and possible adverse effects. *Molecular Nutrition and Food Research*, vol. 56 (1), pp. 30–52. DOI: 10.1002/mnfr.201100165
Koppelman, S.J., Van Koningsveld, G.A., Knulst, A.C., Gruppen, H., Pigmans, I.G.A.J. & De Jongh, H.H.J. (2002). Effect of heat-induced aggregation on the IgE binding of patatin (Sol t 1) is dominated by other potato proteins. *Journal of Agricultural and Food Chemistry*, vol. 50 (6), pp. 1562–1568. https://doi.org/10.1021/jf0106998
Kozukue, N., Yoon, K.S., Byun, G.I.N., Misoo, S., Levin, C.E. & Friedman, M. (2008). Distribution of glycoalkaloids in potato tubers of 59 accessions of two wild and five cultivated Solanum species. *Journal of Agricultural and Food Chemistry*, vol. 56 (24), pp. 11920–11928. DOI: https://doi.org/10.1021/jf802631t
Kwon, C.T., Heo, J., Lemmon, Z.H., Capua, Y., Hutton, S.F., Van Eck, J., Park, S.J. & Lippman, Z.B. (2020). Rapid customization of Solanaceae fruit crops for urban agriculture. *Nature Biotechnology*, vol. 38 (2), pp. 182–188 Springer US. https://doi.org/10.1038/s41587-019-0361-2
Li, H., Li, M., Fan, Y., Liu, Y. & Qin, S. (2022). Antifungal activity of potato glycoalkaloids and its potential to control severity of dry rot caused by Fusarium sulphureum. *Crop Science*, vol. 63 (2), pp. 801–811. DOI: 10.1002/csc2.20874
Li, J.F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M. & Sheen, J. (2013). Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nature Biotechnology*, vol. 31 (8), pp. 688–691. https://doi.org/10.1038/nbt.2654
Li, T., Yang, X., Yu, Y., Si, X., Zhai, X., Zhang, H., Dong, W., Gao, C. & Xu, C. (2018). Domestication of wild tomato is accelerated by genome editing. *Nature Biotechnology*, vol. 36 (12). https://doi.org/10.1038/nbt.4273
Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., Liu, J., Zhang, H., Liu, C., Ran, Y. & Gao, C. (2017). Efficient DNA-free genome editing of bread

wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nature Communications*, vol. 8 (January), pp. 1–5 Nature Publishing Group. DOI: https://doi.org/10.1038/ncomms14261

Liang, Z., Zhang, K., Chen, K. & Gao, C. (2014). Targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system. *Journal of Genetics and Genomics*, vol. 41 (2), pp. 63–68 Elsevier Limited and Science Press. DOI: https://doi.org/10.1016/j.jgg.2013.12.001

Lin, C., Hsu, C.-T., Yuan, Y.-H., Zheng, P.-X., Wu, F.-H., Cheng, Q.-W., Wu, Y.-L., Lin, S., Yue, J.-J., Cheng, Y.-H., Lin, S.-I., Shih, M.-C., Sheen, J., Lin, Y.-C. & Lin, C.-S. (2021). DNA-free CRISPR-Cas9 gene editing of tetraploid tomatoes using protoplast regeneration. Plant physiology, 188(4), 1917–1930. https://doi.org/10.1093/plphys/kiac022

Lowder, L.G., Zhang, D., Baltes, N.J., Paul, J.W., Tang, X., Zheng, X., Voytas, D.F., Hsieh, T.F., Zhang, Y. & Qi, Y. (2015). A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. *Plant Physiology*, vol. 169 (2), pp. 971–985. DOI: 10.1104/pp.15.00636

Maga, J.A. & Fitzpatrick, T.J. (1980). Potato glycoalkaloids. *C R C Critical Reviews in Food Science and Nutrition*, vol. 12 (4), pp. 371–405 Taylor & Francis. DOI: https://doi.org/10.1080/10408398009527281

Matsuura, H.N. & Fett-Neto, A.G. (2015). Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action. *Plant Toxins*, pp. 1–15. https://doi.org/10.1007/978-94-007-6728-7_2-1

McCue, K.F., Allen, P. V., Shepherd, L.V.T., Blake, A., Malendia Maccree, M., Rockhold, D.R., Novy, R.G., Stewart, D., Davies, H. V. & Belknap, W.R. (2007). Potato glycosterol rhamnosyltransferase, the terminal step in triose side-chain biosynthesis. *Phytochemistry*, vol. 68 (3), pp. 327–334. https://doi.org/10.1016/j.phytochem.2006.10.025

McCue, K.F., Allen, P. V., Shepherd, L.V.T., Blake, A., Whitworth, J., Maccree, M.M., Rockhold, D.R., Stewart, D., Davies, H. V. & Belknap, W.R. (2006). The primary in vivo steroidal alkaloid glucosyltransferase from potato. *Phytochemistry*, vol. 67 (15), pp. 1590–1597. DOI: 10.1016/j.phytochem.2005.09.037

McCue, K.F., Shepherd, L.V.T., Allen, P. V., MacCree, M.M., Rockhold, D.R., Corsini, D.L., Davies, H. V. & Belknap, W.R. (2005). Metabolic compensation of steroidal glycoalkaloid biosynthesis in transgenic potato tubers: Using reverse genetics to confirm the in vivo enzyme function of a steroidal alkaloid galactosyltransferase. *Plant Science*, vol. 168 (1), pp. 267–273. https://doi.org/10.1016/j.plantsci.2004.08.006

McGill, C.R., Kurilich, A.C. & Davignon, J. (2013). The role of potatoes and potato components in cardiometabolic health: A review. *Annals of Medicine*, vol. 45 (7), pp. 467–473. https://doi.org/10.3109/07853890.2013.813633

Miedzianka, J., Pęksa, A. & Aniołowska, M. (2012). Properties of acetylated potato protein preparations. *Food Chemistry*, vol. 133 (4), pp. 1283–1291. DOI: 10.1016/j.foodchem.2011.08.080

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*, vol. 59 (8), pp. 3454–3484. DOI: https://doi.org/10.1021/jf200439q

Moehs, C.P., Allen, P. V., Friedman, M. & Belknap, W.R. (1997). Cloning and expression of solanidine UDP-glucose glucosyltransferase from potato. *Plant Journal*, vol. 11 (2), pp. 227–236. DOI: 10.1046/j.1365-313x.1997.11020227.x

Moreau, R.A., Nyström, L., Whitaker, B.D., Winkler-Moser, J.K., Baer, D.J., Gebauer, S.K. & Hicks, K.B. (2018). Phytosterols and their derivatives: Structural diversity, distribution, metabolism, analysis, and health-promoting uses. *Progress in Lipid Research*, vol. 70 (April), pp. 35–61 Elsevier. DOI: https://doi.org/10.1016/j.plipres.2018.04.001

Morgan, A. & Cocking, E.C. (1982). Plant Regeneration from Protoplasts of Lycopersicon esculentum Mill. *Zeitschrift für Pflanzenphysiologie*, vol. 106 (2), pp. 97–104 Gustav Fischer Verlag, Stuttgart. DOI: https://doi.org/10.1016/s0044-328x(82)80071-8

Morikawa, T., Mizutani, M., Aoki, N., Watanabe, B., Saga, H., Saito, S., Oikawa, A., Suzuki, H., Sakurai, N., Shibata, D., Wadano, A., Sakata, K. & Ohta, D. (2006). Cytochrome P450 CYP710A encodes the sterol C-22 desaturase in Arabidopsis and tomato. *Plant Cell*, vol. 18 (4), pp. 1008–1022. DOI: 10.1105/tpc.105.036012

Morikawa, T., Saga, H., Hashizume, H. & Ohta, D. (2009). CYP710A genes encoding sterol C22-desaturase in Physcomitrella patens as molecular evidence for the evolutionary conservation of a sterol biosynthetic pathway in plants. *Planta*, vol. 229 (6), pp. 1311–1322. DOI: 10.1007/s00425-009-0916-4

Morris, S.C. & Lee, T.H. (1984). The toxicity and teratogenicity of Solanaceae glycoalkaloids, particularly those of the potato (Solanum tuberosum): a review. *Food Tech Australia*, vol. 36, pp. 118–124

Nahar, N., Westerberg, E., Arif, U., Huchelmann, A., Guasca, A.O., Beste, L., Dalman, K., Dutta, P.C., Jonsson, L. & Sitbon, F. (2017). Transcript profiling of two potato cultivars during glycoalkaloid-inducing treatments shows differential expression of genes in sterol and glycoalkaloid metabolism. *Scientific Reports*, vol. 7 (October 2016), pp. 1–13. https://doi.org/10.1038/srep43268

Nakayasu, M., Akiyama, R., Jae, H., Osakabe, K. & Osakabe, Y. (2018a). Plant Physiology and Biochemistry Generation of α -solanine-free hairy roots of potato by CRISPR / Cas9 mediated genome editing of the St16DOX gene ☆. *Plant Physiology and Biochemistry*, vol. 131 (February), pp. 70–77 Elsevier. DOI: 10.1016/j.plaphy.2018.04.026

Nakayasu, M., Shioya, N., Shikata, M., Thagun, C., Abdelkareem, A., Okabe, Y., Ariizumi, T., Arimura, G.I., Mizutani, M., Ezura, H., Hashimoto, T. & Shoji, T. (2018b). JRE4 is a master transcriptional regulator of defense-related steroidal glycoalkaloids in tomato. *Plant Journal*, vol. 94 (6), pp. 975–990. DOI: 10.1111/tpj.13911

Nakayasu, M., Umemoto, N., Akiyama, R., Ohyama, K., Lee, H.J., Miyachi, H., Watanabe, B., Muranaka, T., Saito, K., Sugimoto, Y. & Mizutani, M. (2021).

Characterization of C-26 aminotransferase, indispensable for steroidal glycoalkaloid biosynthesis. *Plant Journal*, vol. 108 (1), pp. 81–92. DOI: 10.1111/tpj.15426

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

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J.D.G. & Kamoun, S. (2013). Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nature Biotechnology*, vol. 31 (8), pp. 691–693. https://doi.org/10.1038/nbt.2655

Nicolia, A., Andersson, M., Hofvander, P., Festa, G. & Cardi, T. (2021a). Tomato protoplasts as cell target for ribonucleoprotein (RNP)- mediated multiplexed genome editing. *Plant Cell, Tissue and Organ Culture (PCTOC)*, vol. 144 (2), pp. 463–467 Springer Netherlands. DOI: https://doi.org/10.1007/s11240-020-01954-8

Nicolia, A., Fält, A.-S., Hofvander, P. & Andersson, M. (2021b). Protoplast-Based Method for Genome Editing in Tetraploid Potato. *Methods in Molecular Biology*. pp. 177–186. DOI: 10.1007/978-1-0716-1201-9_12

Nicolia, A., Proux-Wéra, E., Åhman, I., Onkokesung, N., Andersson, M., Andreasson, E. & Zhu, L.H. (2015). Targeted gene mutation in tetraploid potato through transient TALEN expression in protoplasts. *Journal of Biotechnology*, vol. 204, pp. 17–24 Elsevier B.V. DOI: https://doi.org/10.1016/j.jbiotec.2015.03.021

Niedz, R.P., Rutter, S.M., Handley, L.W. & Sink, K.C. (1985). Plant regeneration from leaf protoplasts of six tomato cultivars. *Plant Science*, vol. 39 (3), pp. 199–204. https://doi.org/10.1016/0168-9452(85)90175-X

Nomura, T., Yoshida, S. & Chua, N. (1998). The Arabidopsis DIMINUTO / DWARF1 Gene Encodes a Protein. vol. 10 (October), pp. 1677–1690. DOI: 10.1105/tpc.10.10.1677

Ohyama, K., Okawa, A., Moriuchi, Y. & Fujimoto, Y. (2013). Biosynthesis of steroidal alkaloids in Solanaceae plants: Involvement of an aldehyde intermediate during C-26 amination. *Phytochemistry*, vol. 89, pp. 26–31 Elsevier Ltd. DOI: https://doi.org/10.1016/j.phytochem.2013.01.010

Pan, C., Ye, L., Qin, L., Liu, X., He, Y., Wang, J., Chen, L. & Lu, G. (2016). CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. *Scientific Reports*, vol. 6 (April), pp. 1–10 Nature Publishing Group. https://doi.org/10.1038/srep24765

Panda, S., Jozwiak, A., Sonawane, P.D., Szymanski, J., Kazachkova, Y., Vainer, A., Vasuki Kilambi, H., Almekias-Siegl, E., Dikaya, V., Bocobza, S., Shohat, H., Meir, S., Wizler, G., Giri, A.P., Schuurink, R., Weiss, D., Yasuor, H., Kamble, A. & Aharoni, A. (2022). Steroidal alkaloids defence metabolism and plant growth are modulated by the joint action of gibberellin and jasmonate signalling. *New Phytologist*, vol. 233 (3), pp. 1220–1237. DOI: 10.1111/nph.17845

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*, vol. 30 (11), pp. 876–885. DOI: 10.1094/MPMI-02-17-0033-R

Petersson, E. V., Nahar, N., Dahlin, P., Broberg, A., Tröger, R., Dutta, P.C., Jonsson, L. & Sitbon, F. (2013). Conversion of exogenous cholesterol into glycoalkaloids in potato shoots, using two methods for sterol solubilisation. *PLoS ONE*, vol. 8 (12), pp. 1–11. https://doi.org/10.1371/journal.pone.0082955

Piironen, V., Lindsay, D.G., Miettinen, T.A., Toivo, J. & Lampi, A.M. (2000). Plant sterols: Biosynthesis, biological function and their importance to human nutrition. *Journal of the Science of Food and Agriculture*, vol. 80 (7), pp. 939–966. https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<939::AID-JSFA644>3.0.CO;2-C

Pillai, S.S. & Dandurand, L.M. (2021). Effect of steroidal glycoalkaloids on hatch and reproduction of the potato cyst nematode globodera pallida. *Plant Disease*, vol. 105 (10), pp. 2975–2980. https://doi.org/10.1094/PDIS-02-21-0247-RE

Pnueli, L., Carmel-Goren, L., Hareven, D., Gutfinger, T., Alvarez, J., Ganal, M., Zamir, D. & Lifschitz, E. (1998). The SELF-PRUNING gene of tomato regulates vegetative to reproductive switching of sympodial meristems and is the ortholog of CEN and TFL1. *Development*, vol. 125 (11), pp. 1979–1989. DOI: 10.1242/dev.125.11.1979

Purnhagen, K., Ambrogio, Y., Bartsch, D., Eriksson, D., Jorasch, P., Kahrmann, J., Kardung, M., Molitorisová, A., Monaco, A., Nanda, A.K., Romeis, J., Rostoks, N., Unkel, K. & Schneider, X.T. (2023). Options for regulating new genomic techniques for plants in the European Union. *Nature Plants*, vol. 9 (12), pp. 1958–1961. DOI: https://doi.org/10.1038/s41477-023-01570-2

Roddick, J.G. (1996). Steroidal glycoalkaloids: nature and consequences of bioactivity. *Saponins used in traditional and modern medicine*, pp. 277–295 Springer. Available at: https://doi.org/10.1007/978-1-4899-1367-8_25

Roddick, J.G., Rijnenberg, A.L. & Weissenberg, M. (1990). Membrane-disrupting properties of the steroidal glycoalkaloids solasonine and solamargine. *Phytochemistry*, vol. 29 (5), pp. 1513–1518. DOI: https://doi.org/https://doi.org/10.1016/0031-9422(90)80111-S

Sakata, Y., NISHIO, T. & TAKAYANAGI, K. (1987). Plant regeneration from mesophyll protoplasts of tomato cv. Ponderosa. *Journal of the Japanese Society for Horticultural Science*, vol. 56 (3), pp. 334–338. https://doi.org/10.2503/jjshs.56.334

Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K. & Yamaguchi-Shinozaki, K. (2002). DNA-Binding Specificity of the ERF/AP2 Domain of Arabidopsis DREBs, Transcription Factors Involved in Dehydration- and Cold-Inducible Gene Expression. *Biochemical and Biophysical Research Communications*, vol. 290 (3), pp. 998–1009. DOI: https://doi.org/https://doi.org/10.1006/bbrc.2001.6299

Sánchez-Mata, M.C., Yokoyama, W.E., Hong, Y.J. & Prohens, J. (2010). α-

Solasonine and α-solamargine contents of gboma (solanum macrocarpon l.) and scarlet (solanum aethiopicum l.) eggplants. *Journal of Agricultural and Food Chemistry*, vol. 58 (9), pp. 5502–5508. https://doi.org/10.1021/jf100709g

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. *Plant Cell*, vol. 26 (9), pp. 3763–3774. DOI: https://doi.org/10.1105/tpc.114.130096

Schaller, H. (2003). The role of sterols in plant growth and development. *Progress in Lipid Research*, vol. 42 (3), pp. 163–175. DOI: 10.1016/s0163-7827(02)00047-4

Schoenbeck, I., Graf, A.M., Leuthold, M., Pastor, A., Beutel, S. & Scheper, T. (2013). Purification of high value proteins from particle containing potato fruit juice via direct capture membrane adsorption chromatography. *Journal of Biotechnology*, vol. 168 (4), pp. 693–700 Elsevier B.V. DOI: https://doi.org/10.1016/j.jbiotec.2013.09.018

Schrenk, D., Bignami, M., Bodin, L., Chipman, J.K., del Mazo, J., Hogstrand, C., Hoogenboom, L., Leblanc, J. & Nebbia, C.S. (2020). Risk assessment of glycoalkaloids in feed and food, in particular in potatoes and potato-derived products. *EFSA Journal*, vol. 18 (8), p. e06222 Wiley Online Library.
https://doi.org/10.2903/j.efsa.2020.6222

Shakya, R. & Navarre, D.A. (2008). LC-MS analysis of solanidane glycoalkaloid diversity among tubers of four wild potato species and three cultivars (Solanum tuberosum). *Journal of Agricultural and Food Chemistry*, vol. 56 (16), pp. 6949–6958. DOI: https://doi.org/10.1021/jf8006618

Shimizu, K., Kushida, A., Akiyama, R., Lee, H.J., Okamura, Y., Masuda, Y., Sakata, I., Tanino, K., Matsukida, S., Inoue, T., Sugimoto, Y. & Mizutani, M. (2020). Hatching stimulation activity of steroidal glycoalkaloids toward the potato cyst nematode, globodera rostochiensis. *Plant Biotechnology*, vol. 37 (3), pp. 1–7. DOI: 10.5511/plantbiotechnology.20.0516a

Sidorov, V., Wang, D., Nagy, E.D., Armstrong, C., Beach, S., Zhang, Y., Groat, J., Yang, S., Yang, P. & Gilbertson, L. (2021). Heritable DNA-free genome editing of canola (Brassica napus L.) using PEG-mediated transfection of isolated protoplasts. *In Vitro Cellular and Developmental Biology - Plant*, (0123456789) Springer US. DOI: https://doi.org/10.1007/s11627-021-10236-7

Sinden, S.L., Sanford, L.L. & Osman, S.F. (1980). Glycoalkaloids and resistance to the Colorado potato beetle in Solanum chacoense Bitter. *American Potato Journal*, vol. 57 (7), pp. 331–343. DOI: https://doi.org/10.1007/BF02854028

Sinden, S.L., Sanford, L.L. & Webb, R.E. (1984). Genetic and environmental control of potato glycoalkaloids. *American Potato Journal*, vol. 61 (3), pp. 141–156. DOI: https://doi.org/10.1007/BF02854035

Sonawane, P.D., Jozwiak, A., Panda, S. & Aharoni, A. (2020). ScienceDirect ' Hijacking ' core metabolism : a new panache for the evolution of steroidal

glycoalkaloids structural diversity Prashant D Sonawane , Adam Jozwiak , Sayantan Panda and Asaph. *Current Opinion in Plant Biology*, vol. 55, pp. 118–128 Elsevier Ltd. DOI: 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., Giri, A.P., Goossens, A. & Aharoni, A. (2016). Plant cholesterol biosynthetic pathway overlaps with phytosterol metabolism. *Nature Plants*, vol. 3 (December) Nature Publishing Group. https://doi.org/10.1038/nplants.2016.205

Soyk, S., Müller, N.A., Park, S.J., Schmalenbach, I., Jiang, K., Hayama, R., Zhang, L., Van Eck, J., Jiménez-Gómez, J.M. & Lippman, Z.B. (2017). Variation in the flowering gene SELF PRUNING 5G promotes day-neutrality and early yield in tomato. *Nature Genetics*, vol. 49 (1), pp. 162–168. DOI: 10.1038/ng.3733

Stermer, B.A., Bianchini, G.M. & Korth, K.L. (1994). Regulation of HMG-CoA reductase activity in plants. *Journal of Lipid Research*, vol. 35 (7), pp. 1133–1140 Â© 1994 ASBMB. Currently published by Elsevier Inc; originally published by American Society for Biochemistry and Molecular Biology. DOI: https://doi.org/10.1016/s0022-2275(20)39958-2

Storey, M. (2007). The Harvested Crop. (Vreugdenhil, D., ed.) *Potato Biology and Biotechnology Advances and Perspectives, Elsevier, Oxford*, pp. 441–470

Svitashev, S., Schwartz, C., Lenderts, B., Young, J.K. & Mark Cigan, A. (2016). Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nature Communications*, vol. 7, pp. 1–7 Nature Publishing Group. DOI: https://doi.org/10.1038/ncomms13274

Swinnen, G., De Meyer, M., Pollier, J., Molina-Hidalgo, F.J., Ceulemans, E., Venegas-Molina, J., De Milde, L., Fernández-Calvo, P., Ron, M., Pauwels, L. & Goossens, A. (2022). The basic helix–loop–helix transcription factors MYC1 and MYC2 have a dual role in the regulation of constitutive and stress-inducible specialized metabolism in tomato. *New Phytologist*, vol. 236 (3), pp. 911–928. DOI: 10.1111/nph.18379

Tan, M.M.C., Rietveld, E.M., Marrewijk, V. & Al., G.A.M. et (1987). Regeneration of leaf mesophyll protoplasts of tomato cultivars (L. esculentum): factors important for efficient protoplast culture and plant regeneration. *Plant Cell Reports*, vol. 6 (67), pp. 172–175. DOI: 10.1007/BF00268470

Thagun, C., Imanishi, S., Kudo, T., Nakabayashi, R., Ohyama, K., Mori, T., Kawamoto, K., Nakamura, Y., Katayama, M., Nonaka, S., Matsukura, C., Yano, K., Ezura, H., Saito, K., Hashimoto, T. & Shoji, T. (2016). Jasmonate-Responsive ERF Transcription Factors Regulate Steroidal Glycoalkaloid Biosynthesis in Tomato. *Plant and Cell Physiology*, vol. 57 (5), pp. 961–975. DOI: 10.1093/pcp/pcw067

The Potato Genome Sequencing Consortium (2011). Genome sequence and analysis of the tuber crop potato. *Nature*, vol. 475, pp. 189–195. DOI: https://doi.org/10.1038/nature10158

Tingey, W.M. (1984). Glycoalkaloids as pest resistance factors. *American Potato*

Journal, (61), pp. 157–167. Available at: https://doi.org/10.1007/BF02854036
Tomato Genome Consortium (2012). The tomato genome sequence provides insights into fleshy fruit evolution. *Nature*, vol. 485 (7400), pp. 635–641 England. DOI: https://doi.org/10.1038/nature11119
Ueta, R., Abe, C., Watanabe, T., Sugano, S.S., Ishihara, R., Ezura, H., Osakabe, Y. & Osakabe, K. (2017). Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. *Scientific Reports*, vol. 7 (1), pp. 1–8 Springer US. DOI: https://doi.org/10.1038/s41598-017-00501-4
Umemoto, N., Nakayasu, M., Ohyama, K., Yotsu-Yamashita, M., Mizutani, M., Seki, H., Saito, K. & Muranaka, T. (2016). Two cytochrome P450 monooxygenases catalyze early hydroxylation steps in the potato steroid glycoalkaloid biosynthetic pathway. *Plant Physiology*, vol. 171 (4), pp. 2458–2467. https://doi.org/10.1104/pp.16.00137
Upadhyay, S.K., Kumar, J., Alok, A. & Tuli, R. (2013). RNA-Guided genome editing for target gene mutations in wheat. *G3: Genes, Genomes, Genetics*, vol. 3 (12), pp. 2233–2238
Valitova, J.N., Sulkarnayeva, A.G. & Minibayeva, F. V. (2016). Plant sterols: Diversity, biosynthesis, and physiological functions. *Biochemistry (Moscow)*, vol. 81 (8), pp. 819–834. DOI: 10.1134/S0006297916080046
Vicente, M.H., Zsögön, A., de Sá, A.F.L., Ribeiro, R. V. & Peres, L.E.P. (2015). Semi-determinate growth habit adjusts the vegetative-to-reproductive balance and increases productivity and water-use efficiency in tomato (Solanum lycopersicum). *Journal of Plant Physiology*, vol. 177, pp. 11–19 Elsevier GmbH. DOI: https://doi.org/10.1016/j.jplph.2015.01.003
Waglay, A., Karboune, S. & Alli, I. (2014). Potato protein isolates : Recovery and characterization of their properties. *Food Chemistry*, vol. 142, pp. 373–382 Elsevier Ltd. DOI: https://doi.org/10.1016/j.foodchem.2013.07.060
Wang, S., Zhang, S., Wang, W., Xiong, X., Meng, F. & Cui, X. (2015). Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. *Plant Cell Reports*, vol. 34 (9), pp. 1473–1476 Springer Berlin Heidelberg. DOI: 10.1007/s00299-015-1816-7
Woo, J.W., Kim, J., Kwon, S. Il, Corvalán, C., Cho, S.W., Kim, H., Kim, S., Kim, S., Choe, S. & Kim, J. (2015). DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. vol. 33 (11), pp. 1162–1165. DOI: 10.1038/nbt.3389
Yasumoto, S., Sawai, S., Lee, H.J., Mizutani, M., Saito, K., Umemoto, N. & Muranaka, T. (2020). Targeted genome editing in tetraploid potato through transient talen expression by agrobacterium infection. *Plant Biotechnology*, vol. 37 (2), pp. 205–211. doi: 10.5511/plantbiotechnology.20.0525a
Yasumoto, S., Umemoto, N., Lee, H.J., Nakayasu, M., Sawai, S., Sakuma, T., Yamamoto, T., Mizutani, M., Saito, K. & Muranaka, T. (2019). Efficient genome engineering using platinum talein potato. *Plant Biotechnology*, vol. 36 (3), pp. 167–173. DOI: 10.5511/plantbiotechnology.19.0805a
Zhang, Z., Mao, Y., Ha, S., Liu, W., Botella, J.R. & Zhu, J.K. (2016). A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in Arabidopsis. *Plant Cell Reports*, vol. 35 (7), pp. 1519–1533. DOI:

10.1007/s00299-015-1900-z
Zhao, D.K., Zhao, Y., Chen, S.Y. & Kennelly, E.J. (2021). Solanum steroidal glycoalkaloids: Structural diversity, biological activities, and biosynthesis. *Natural Product Reports*, vol. 38 (8), pp. 1423–1444 Royal Society of Chemistry. https://doi.org/10.1039/d1np00001b
Zheng, Z., Ye, G., Zhou, Y., Pu, X., Su, W. & Wang, J. (2021). Editing sterol side chain reductase 2 gene (StSSR2) via CRISPR/Cas9 reduces the total steroidal glycoalkaloids in potato. *All Life*, vol. 14 (1), pp. 401–413. DOI: https://doi.org/10.1080/26895293.2021.1925358
Zhou, H., Liu, B., Weeks, D.P., Spalding, M.H. & Yang, B. (2014). Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Research*, vol. 42 (17), pp. 10903–10914. DOI: 10.1093/nar/gku806
Zwijnenberg, H.J., Kemperman, A.J.B., Boerrigter, M.E., Lotzb, M., Dijksterhuisb, J.F., Paulsen, P.E. & Koops, G. (2002). Native protein recovery from potato fruit juice by ultrafiltration. *Desalination*, vol. 144, pp. 331–334. Available at: https://doi.org/10.1016/S0011-9164(02)00338-7

Popular science summary

Have you ever wondered if those sprouted potatoes in your kitchen are safe to eat? It seems that this might not be the case. Sprouted potatoes can accumulate glycoalkaloids, natural toxins found in the Solanaceae family, i.e. the potato and tomato. Consuming too much glycoalkaloids can lead to unpleasant symptoms, ranging from stomach upset to more severe issues like headaches and confusion.

While glycoalkaloid content in table potato cultivars has received high attention due to the consideration of food safety, glycoalkaloids in starch potato cultivars have received less investigation. The total production of starch potatoes is estimated at 336, 200 tonnes in the year 2020 in Sweden, showing an increasing trend of total production over the last five years. However, the processing of starch potatoes generates substantial by-products, such as potato fruit juice and pulp, often relegated to low-value animal feed or waste. The challenge of turning the by-products into economically valuable compounds, e.g. food-grade proteins and fibre, is in removing the accumulated glycoalkaloids in the by-products while extracting proteins and fibre, which is complex and costly.

In this thesis, we investigated the differences in glycoalkaloid metabolism in starch potato cultivars compared to table potato cultivars, which are commonly used cultivars in Sweden. We also obtained glycoalkaloid-free starch potatoes, using the modern breeding tool CRISPR/Cas9. These glycoalkaloid-free starch potatoes offer a novel approach for the production of food-grade potato protein and fibre products from industrial by-products. Beyond ensuring a safer potato consumption experience, this innovation transforms waste into valuable resources, aligning with the global shift towards sustainability and environmentally conscious practices.

Populärvetenskaplig sammanfattning

Har du någonsin undrat om den där grodda potatisen i ditt kök är säker att äta? Det verkar som att så kanske inte är fallet. Grodd potatis kan ackumulera glykoalkaloider, naturliga gifter som finns i familjen Solanaceae, där även tomat ingår. Att konsumera för mycket glykoalkaloider kan leda till obehagliga symtom som spänner från magbesvär till mer allvarliga problem som huvudvärk och förvirring.

Medan glykoalkaloidhalten i matpotatissorter har fått stor uppmärksamhet på grund av hänsyn till livsmedelssäkerhet, har glykoalkaloider i stärkelsepotatissorter undersökts mindre. Den totala produktionen av stärkelsepotatis, i Sverige, uppskattas till 336 200 ton år 2020 med en ökande trend av den totala produktionen de senaste fem åren. Men bearbetningen av stärkelsepotatis genererar betydande biprodukter, såsom potatisfruktjuice och fruktkött, ofta förpassat till djurfoder eller avfall av lågt värde. Utmaningen för att omvandla biprodukterna till ekonomiskt värdefulla produkter, t.ex. livsmedelsklassade proteiner och fibre, är att ta bort de ackumulerade glykoalkaloiderna i biprodukterna samtidigt som man extraherar proteiner och fibre, vilket är komplext och kostsamt.

I denna avhandling undersökte jag skillnaden mellan glykoalkaloidmetabolism hos vanligt förekommande potatissorter i Sverige som matpotatissorter, jämfört med sorter som är odlade för stärkelse. Jag erhöll också glykoalkaloidfri stärkelsepotatis med det moderna växtförädlings verktyget CRISPR/Cas9. Dessa glykoalkaloidfria stärkelsepotatisar kommer att erbjuda ett nytt tillvägagångssätt för produktion av livsmedelsklassat potatisprotein och fiberprodukter från industriella biprodukter. Förutom att säkerställa en säkrare potatiskonsumtion, förvandlar denna innovation sidoströmmar till värdefulla resurser, i linje med den globala förändringen mot hållbarhet och miljömedvetna metoder.

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IV

Plant Cell Reports
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ORIGINAL ARTICLE

Establishment of a DNA-free genome editing and protoplast regeneration method in cultivated tomato (*Solanum lycopersicum*)

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Abstract

Key message **We have established a DNA-free genome editing method via ribonucleoprotein-based CRISPR/Cas9 in cultivated tomato and obtained mutant plants regenerated from transfected protoplasts with a high mutation rate.**

Abstract The application of genome editing as a research and breeding method has provided many possibilities to improve traits in many crops in recent years. In cultivated tomato (*Solanum lycopersicum*), so far only stable *Agrobacterium*-mediated transformation carrying CRISPR/Cas9 reagents has been established. Shoot regeneration from transfected protoplasts is the major bottleneck in the application of DNA-free genome editing via ribonucleoprotein-based CRISPR/Cas9 method in cultivated tomato. In this study, we report the implementation of a transgene-free breeding method for cultivated tomato by CRISPR/Cas9 technology, including the optimization of protoplast isolation and overcoming the obstacle in shoot regeneration from transfected protoplasts. We have identified that the shoot regeneration medium containing 0.1 mg/L IAA and 0.75 mg/L zeatin was the best hormone combination with a regeneration rate of up to 21.3%. We have successfully obtained regenerated plants with a high mutation rate four months after protoplast isolation and transfection. Out of 110 regenerated M_0 plants obtained, 35 (31.8%) were mutated targeting both *SP* and *SP5G* genes simultaneously and the editing efficiency was up to 60% in at least one allele in either *SP* or *SP5G* genes.

Keywords *Solanum lycopersicum* · Mesophyll protoplast regeneration · CRISPR/Cas9 · Ribonucleoprotein · *SP* and *SP5G* genes

Introduction

Variations of CRISPR/Cas9 technology have been applied for genome editing in recent years (Gao 2021). This technology has surpassed the other genome editing tools, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), as it is more easily and cheaply customized and yields high mutation efficiency in some species (Lowder et al. 2015). So far, successful applications using CRISPR/Cas9 have been reported in model plants, such as *Arabidopsis thaliana* (Li et al. 2013; Yan et al. 2015) and *Nicotiana benthamiana* (Li et al. 2013; Nekrasov et al. 2013), and many commercial crops, such as potato (Wang et al. 2015; Andersson et al. 2017), wheat (Upadhyay et al. 2013; Zhang et al. 2016), rice (Jiang et al. 2013; Zhou et al. 2014), maize (Liang et al. 2014; Char et al. 2016), tomato (Brooks et al. 2014; Ito et al. 2015) and many others (Gao 2021).

In general, CRISPR/Cas9 reagents, usually as DNA plasmids, can be delivered to cell-wall-free protoplasts by polyethylene glycol (PEG) transformation, or to plant tissues by stable *Agrobacterium*-mediated transformation, or by other means, such as particle bombardment and electroporation (Chen et al. 2019). To avoid foreign DNA integrated into plant cells, Woo et al. (2015) were the first to develop the

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delivery of Ribonucleoprotein (RNP) complexes into plant protoplasts using in vitro preassembled complexes of purified Cas9 protein and guide RNA (gRNA) in *Arabidopsis*, tobacco, lettuce and rice. Subsequently, studies on the application of RNPs for genome editing in plant species have been reported in crop plants, such as maize (Svitashev et al. 2016), wheat (Liang et al. 2017), potato (Andersson et al. 2018) and canola (Sidorov et al. 2021).

Tomato (*Solanum lycopersicum*) is an important commercial agricultural crop which is extensively cultivated all over the world as well as being a model plant used in scientific research due to its simple diploid genetics (2n = 2x = 24) and short life cycle (Ito et al. 2015). It has been demonstrated that the CRISPR/Cas9 technology can be used to generate mutated tomato plants for crop improvement such as improved disease resistance (Ito et al. 2015; Pan et al. 2016). Hitherto, most reports about CRISPR/Cas9 applied to tomato were based on stable *Agrobacterium tumefaciens*-mediated transformation and usually the homozygous mutation rate was low and segregation in the next generation needed to eliminate foreign DNA integrated into the plant genome. An attractive alternative technology would be the use of RNPs to deliver the CRISPR/Cas9 reagents into protoplasts, resulting in transgene-free plants. Although a high editing efficiency has been reported on tomato calli from transfected protoplasts of cultivated tomato, the shoot regeneration from RNP-transfected protoplasts is a bottleneck (Nicolia et al. 2021a). Very recently, (Lin et al. 2022) reported the successful protoplast regeneration of wild tomato (*Solanum peruvianum*) harboring CRISRP/Cas9 mutations, with a mutation rate varying from 8.3% to 63.6%. However, for cultivated tomato, there are only a few old reports published on plant regeneration from unedited protoplasts (Morgan and Cocking 1982; Sakata et al. 1987; Tan et al. 1987). Thus, establishing a protocol with high editing efficiency and regeneration rate in cultivated tomato would be beneficial regarding genetic studies as well as for breeding purposes. From this aspect, such a protocol could also be further adapted to wild relatives of the tomato, that represent a precious source of variability (e.g., *Solanum pennellii*, *Solanum pimpinellifolium*), to speed up programs of "de novo" domestication and/or introgression (Li et al. 2018).

The vegetative-to-reproductive phases in tomato are altered in the sympodial shoots and the switch between those two phases is controlled by the flowering repressor gene *SELF PRUNING* (*SP*). Genetic variation and mutations in this gene yields tomato genotypes that are classified into two categories: 'determinate' and 'indeterminate' varieties due to different growth habits (Pnueli et al. 1998; Carmel-Goren et al. 2003). Another flowering repressor *SELF PRUNING 5G* (*SP5G*), which is a paralog of the *SP* gene, is mainly responsible for flower repression in primary and canonical axillary shoots (Soyk et al. 2017). Tomato plants with mutation in either the *SP* gene or both the *SP* and *SP5G* genes showed the determinate phenotype, which resulted in acceleration of flowering, short internodes, bushy appearance and rapid life cycling (Soyk et al. 2017; Kwon et al. 2020). Those mutated plants would be suitable for urban vertical farming and greenhouse cultivation since the agricultural productivity can be increased due to their fast growth habit and compact size, especially in a confined environment. It is also beneficial for open field cultivation in that they grow as small bushes that need less attention compared to indeterminate varieties needing support. Besides, all fruits from determinate cultivars usually ripen in a short period from simultaneous flowering, which is beneficial for facilitating mechanical harvest.

In this study, we have successfully regenerated plants from cultivated tomato transfected protoplasts within four months after transfection. Furthermore, the regenerated plants have a high editing rate when targeting both *SP* and *SP5G* genes simultaneously. Hence, we have improved the process of tomato protoplast isolation and solved the challenge of shoot regeneration from RNP-transfected protoplasts using CRISPR/Cas9 technology.

Materials and methods

Plant material and in vitro culture conditions

Seeds of tomato (*S. lycopersicum*) cultivars (cvs) Red Setter, Ailsa Craig, M82 and Moneymaker were used in this study. Seeds were surface sterilized by washing with 70% ethanol for 5 min, followed by 15% (w/v) calcium hypochlorite ($CaCl_2O_2$) for 3 min and then rinsed 5 times with sterile distilled water. Sterilized seeds were placed in Plante Containers (Sakata Ornamentals Europe A/S, Denmark) with germination medium containing 0.2 mg/L Indole Acetic acid (IAA), 15 g/L sucrose, 8 g/L phyto agar, and half-strength Murashige & Skoog (MS) with vitamins (Duchefa Biochemie M0222, Haarlem, Netherlands) (2.2 g/L) with additional 0.2 mg/L Thiamine and 50 mg/L Myo-Inositol at pH 5.9.

In vitro culture mentioned in this study was carried out in a controlled chamber at a temperature of 24 °C/18 °C (light/dark), under a photoperiod of 16 h at 120–140 µE m^{-2} s^{-1} light and 8 h dark.

Protoplast isolation, transfection and callus induction in liquid medium

Protoplast isolation, transfection and early callus induction in liquid medium were done as previously described by Nicolia et al. (2021b) with some modifications to improve the yield of isolated protoplasts and regeneration. The components of Medium C, E, F, wash solution, PEG solution,

alginate solution and transient expression solution mentioned below can be found in Nicolia et al. (2021b).

In brief, the modifications in protoplast isolation were as follows: preconditioning treatment of in vitro cultured seedlings prior to protoplast isolation was done by placing the Plante Containers in a fridge (4 °C) in darkness one day before isolation. Cotyledons and first true leaves at different ages (14, 17 and 21 d) were used for protoplast isolation. The leaf tissues were sliced and treated with enzyme solution (medium C) at different temperatures (15 and 25 °C) and different time durations of enzyme digestion (14 and 16 h). Protoplasts were collected after centrifugation and the protoplast yield was quantified immediately after isolation by a hemocytometer (FuchsRosenthal 0.2 mm chamber, Horsham, UK) under microscope. The optimization of protoplast isolation was carried out with two cvs (Red Setter and Ailsa Craig) and optimized conditions were confirmed in all four cvs.

Freshly isolated protoplasts were transfected via PEG mediated delivery of RNPs. For each transfection, two different RNP complexes were assembled by mixing two 0.1 nmol synthetic sgRNAs (Synthego) with 10 µg TrueCut™ Cas9 v.2 (Thermo Fisher, Waltham, USA) in a 15 ml tube at room temperature for 15 min. The sgRNA was used as one synthetically produced component including the 20 bp target and an 80-mer SpCas9 scaffold from the suppliers' standard products. Then, 100 µl of protoplast suspension (1.0×10^6 protoplasts/ml) was added to the same tube and gently mixed before and after adding 120 µl 25% (w/v) PEG solution. The transfection was stopped after 3 min by 5 ml wash solution. Two control experiments, one with and one without PEG solution were also conducted. For estimation of transfection efficiency, protoplasts were transfected by replacing RNP with 20 µg plasmid vector expressing Green Fluorescent Protein (GFP) (pCW498-35S-GFiP-OcsT) and incubated with transient expression solution at room temperature in darkness. After 24 h, the expression of GFP signal was detected under a confocal microscope (Zeiss LSM 880 Airyscan confocal laser scanning microscope, Oberkochen, Germany).

After transfection, protoplasts were embedded in alginate and incubated in Medium E at 25 °C in darkness for 5 d where after the light was gradually increased by replacing the aluminum foil with a white paper sheet under the light intensity at ca. 10 µE m^{-2} s^{-1}. After two weeks, Medium E was replaced by Medium F and calli were exposed to full light with fresh Medium F changed every week.

Shoot and root regeneration on solid medium

After two weeks of incubation in Medium F, calli of 1–3 mm in size were released from the alginate using forceps and transferred directly to solid media for further shoot regeneration. Different solid media were designed to test their respective potential for tomato protoplast shoot regeneration. The composition of each medium is listed in Supp. Table 1. Solid shoot media were renewed every two weeks until shoots were regenerated. The number of regenerated shoots was evaluated continuously on different regeneration media until six months after protoplast isolation.

Individual regenerated shoots were excised from calli and moved to root regeneration medium containing 4.405 g/L MS medium with vitamins, 30 g/L sucrose and 6 g/L phyto agar at pH 5.8 in Plante Containers. Regenerated plants with roots were moved to soil for subsequent seed production and phenotypic observation.

Identification of *SP* and *SP5G* genes and sgRNA design

Genomic DNA was extracted from tomato leaf tissue of the four tomato cultivars using the GeneJet Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, Waltham USA) for amplification of the target regions in *SP* and *SP5G* genes. For each gene, two pairs of primers were designed based on the sequence of Solyc06g074350 (*SP*) and Solyc05g053850 (*SP5G*) (https://solgenomics.net/). Amplification of the target regions was conducted in a total reaction of 10 µl containing 5X Phusion HF buffer, 0.2 mM dNTPS, 0.15 µM primers, 0.02 U/µl Phusion DNA polymerase and 1 µl of extracted gDNA. PCR was conducted as follows: 98 ℃ for 1 min, 35 cycles of 98 °C 10 s, 59 °C 15 s, 72 °C 15 s and a final extension of 72 °C for 10 min. PCR products were cloned using the CloneJET PCR cloning Kit (Thermo Fisher Scientific, Waltham USA) and six random clones from each amplicon were selected for Sanger Sequencing (Eurofins). sgRNAs were designed to target all alleles in the four tomato cvs. according to the Sanger Sequencing using CRISPR RGEN Cas-Designer (Bae et al. 2014) and CRISPOR (Concordet and Haeussler 2018). All primers and sgRNAs used in this study are summarized in Supp. Table 2.

Genotyping of *SP* and *SP5G* mutants

Initial screening of mutations was performed with High Resolution Fragment Analysis (HRFA) according to Andersson et al. (2017). Genomic DNA was extracted from single young leaf tissue from each in vitro regenerated plant using GeneJet Plant Genomic DNA Purification Mini Kit. Multiplexing PCR was applied to amplify the regions covering target sites of both *SP* and *SP5G* genes simultaneously with forward primers labeled with FAM and HEX fluorescent dye (Thermo Fisher Scientific, Waltham USA), respectively. Labeled amplicons were analyzed in a 3500 Genetic Analyzer (Applied Biosystems) and the size of fragments

was determined with GeneMarker Software (SoftGenetics, Pennsylvania, USA) compared with the size of wild type amplicons. Sanger Sequencing was conducted for further characterization of mutations using unlabeled primers.

Statistical analysis

To evaluate the effect of different media on shoot regeneration rate, the number of shoots on each medium was recorded until six months after protoplast isolation. On the representative media (Medium TRS-a, b and c), the mean regeneration rate of each treatment was calculated with three replicates on individual culture dishes, containing ca. 40–60 calli per replicate. For other shoot regeneration media, the regeneration rate of each treatment was calculated based on one replicate containing 50 calli. Data were analyzed with one-way ANOVA and Tukey's test using software IBM SPSS Statistics version 27.

Results

Improvement on protoplast isolation

To improve yield, viability and regenerability of isolated protoplasts, the process was optimized in this study based on a previously published method (Nicolia et al. 2021a). The key steps of protoplast isolation are shown in Fig. 1a–d. The yield of protoplasts with initial isolation conditions (14 d seedlings, enzyme treatment: 15 °C for 17 h) from the four cultivars is shown in Fig. 2 and the optimization efforts were made on two cvs, Red Setter and Ailsa Craig, using variables, such as different seedling age, enzyme digestion temperature and duration, and preconditioning treatment.

We found that the yield of extracted protoplasts was improved using older seedlings, higher enzyme treatment temperature and longer incubation time. The number of isolated protoplasts from seedlings at the age of 21 d with the enzyme digestion at 25 °C for 16 h was 15–25 times higher than when using 14-d-old seedlings with the enzyme treatment at 15 °C for 17 h in all four tested cultivars, where a thick dark green band was formed after purification using sucrose density gradient centrifugation (Fig. 1c). An extra pretreatment step of in vitro seedlings (cv. Red Setter) before protoplast isolation did not increase the yield of protoplasts, as shown in Fig. 2.

To confirm our findings for optimized protoplast isolation, we tested two additional tomato cvs, M82 and Moneymaker, under the optimized protoplast isolation conditions. The results illustrated that the yield of protoplasts obtained from cvs M82 and Moneymaker was also improved using the new protoplast isolation conditions.

Cell division and callus formation from RNP-transfected protoplasts (week 1–4)

Freshly isolated protoplasts (Fig. 1d) were used for PEG transfection with RNP complexes or a vector harboring GFP. Expression of GFP was observed under microscope after 24 h incubation at room temperature and the estimated transfection efficiency was 30–50% (Supp. Figure 1a). RNP-transfected protoplasts were embedded in alginate and incubated at 25 °C. After 4–5-d incubation in the dark, initial cell division was observed under microscope (Fig. 1e). Light was gradually increased, and the mini-calli were usually visible to the naked eye 2 weeks after transfection (Fig. 1f).

Shoot regeneration on different solid media (week 4–10) and root formation (week 9–12)

To find the optimal solid tomato shoot regeneration (TSR) medium, different media compositions were assessed. Calli were released from alginate when the size reached 1–3 mm (usually 5 weeks after transfection) and moved to the various solid media for assessment of shoot regeneration. Media were designed to study the effect of different combinations or concentrations of plant hormones, different gelling agents and different carbon sources on shoot regeneration. The results of shoot regeneration rate from non-treated and treated protoplasts of the cv. Red Setter on three different shoot regeneration media, are summarized in Table 1 (for results with all tested media see Supp. Table 3). Shoot primordia were observed 2–12 weeks after moving to different shoot regeneration media. The highest shoot regeneration rate (from RNP-transfected protoplasts) was on Medium TSR-a (31.4%) and Medium TSR-b (21.3%), without significant difference. While there was no significant difference among the three treatments ($p = 0.844$), differences for the various media ($p = 0.007$) and the interaction between treatments and media were both significant ($p < 0.001$).

When the shoots reached a length of 1–2 cm and at least two leaves had developed, they were excised from the calli (the calli was discarded after picking one shoot) and transferred to root regeneration medium. Usually well-developed roots were formed within two weeks.

Interestingly, there was morphological difference among shoots regenerated on different shoot regeneration media using cultivar Red Setter (Supp. Figure 1), which had an effect on root formation. For example, the shoots from Medium TSR-b (Fig. 1g) were green and healthy without any evident defect and usually produced well and fast developed roots (Fig. 1h) with normal rooting and acclimation in pots (Fig. 1i). On the other hand, the shoots regenerated from Medium TSR-a were curved, less green, with a grass-like shape (Supp. Figure 1b). When moved to root regeneration medium, roots developed more

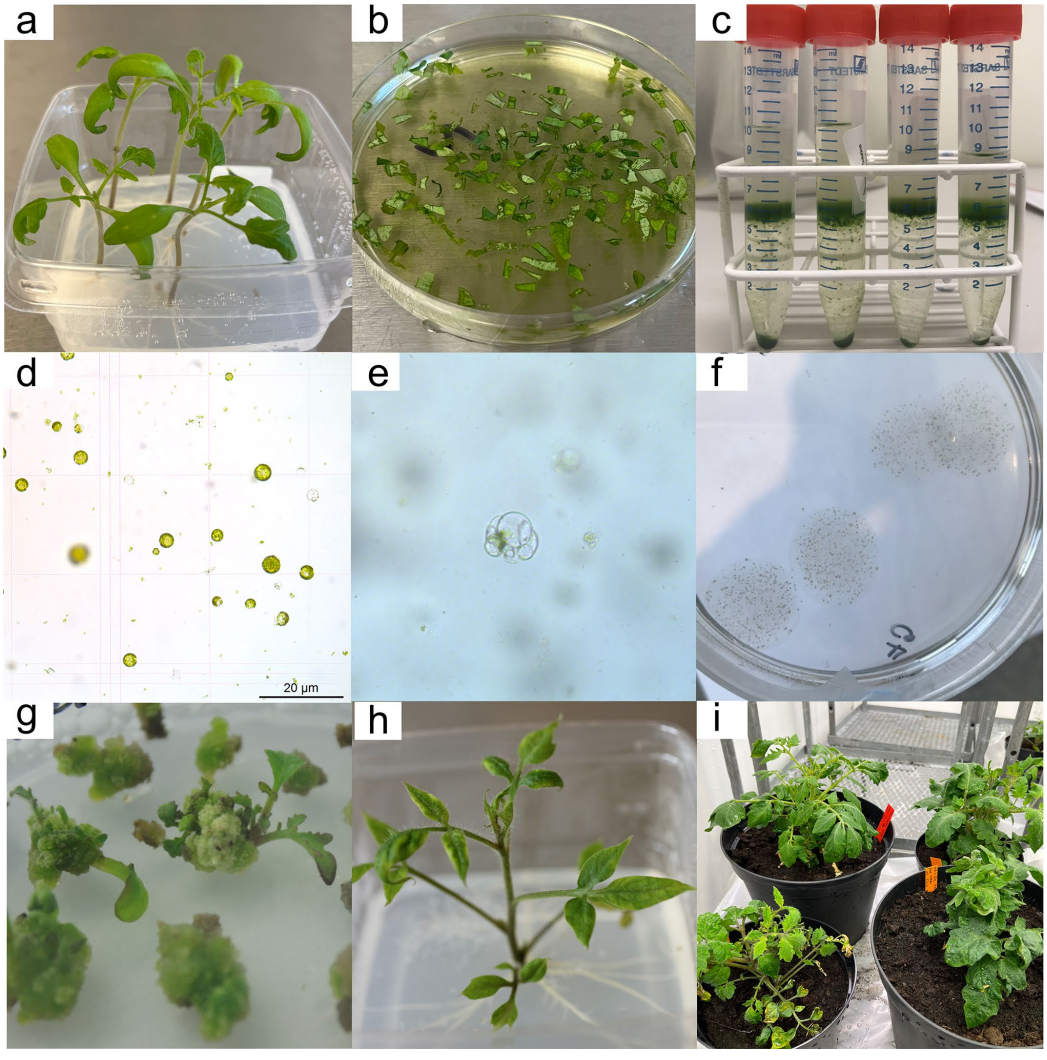

Fig. 1 Protoplast isolation and regeneration from tomato (*S. lycopersicum*) cv. Red Setter. **a** Cotyledons and first true leaves from 21-d-old in vitro seedlings used for protoplast isolation. **b** Sliced cotyledons and first true leaves incubated in enzyme solution after 16 h under 25 °C before protoplast purification. **c** Dark green bands containing released intact protoplasts appeared at the interface of sucrose solution and wash solution after centrifugation. **d** Freshly isolated green protoplasts under microscope. **e** Cell division 5 d after protoplast isolation. **f** Callus formation derived from protoplasts embedded in alginate after 12 d from protoplast isolation. **g** Calli released from alginate and cultured on solid shoot regeneration Medium TSR-b with first regenerated shoots observed three months after protoplast isolation. **h** A regenerated plant with well-developed roots on root regeneration medium three months after protoplast isolation. **i** Regenerated plants moved to soil in biotron four months after protoplast isolation

slowly and shoots even failed to survive. Contrasting with this, the shoots on Medium TSR-c (Supp. Figure 1c) were thicker, and faced the same rooting issue as using Medium TSR-a.

SP and *SP5G* allele sequencing and sgRNA design

For the determination of *SP* and *SP5G* allele gene sequences, we designed two different primer pairs for

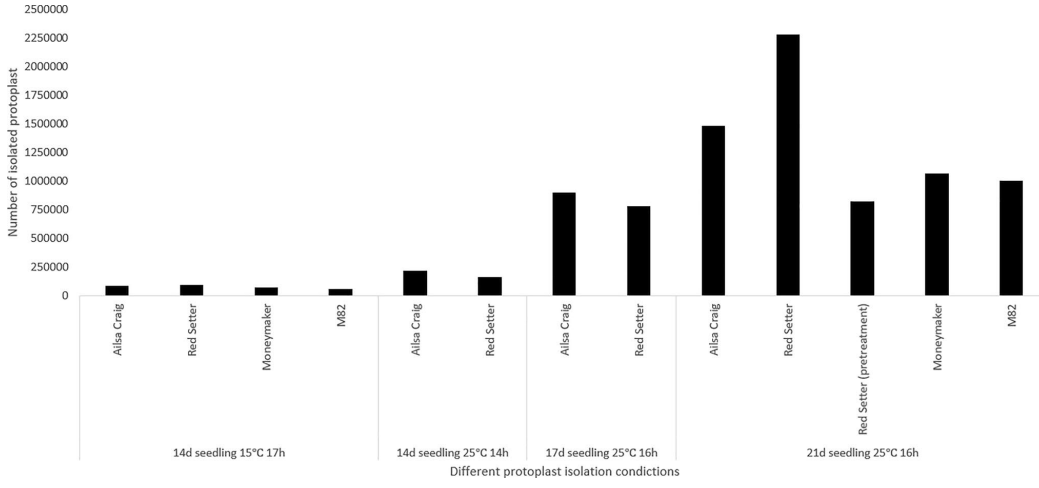

Fig. 2 Comparison of effects of different protoplast isolation conditions on the protoplast yield from four different tomato cultivars (Red Setter, Ailsa Craig, M82 and Moneymaker). The number of isolated protoplasts was calculated from the extraction and sampling of 1 g seedlings (results are normalized)

Table 1 Shoot regeneration rate[1] (%) on three media, TSR a-c (cv. Red Setter)

Treatment	Regeneration rate (%) on TSR Media[2]		
	TSR-a	TSR-b	TSR-c
Protoplasts + PEG + RNPs	31.4 a	21.3ab	18.4b
Protoplasts + PEG	19.3a	30.3a	25.4a
Protoplasts	8.1b	44.6a	24.5ab

[1]The calculation of regeneration rate is described in detail in material and methods. Values in a row followed by the same letters were not statistically different at $p = 0.05$ ($n = 3$)

[2]TSR Medium a, b and c are different media for shoot regeneration and the components of each medium are shown in Supp. Table 3

PCR amplification covering the exon 1 region of all four used cultivars (Fig. 3a). Sequence results showed that in the amplified region, they were identical to the public tomato reference genomic sequences, except for the *SP* gene, where one single-nucleotide polymorphism (SNP) was identified among the four cultivars (Supp. Figure 2).

One sgRNA (sgRNA1) for *SP* and two sgRNAs for *SP5G* (sgRNA2 and sgRNA3) were designed (Fig. 3a) and used for multiplexing of the targets in two different combinations, sgRNA1 + 2 and sgRNA1 + 3.

Identification of mutants after multiplexed targeting of *SP* and *SP5G*

In total we analyzed 110 regenerated shoots (events, M_0 plants) by HRFA analysis (for HRFA results on all mutants see Supp. Table 4), where *SP* and *SP5G* genes were targeted simultaneously by either sgRNA1 + 2 or sgRNA1 + 3. Among all 110 events, 66 (60.0%) were identified with mutations (indels found in at least one allele) in either *SP* or *SP5G* genes (Table 2). Of mutated events, 10 (9.1%) and 21 (19.1%) events were edited only in *SP* and *SP5G*, respectively, while the remaining were mutated in both genes. Furthermore, 34 (30.9%) events were found to be potentially chimeric from the observation that more than two allelic variants of either gene were detected during HRFA analysis. We selected 20 events for genotyping by Sanger Sequencing and the results were in line with the indel sizes identified with HRFA analysis, as can be seen in Fig. 3b and Supp. Table 4. We selected 14 representative regenerated M_0 plants and five unedited regenerated plants and grew them in the biotron for further phenotypical assessment (Supp. Figure 3).

Discussion

Genome editing has become a complementary method to traditional breeding of many crops including tomato and the CRISPR/Cas9 technology is the most utilized tool in recent years. Tomato, as an important horticultural crop with high commercial value, has already been well studied genetically,

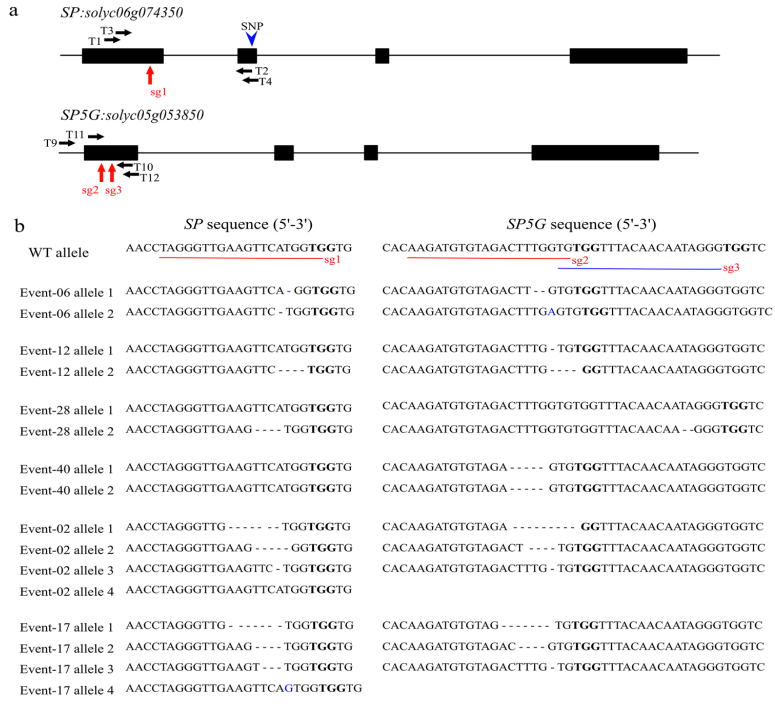

Fig. 3 DNA-free CRISPR/Cas9 mediated genome editing in tomato multiplexing of *SP* and *SP5G* genes. **a** Structure of *SP* and *SP5G* genes. Exons are indicated in black boxes. Primers used for genotyping and sequencing are noted with black arrows. For each gene, sgRNAs (red arrows) were designed, all targeting exon 1. Only one SNP (blue arrow) was found within the amplification region of the *SP* gene. **b** Genotyping of first-generation events (M_0) by Sanger Sequencing. The DNA sequence of each allele was aligned to wild type (WT) allele and deletions are shown with hyphens and insertions marked with blue color. Protospacer Adjacent Motif (PAM) is shown in bold

Table 2 Mutation rate[1] of regenerated events (M_0) from transfected protoplasts, tomato cv. Red Setter

total # of events analyzed	# of events with mutation[2]	# of events with mutation only in *SP*	# of events with mutation only in *SP5G*	# of events with mutation in both SP and *SP5G*	# of events possibly chimeric[3]
110	66 (60.0%)	10 (9.1%)	21 (19.1%)	35 (31.8%)	34 (30.9%)

[1]Mutations (indels) were determined on a single leaf from 110 regenerated plants by HRFA analysis where both *SP* and *SP5G* genes were targeted simultaneously

[2]Mutations in at least one allele in either *SP* or *SP5G* genes. The results of HRFA analysis of all 66 mutated events are shown in Supp. Table 4

[3]More than two allelic variants for either *SP* or *SP5G* detected in an event

which makes the application of modern molecular breeding possible (Foolad 2007). Currently, there is no DNA-free genome editing method established for cultivated tomato, which is an important drawback when utilizing this important technology for breeding or in research. A DNA-free genome editing method requires efficient and reproducible shoot regeneration from single cells, which is still a challenge and can be highly genotype dependent (Peres et al. 2001). A recent study reported protoplast regeneration via a DNA-free method on wild tomato, which is the closest study so far to cultivated tomato (Lin et al. 2022). Based on a previously published protocol for cultivated tomato protoplast genome editing via RNP-based CRISPR/Cas9 (Nicolia et al. 2021a), we have improved the protoplast isolation process and solved the challenge of shoot regeneration from RNP-transfected protoplasts.

The yield and quality of isolated protoplasts further affect shoot regeneration. Here we have optimized the process of protoplast isolation based on seedling age and enzyme digestion temperature and duration. With the optimized conditions (21 d seedlings, enzyme treatment: 25 °C for 16 h), we successfully increased the yield of protoplasts to $1.0–2.2 \times 10^6$ per gram of leaf materials, which were comparable results to previous reports on tomato (Morgan and Cocking 1982; Niedz et al. 1985; Tan et al. 1987). We also found in this study that preconditioning treatment of donor plants under 4 °C prior to protoplast isolation had a negative effect on protoplast yield. By contrast, Tan et al. (1987) got

the opposite result from preconditioning treatment, where they found that cold treatment increased the stability of protoplasts and thus yielded more viable protoplasts.

The formation of callus from transfected protoplasts is achieved by stimulation of cell wall development and cell divisions. Moreover, there are many factors that can affect the success of shoot regeneration, such as osmotic pressure, different types and concentration of hormones, carbon sources and gelling reagents. In this study, shoot regeneration was analyzed on ten different shoot regeneration media, but with extra focus on three of them. Cytokinins, such as zeatin and 6-Benzylaminopurine (6-BAP), are involved in early cell division as well as initiation and elongation of shoots, and are widely used in protoplast-derived shoot regeneration in many plant species. Previous studies showed that zeatin was necessary for tomato shoot regeneration (Morgan and Cocking 1982) and we found that the 0.1 mg/L IAA and 0.75 mg/L zeatin was the most suitable combination for shoot regeneration in all ten media tested with cv. Red Setter. On the other hand, when the calli were cultured on 6-BAP-based media together with IAA or 1-Naphthaleneacetic acid (NAA) (Medium TSR-d and TSR-e) (Supp. Figure 1d, e), the browning of calli seemed to accelerate or smaller calli were generated and no shoots were regenerated after six months. Gibberellins such as Gibberellic acid (GA_3) have been proven to be beneficial for stimulating shoot elongation (Niedz et al. 1985). Shahin (1985) observed higher regeneration rate when using both zeatin and GA_3 compared with using zeatin alone. In contrast, we found that when GA_3 was added in early shoot induction process, it had an adverse effect on shoot morphology which was curved, thin and grass-like as observed from most of the shoots regenerated from Medium TSR-a, TSR-f and TSR-g (Supp. Figure 1b, f and g) where GA_3 concentration varied from 0.34 mg/L to 1 mg/L. Auxins are also an essential component in shoot regeneration medium, such as the frequently used IAA and NAA. We found that when IAA was replaced by NAA, the calli on Medium TSR-h were inflated, less green and not able to generate shoots (Supp Fig. 1h), which did not concur with the conclusions from Niedz et al. (1985). We did not find an obvious difference between two carbon sources and gelling agent in this study.

In this study, four different tomato cvs Red Setter, Ailsa Craig, M82 and Moneymaker were used to study protoplast regeneration from RNP-transfected protoplasts. Cultivar Red Setter was superior to other cvs with a regeneration rate up to 31.4% from RNP-transfected protoplasts. Five shoots were obtained from 200 RNP-transfected protoplast-derived calli from cv. M82, with a high mutation rate (80%), although all four mutant regenerated events were chimeras (Supp. Figure 4). On the contrary, plating efficiency was low on both cvs Ailsa Craig and Moneymaker and all attempts to regenerate shoots from RNP-transfected protoplasts failed, indicating that a further adaptation of the protocol will be required for these cultivars. In this study, we clearly observed genotype differences among the four tested cultivars, which was in line with previous reports where variable regeneration rate among cultivars was found (Morgan and Cocking 1982; Niedz et al. 1985; Tan et al. 1987).

The mutations identified in M_0 events were a mix of bi-allelic, mono-allelic and chimeric with small deletions or insertions at the target site with an editing efficiency up to 60% in considering at least one allele mutated in either *SP* or *SP5G* genes and 31.8% considering on both genes simultaneously targeted. Previously, 30 and 90% of protoplast-derived calli were found to be mutated in at least one allele of *CCD7* or *CCD8* genes, respectively, after multiplex RNP delivery (Nicolia et al. 2021a). Such results indicate that the CRISPR/Cas9 system using RNP-transfected protoplasts can be highly efficient to generate desired mutations in cultivated tomato, without any stable integration of foreign DNA. Brooks et al. (2014) were the first to report the successful application of the CRISPR/Cas9 system on tomato via stable *Agrobacterium tumefaciens*-mediated transformation with an editing efficiency of 48% on T_0 plants with two sgRNAs targeting at the same gene. More studies using CRISPR/Cas9 via *Agrobacterium tumefaciens*-mediated transformation have been published in recent years and some of these studies had a very high editing efficiency of up to 100% of the transgenic shoots (Ito et al. 2015; Ueta et al. 2017; Dahan-Meir et al. 2018). With the latter method, however, comes the use of antibiotic selection as well as either selfing or backcrossing to remove T-DNA insertions if a transgene-free plant is desirable.

We observed a higher rate of potential chimeras in tomato than for example, potato, using RNP complexes and similar protoplast density (Andersson et al. 2018). This might be because the ratio between RNP complexes and protoplasts was not optimal in our study, as Sidorov et al. (2021) also observed high number of chimeras (33.3%) on regenerated calli from RNP-transfected protoplasts in canola (*Brassica napus* L.). It might be possible to address it by testing higher concentrations of RNPs as the efficiency of RNP is dose dependent (Zhang et al. 2022). Another possible reason might be endopolyploidy according to a report by Smulders et al. (1994) on varying ploidy level in different tomato somatic tissues. A high chimeras using CRISPR/Cas9 on tomato was also identified in earlier studies on *Agrobacterium tumefaciens*-mediated transformation (Brooks et al. 2014; Dahan-Meir et al. 2018). However, a high frequency of chimeric events is less important in tomato than in clonally propagated crops, due to sexual generation and the possibility of selecting and producing homozygous mutations in the next generation. Moreover, the use of protoplasts allows to scale-up the experiments of mutagenesis easily, so that

among a high number of regenerated mutant M_0 plants those indicating chimerism can be discarded.

Our findings illustrate that the challenge of shoot regeneration from transfected protoplasts of cultivated tomato has been overcome and we have successfully obtained regenerated plants from non-treated protoplasts from all four studied cultivars, as well as regenerated plants with induced mutations in two cultivars (Red Setter and M82) via DNA-free CRISPR/Cas9. The availability of this reported method in the determinate cvs Red Setter or M82 provides opportunities for important research and breeding efforts oriented toward tomato field cultivation and industrial processing, such as improving the fruit quality (e.g., flavor, sugar content, acidity) and plant resistance to biotic (e.g., soil born, virus, parasitic plants) and abiotic stress (e.g., water deficiency, salinity).

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Author contributions YL, MA, TC, PH and AN conceived the project and contributed to the study conception. YL designed and conducted the experiment and wrote the first draft of the manuscript and all authors commented on previous versions of the manuscript. AG contributed with design of shoot regeneration Medium TSR-b. All authors read and approved the manuscript.

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Data availability All the data in this study are included in this manuscript and supplementary data file.

Declarations

Conflict of interest The authors declare non-financial interests that are directly or indirectly related to the work submitted for publication.

References

Andersson M, Turesson H, Nicolia A et al (2017) Efficient targeted multiallelic mutagenesis in tetraploid potato (Solanum tuberosum) by transient CRISPR-Cas9 expression in protoplasts. Plant Cell Rep 36:117–128. https://doi.org/10.1007/s00299-016-2062-3

Andersson M, Turesson H, Olsson N et al (2018) Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. Physiol Plant 164:378–384. https://doi.org/10.1111/ppl.12731

Bae S, Kweon J, Kim HS, Kim J (2014) Microhomology-based choice of Cas9 nuclease target sites. Nat Publ Gr 11:705–706. https://doi.org/10.1038/nmeth.3015

Brooks C, Nekrasov V, Lipppman ZB, 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 Physiol 166:1292–1297. https://doi.org/10.1104/pp.114.247577

Carmel-Goren L, Liu YS, Lifschitz E, Zamir D (2003) The self-pruning gene family in tomato. Plant Mol Biol 52:1215–1222. https://doi.org/10.1023/B:PLAN.0000004333.96451.11

Char SN, Neelakandan AK, Nahampun H et al (2016) An Agrobacterium-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. Plant Biotechnol J 15:257–268. https://doi.org/10.1111/pbi.12611

Chen K, Wang Y, Zhang R et al (2019) CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu Rev Plant Biol 70:667–697. https://doi.org/10.1146/annurev-arplant-050718-100049

Concordet J, Haeussler M (2018) CRISPOR : intuitive guide selection for CRISPR / Cas9 genome editing experiments and screens. Nucleic Acids Res 46:242–245. https://doi.org/10.1093/nar/gky354

Dahan-Meir T, Filler-Hayut S, Melamed-Bessudo C et al (2018) Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. Plant J 95:5–16. https://doi.org/10.1111/tpj.13932

Foolad MR (2007) Genome mapping and molecular breeding of tomato. Int J Plant Genomics 1:52. https://doi.org/10.1155/2007/64358

Gao C (2021) Genome engineering for crop improvement and future agriculture. Cell 184:1621–1635. https://doi.org/10.1016/j.cell.2021.01.005

Ito Y, Nishizawa-Yokoi A, Endo M et al (2015) CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. Biochem Biophys Res Commun 467:76–82. https://doi.org/10.1016/j.bbrc.2015.09.117

Jiang W, Zhou H, Bi H et al (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res 41:1–12. https://doi.org/10.1093/nar/gkt780

Kwon CT, Heo J, Lemmon ZH et al (2020) Rapid customization of Solanaceae fruit crops for urban agriculture. Nat Biotechnol 38:182–188. https://doi.org/10.1038/s41587-019-0361-2

Li JF, Norville JE, Aach J et al (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat Biotechnol 31:688–691. https://doi.org/10.1038/nbt.2654

Li T, Yang X, Yu Y et al (2018) Domestication of wild tomato is accelerated by genome editing. Nat Biotechnol. https://doi.org/10.1038/nbt.4273

Liang Z, Zhang K, Chen K, Gao C (2014) Targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system. J Genet Genomics 41:63–68. https://doi.org/10.1016/j.jgg.2013.12.001

Liang Z, Chen K, Li T et al (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat Commun 8:1–5. https://doi.org/10.1038/ncomms14261

Lin C-S, Hsu C-T, Yuan Y-H et al (2022) DNA-free CRISPR-Cas9 gene editing of wild tetraploid tomato Solanum peruvianum using protoplast regeneration. Plant Physiol. https://doi.org/10.1093/plphys/kiac022

Lowder LG, Zhang D, Baltes NJ et al (2015) A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. Plant Physiol 169:971–985. https://doi.org/10.1104/pp.15.00636

Morgan A, Cocking EC (1982) Plant Regeneration from protoplasts of Lycopersicon esculentum Mill. Z Pflanzenphysiol 106:97–104. https://doi.org/10.1016/s0044-328x(82)80071-8

Nekrasov V, Staskawicz B, Weigel D et al (2013) Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat Biotechnol 31:691–693. https://doi.org/10.1038/nbt.2655

Nicolia A, Andersson M, Hofvander P et al (2021a) Tomato protoplasts as cell target for ribonucleoprotein (RNP)- mediated multiplexed genome editing. Plant Cell Tissue Organ Cult 144:463–467. https://doi.org/10.1007/s11240-020-01954-8

Nicolia A, Fält A-S, Hofvander P, Andersson M (2021b) Protoplast-Based Method for Genome Editing in Tetraploid Potato Methods in Molecular Biology. Springer, New york, pp 177–186. https://doi.org/10.1007/978-1-0716-1201-9_12

Niedz RP, Rutter SM, Handley LW, Sink KC (1985) Plant regeneration from leaf protoplasts of six tomato cultivars. Plant Sci 39:199–204. https://doi.org/10.1016/0168-9452(85)90175-X

Pan C, Ye L, Qin L et al (2016) CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. Sci Rep 6:1–10. https://doi.org/10.1038/srep24765

Peres LEP, Morgante PG, Vecchi C et al (2001) Shoot regeneration capacity from roots and transgenic hairy roots of tomato cultivars and wild related species. Plant Cell Tissue Organ Cult 65:37–44. https://doi.org/10.1023/A:1010631731559

Pnueli L, Carmel-Goren L, Hareven D et al (1998) The SELF-PRUNING gene of tomato regulates vegetative to reproductive switching of sympodial meristems and is the ortholog of CEN and TFL1. Development 125:1979–1989. https://doi.org/10.1242/dev.125.11.1979

Sakata Y, NISHIO T, TAKAYANAGI K, (1987) Plant regeneration from mesophyll protoplasts of tomato cv. Ponderosa J Jpn Soc Hortic Sci 56:334–338. https://doi.org/10.2503/jjshs.56.334

Shahin EA (1985) Totipotency of tomato protoplasts. Theor Appl Genet 69:235–240. https://doi.org/10.1007/BF00662431

Sidorov V, Wang D, Nagy ED et al (2021) Heritable DNA-free genome editing of canola (Brassica napus L) using PEG-mediated transfection of isolated protoplasts. Vitr Cell Dev Biol Plant. https://doi.org/10.1007/s11627-021-10236-7

Smulders MJM, Rus-Kortekaas W, Gilissen LJW (1994) Development of polysomaty during differentiation in diploid and tetraploid tomato (Lycopersicon esculentum) plants. Plant Sci 97:53–60. https://doi.org/10.1016/0168-9452(94)90107-4

Soyk S, Müller NA, Park SJ et al (2017) Variation in the flowering gene SELF PRUNING 5G promotes day-neutrality and early yield in tomato. Nat Genet 49:162–168. https://doi.org/10.1038/ng.3733

Svitashev S, Schwartz C, Lenderts B et al (2016) Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. Nat Commun 7:1–7. https://doi.org/10.1038/ncomms13274

Tan MMC, Rietveld EM et al (1987) Regeneration of leaf mesophyll protoplasts of tomato cultivars (L. esculentum): factors important for efficient protoplast culture and plant regeneration. Plant Cell Rep 6:172–175. https://doi.org/10.1007/BF00268470

Ueta R, Abe C, Watanabe T et al (2017) Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. Sci Rep 7:1–8. https://doi.org/10.1038/s41598-017-00501-4

Upadhyay SK, Kumar J, Alok A, Tuli R (2013) RNA-Guided genome editing for target gene mutations in wheat. G3 Genes. Genomes Genet 3:2233–2238. https://doi.org/10.1534/g3.113.008847

Wang S, Zhang S, Wang W et al (2015) Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. Plant Cell Rep 34:1473–1476. https://doi.org/10.1007/s00299-015-1816-7

Woo JW, Kim J, Il KS et al (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33:1162–1165. https://doi.org/10.1038/nbt.3389

Yan L, Wei S, Wu Y et al (2015) High-Efficiency genome editing in Arabidopsis using YAO promoter-driven CRISPR/Cas9 system. Mol Plant 8:1820–1823. https://doi.org/10.1016/j.molp.2015.10.004

Zhang Y, Liang Z, Zong Y et al (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat Commun 7:1–8. https://doi.org/10.1038/ncomms12617

Zhang Y, Cheng Y, Fang H et al (2022) Highly efficient genome editing in plant protoplasts by ribonucleoprotein delivery of CRISPR-Cas12a nucleases. Front Genome Ed 4:1–11. https://doi.org/10.3389/fgeed.2022.780238

Zhou H, Liu B, Weeks DP et al (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res 42:10903–10914. https://doi.org/10.1093/nar/gku806

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Concerns about toxic steroidal glycoalkaloids (SGAs) have been raised in potatoes. Aiming at reducing the SGA hazard in potato, the SGA metabolism was investigated in starch- compared to table potato cultivars. Further, seven types of gene-edited starch potato mutants with reduced SGA levels were generated, targeting key genes involved in SGA biosynthesis. Several mutants displayed close to SGA-free tubers, offering an interesting opportunity for industrial applications. Additionally, a DNA-free genome editing method was established for cultivated tomato.

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