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Functional analysis of the *SlGRAS10* gene in tomato fruit ripening.

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Resumo detalhado

A família Solanaceae, género *Solanum* é um dos mais representativos grupos taxonómicos de angiospérmicas e inclui, entre outras, a espécie *Solanum lycopersicum*, comumente reconhecida pelo seu fruto (tomate). À espécie, é atribuído o México como centro de domesticação. Daí, através de rotas comerciais decorrentes no séc. XVI, a cultura foi introduzida primariamente nas colónias europeias e sofreu, posteriormente, uma gradual dispersão geográfica. O melhoramento tradicional, com foco na seleção diferencial de características quantitativas, dotou a espécie de uma variabilidade fenotípica, plasticidade adaptativa e superioridade agronómica. Em concordância, o tomate integra presentemente a dieta de uma fração significativa da população, traduzindo-se numa mercadoria alimentar comercial e nutricional indispensável e, elevando-o a produto hortícola de referência agrícola e científica. No que concerne ao último, a sua particularidade associa-se à categorização como fruto carnudo climatérico no qual, após a colheita, o processo de amadurecimento mantém-se ininterrupto, levando à diminuição do tempo de armazenamento e distribuição e, ao aumento da suscetibilidade a fungos patogénicos. O amadurecimento é um evento fisiológico complexo, desencadeado por sinais ambientais. A ele está inerente a reprogramação do desenvolvimento do fruto, através da modulação de vias regulatórias específicas e, finamente controladas por fatores de transcrição. Em tomate, r*ipening-inhibitor* (rin), *nonripening* (nor) e *Colorless nonripening* (Cnr) correspondem a mutantes comprometidos no amadurecimento. Nestes mutantes, o desenvolvimento progride até à fase do verde maduro (*mature green*), onde o fruto atinge o tamanho máximo e as sementes amadurecem, mas as subsequentes fases de desenvolvimento não ocorrem. A caracterização funcional de *rin*, *nor*, *Cnr* demonstrou tratar-se de três genes codificadores de fatores de transcrição, que atuam como reguladores centrais no processo de amadurecimento. Ademais, a presença de homólogos de *RIN-MADS* em frutos não climatéricos evidenciou a sua conservação evolutiva e transversalidade interespecífica. Posteriores *screenings* genéticos permitiram a identificação de novos integrantes da cascata transcricional alocada ao amadurecimento. Dos quais, *SlGRAS38* foi referido como alvo da regulação por RIN, através da interação nos locais de ligação putativamente identificados. *GRAS* corresponde a uma família génica de fatores de transcrição exclusivos de plantas e, com papéis-chave na regulação do desenvolvimento de órgãos da planta. Em paralelo com a identificação de novos *GRAS* como alvos diretos de RIN, a atuação igualmente crucial destes no amadurecimento tem sido demonstrada. Em morango, um gene da família *GRAS*, *FaSCL8*, demonstrou influenciar a expressão de genes relacionados com a produção de antocianinas, flavonoides e a iniciação da maturação do fruto. Através da comparação genómica entre tomate e videira, Grimplet et al. (2016) identificou dois *GRAS (SlGRAS10* e o seu duplicado *SlGRAS9)* como possíveis reguladores transcricionais conservados do amadurecimento de frutos climatéricos e não climatéricos. Ainda, identificou um ortólogo de *SIGRAS10* (*VviPAT6)* em videira, cujo padrão de expressão variou durante o amadurecimento do bago. Um subsequente estudo de *Genome Wide Association* mostrou que *SlGRAS10* e *SlGRAS9* têm uma regulação positiva na sua expressão nos frutos nos estágios de 7 e 10 dias após o *Breaker* (Br +7 e Br+10), assim como na flor em antese, sugerindo o papel putativo de ambos os genes no amadurecimento do tomate e, reforçando o interesse na sua análise (Huang et al., 2015a). Anteriormente ao presente estudo, foi utilizada a tecnologia da CRISPR-cas9 para gerar linhas mutantes com *knock-out* para o gene *SlGRAS10*. No presente estudo estas linhas foram estabilizadas da geração T0 para a geração T1 com altas taxas de mutação, determinadas através da ferramenta informática TIDE. Os frutos das plantas T1 foram colhidos em estágios específicos do amadurecimento, nomeadamente Br+7 e Br+10 e a expressão relativa de genes envolvidos no amadurecimento ocorreu por PCR quantitativo em tempo real. A maioria das diferenças foi encontrada nos frutos

colhidos na fase Br+10, nos quais a expressão relativa de *RIN*, *PYL9*, *ACO1* e *PSY*, genes associados ao amadurecimento, foi significativamente menor comparada com a do *Wild-type*. *PYL9* (recetor de ácido abscísico PYL9) e *ACO1 (*ácido 1-carboxílico-1-amino ciclo propano oxidase) são genes envolvidos nas vias de sinalização ácido abscísico e na síntese de etileno. Ambas as hormonas, o ácido abscísico e o etileno, são classicamente descritas como estando envolvidas no amadurecimento dos frutos, atuando a jusante na cascata transcricional de forma a ativar e reprimir processos determinantes. Particularmente, o etileno, que é considerado a hormona incitadora fulcral do início do amadurecimento dos frutos climatéricos, uma vez que tem um aumento muiti significativo na fase inicial e seguinte de amadurceimneto. A biossíntese autocatalítica de etileno via ACO e ACS (ácido 1-carboxílico-1-amino ciclo propano sintase) no Sistema 2 resulta num pico na respiração concomitante com o amadurecimento do fruto. Entre o início e o alcance máximo do amadurecimento, alterações na pigmentação do tomate refletem acumulações diferenciais de carotenoides. *PSY*, um dos genes analisados neste estudo, integra a via de síntese dos carotenoides. Concordante com a sua expressão, a análise do teor de carotenoides totais no pericarpo dos frutos evidenciou uma redução significativa no seu conteúdo total nos mutantes *SlGRAS10* no estágio Br + 10 face ao *Wild-type*. Nos estágios de amadurecimento Br + 7 os frutos aparentaram ter uma concentração semelhante ao *Wild-type*, contudo a redução do teor de carotenoides totais nos frutos Br + 10 representa um atraso significativo de 3 a 5 dias. Nas linhas 1A2, 2A2, 2A3, 1B1, 1B2 e 2B5 *SlGRAS10*, surgiu um fenótipo imprevisto onde um menor número de sementes face ao *Wild-type*. Este fenótipo não estava relacionado com menores dimensões dos frutos em todas as linhas mutantes. Nas linhas com maior significância (*p-value* <0,001) deste fenótipo, nomeadamente 1A2 e 1B2, realizou-se um ensaio preliminar de germinação de pólen. Destas, verificou-se que em 1A2, as flores produziam pólen que dispunha de taxas de germinação significativamente mais baixas. Com base em estudos anteriores que investigaram as homologias das sequencias, julga-se que *SlGRAS9* e *SlGRAS10* resultaram de uma duplicação génica e dispõem de padrões de expressão conservados, o que implica uma retenção por redundância genética. Devido à possibilidade de compensação por *SlGRAS9* nas linhas *knock-out SlGRAS10* e, de forma a eliminar a redundância funcional, novas linhas *knock-out* para o gene *SlGRAS10* e *SlGRAS9 (double knock-out)* foram desenvolvidas. O genótipo das novas linhas T0 foi analisado por sequenciação de *sanger*, seguido de posterior análise de TIDE para determinação do tipo e taxa de mutação presente. Para *SlGRAS10*, as taxas de mutação foram semelhantes aos primeiros mutantes desenvolvidos e, para *SlGRAS9* foram ligeiramente mais baixas. Contudo, prevê-se um aumento destas últimas com a segregação na geração T1, assim como um fenótipo mais marcado que o presente nas linhas *SlGRAS10*. Em suma, a regulação negativa de genes envolvidos em vias reguladoras do amadurecimento em paralelo com um ligeiro atraso no amadurecimento de tomate, sem redução no peso total do fruto e a redundância funcional entre *SlGRAS10* e *SlGRAS9* apontam para um possível papel fundamental destes na regulação do amadurecimento e desenvolvimento do tomate. Os resultados aqui dispostos poderão ser utilizados em programas de melhoramento de tomate que visam a produção de plantas geneticamente melhoradas adequadas às políticas europeias de redução do desperdício alimentar e adequação dos recursos genéticos. Neste âmbito, prevê-se a sua aplicabilidade no desenvolvimento de novas variedades com maior resistência aos fenómenos de senescência e consequentemente um maior tempo de prateleira e sem sementes.

Palavras chave: edição génica por CRISPR-cas9, fatores de transcrição, carotenoides totais, expressão génica

Abstract

Fleshy fruits, like Solanum lycopersicum (tomato), comprise an indispensable commercial and nutritional food commodity, being an essential crop for agriculture and being part of the diet of a significant portion of the population.

Tomato is a climacteric fruit; one feature of climacteric fruits is that these fruits continue their process of ripening after harvest making it much more challenging to store and distribute than other fruits due to easier fungal contaminations. This process of ripening is very complex and involves several mechanisms. The trigger to this reprogramming of fruit development and ripening comprises several transcription factors, among them the *GRAS* transcription factors have been suggested to play a role. In a previous work, the *SlGRAS*9 and *SlGRAS10* have exhibited transcriptional up-regulation in post-breaker stage fruits (Grimplet et al., 2016a), suggesting these genes may be involved in tomato ripening.

There are a few tomato ripening mutants that have been identified, and characterization of these mutants suggests that they may have defects in ripening regulatory systems. These mutations are the *ripening-inhibitor* (*rin*), *nonripening* (*nor*), and *Colorless nonripening* (*Cnr*) mutations. All three genes encode transcription factors, and the associated phenotypes suggest that the *rin*, *nor* and *Cnr* mutants are impacted in the central ripening phenomena. It is hypothesized that such genes might represent conserved regulators affecting ripening even in non-climacteric fruits, since homologs of the *RIN-MADS* gene have been found in climacteric species such as tomato but also in non-climacteric species, such as strawberry.

The *GRAS* gene family is a plant-specific family of transcription factors with fundamental roles in the regulation of plant organ development and some *GRAS* genes have been identified as direct targets of *RIN*. By comparing the information available for tomato and grapevine *GRAS* genes, (Grimplet et al., 2016) identified candidate genes that might constitute conserved transcriptional regulators of both climacteric and non-climacteric fruit ripening, and that deserve further functional analysis, namely *SlGRAS10* and its orthologue in Vitis vinifera *PAT6.*

In our study, the *SlGRAS10* mutant, developed by CRISPR-cas9 technology was stabilized from T0 to T1 with high mutation rates that were analyzed through TIDE analysis. The fruits from the T1 plants were collected in specific time-points and through Quantitative Real Time PCR (qPCR) the expression of a few genes involved in fruit ripening were analyzed. Most differences were found at fruits collected 10 days post Breaker (Br+10), where the relative expression of the *RIN*, *PYL9*, *ACO1* and *PSY* was significantly lower than the Wild-type in specific knock-out lines. The analysis of total carotenoids content in the fruits pericarp also showed significant lower content of total carotenoids in the *SlGRAS10* mutants, specifically at the Br+10 stage again, the fruits appear to have a concentration similar to the Wild-type at the Br+5 and Br+7 stages of ripening, which is a significant delay of 3 to almost 5 days.

Interestingly, the 1A2, 2A2, 2A3, 1B1, 1B2 and 2B5 *SlGRAS10* lines had an unanticipated phenotype of low seeds, that was related to smaller fruits in some lines. Due to this phenotype a germination assay was performed with pollen from selected lines. The most significant reduction in seed number (1A2 and 1B2) and most lines descendent from de 1A2 line had significantly lower pollen germination rates.

In previous studies the *SlGRAS9* and *SlGRAS10* genes showed to have conserved expression patterns, which implied their retention of genetic redundancy. Therefore, it is suggested that *SlGRAS9* and *SlGRAS10* can compensate each other in a redundant functional way. Therefore, posteriorly to the *SlGRAS10* mutants were established, other mutant plants were developed with

a knock-out both in the *SlGRAS10* and *SlGRAS9* genes. These T0 lines were genotyped by Sanger Sequencing and later TIDE analysis to determine the type and mutation rate on those lines, the mutations rates for the *SlGRAS10* were like the first single mutants developed and the mutation rates for the *SlGRAS9* were slightly lower, but mutation rate is expected to grow on the T1 generation, since cas9 is still present.
Altogether the data indicated that *SlGRAS10* and *SlGRAS9* play an important role in tomato fertilization and ripening. These mutants could be applied to produce seedless tomatoes with longer shelf-life as part of a breeding program to develop a new variety commercially valuable.

Keywords: CRISPR, transcription factors, carotenoids, gene expression

Declaração

De acordo com o disposto no artigo n.o 19 do Regulamento de Estudos de Pós-Graduação da Universidade de Lisboa, Despacho n.o 2950/2015, publicado no Diário da República, 2.a série — N.o 57 — 23 de março de 2015, foram incluídos nesta dissertação os resultados apresentados em:

Paper in a magazine

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Oral and Poster presentations

Soares, Flávio; Pimentel, Diana; **Neves, Catarina**; Amaro, R.; Lecourieux, D.; Granell, Antonio; Fortes, Ana Margarida. "CRISPR-based assessment of SlGRAS10 function during tomato fruit development and ripening". Poster presentation- Ciências Research Day (online), 10/2020.

Soares, Flávio; Pimentel, Diana; **Neves, Catarina**; Amaro, R.; Lecourieux, D.; Granell, Antonio; Fortes, Ana Margarida. "CRISPR/Cas9-mediated mutagenesis of SlGRAS10: role during tomato fruit development and ripening". Poster presentation - Sol International Online Meeting (online), 11/2020.

Functional analysis of SlGRAS9 and SlGRAS10 during tomato fruit ripening, Mini-workshop - Auxins linking Universities of Lisbon and Freiburg. Oral presentation (online), 03/2021.

Neves, Catarina; Soares, Flávio; Pimentel, Diana; Amaro, R.; Lecourieux, D.; Granell, Antonio; Fortes, Ana Margarida. "Role of SIGRAS9 and SIGRAS10 in tomato fruit ripening". Poster presentation - Plant Biology Europe 2021 (online), 07/2021.

Neves, Catarina; Soares, Flávio; Pimentel, Diana; Amaro, R.; Lecourieux, D.; Granell, Antonio; Fortes, Ana Margarida. "CRISPR-based functional analysis of SlGRAS9 and SlGRAS10 genes in tomato fruit ripening." Poster presentation - RAFV 2021 (online), 09/2021.

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Em cumprimento com o suprarreferido despacho, confirma-se ser da minha total responsabilidade (exceto quando indicado em contrário) a execução das experiências que estiverem na base dos resultados apresentados, bem como a interpretação e discussão dos mesmos.

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1 – INTRODUCTION

1.1 - Tomato – Economic importance

Fleshy fruits, like tomato, are a significant commercial crop and are essential for nutrition. Tomato is a fruit of high economic value worldwide due to its ability to produce high yields (37.6 tons/hectare) of its tasty fruit and for being rich in healthy nutrients like lycopene and carotenoids (Ronga et al., 2021). As of 2018, tomatoes' total world production was 182 million tonnes being one of the most extensively cultivated and broadly consumed agricultural crops worldwide (Arah et al., 2016). However, the reduced shelf life of tomatoes is one of the problems that leads to substantial losses. So, the understanding of fleshy fruit development and ripening is paramount for crop improvement.

Fleshy fruits, like tomato, are an essential commercial crop and are vital for the nutrition of many people worldwide. In many developing countries, grain, tuber and seed crops are the most explored crops. Seeing as these crops are so crucial for nutrition, these crops are the ones that have been most studied in recent years. These crops have been bred and engineered to be more productive and resistant to biotic and abiotic stresses. Fleshy fruits are a staple in nutrition, and even though cereals are one of the most important crops offering sustenance and nourishment, fleshy fruits are a source of fibre and necessary phytonutrients, which are also very important to lead a healthy life. Lycopene, a type of carotenoid present in tomato has antioxidant properties that help reduce the incidence of certain chronic diseases (Agarwal & Rao, 2000). Fleshy fruits, like tomato, are vital to lead a balanced diet and provide vitamins and nutrients to everyone worldwide and are starting to be explored to produce new varieties that can be richer in nutrients but also last longer.

There are numerous uses for tomatoes, which can contribute to its overall production worldwide (Agarwal & Rao, 2000). Research in the production aspect has improved cultivars or varieties, obtaining high yielding varieties and more resistant to biotic and abiotic stresses (Kader, 2005). However, scientific research has focused on yield mainly whilst neglecting postharvest quality. This allows tomato producers to have abundant harvests in recent years; however, this does not result in profit as most are lost after harvest (Arah et al., 2016).

Postharvest handling methods are needed to extend the crop's shelf life after harvest. Tomato being a perishable crop due to its high moisture content has a short shelf life has its quality compromised when exposed to high temperatures, and high relative humidity, causing over-ripening. However, low-temperature storage is detrimental to the shelf life and quality of tomatoes. Still, chilling can impact fruit quality, altering flavour, a quality trait of tomatoes which is mostly determined by the total soluble solids and fruit pH (Parker & Maalekuu, 2013).

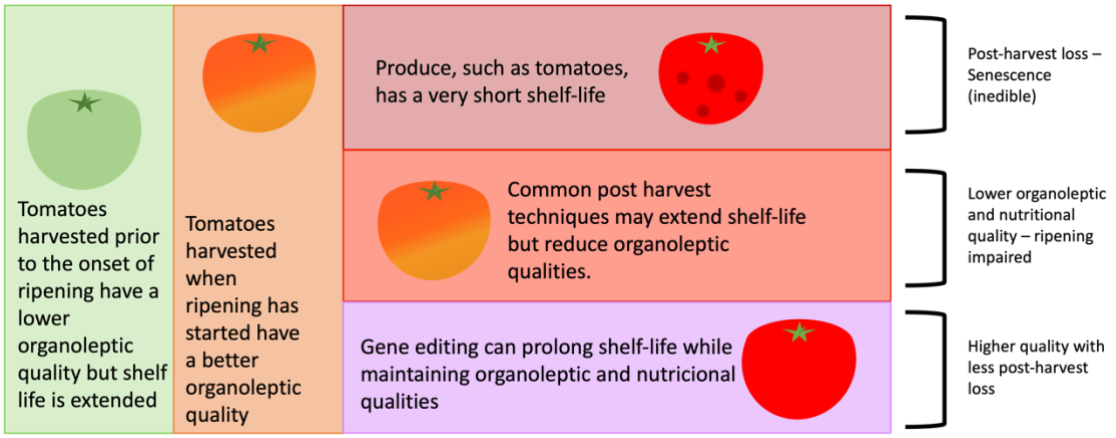

Figure 1.1.1 - Outcomes of tomato ripening post-harvest adapted from 'Can gene editing reduce postharvest waste and loss of fruit, vegetables, and ornamentals?' Emma N. Shipman 2021

Over the years, consumers have become more demanding about the food products they consume. Consumers expect tasty and nutritious produce. These qualities depend on a good sensory quality, high concentration in sugars, the right stage of ripeness, and specific nutritional properties such as accumulation of specific antioxidant components, like carotenoids and lycopene, which are dependent on intrinsic developmental characteristics but also on harvesting techniques (Fig.1.1.1). In this regard, food technology has evolved to create deliver food products that cater to the particular consumer requirements. Consequently, research focused on fruit quality and better shelf life is paramount to improve this crop.

1.2 - Tomato – model fruit, genetic resources, and omics-assisted breeding

Tomato is the main model for climacteric fruit ripening for scientific and agricultural reasons. Wild tomato species originated in western South America being later cultivated worldwide (Bergougnoux, 2014). The tomato wild relatives are thought to have originated in western South America, and, to this day, wild tomatoes are still found along the western coast of South America (Caspermeyer, 2020). And while the centre of origin for tomatoes is in Peru, it is thought that it was in Mexico that domestication occurred because it is where the centre of diversity for cultivated tomatoes is found (Bergougnoux, 2014). During the 16th century, Europeans came to South America and distributed many crops around the world (McCue, 1952). Because of that, tomatoes started being cultivated all around the world. Nowadays, tomatoes have become one of the major vegetable crop plants being produced year-round worldwide. In the wild, tomatoes can be found in many environments, and it is this adaptability to different conditions that have induced variability in the wild tomato varieties. Because of the environmental pressures, wild tomatoes have developed an abundance of phenotypes, from abiotic stress resistance, disease susceptibility, and stress resistance. In traditional breeding, these wild tomato resources were used as a source for desirable agronomical traits such as increased yield, fruit quality, and resistance to pathogens and abiotic stress(Stevens & Rick, 1986). Because of its importance as a food commodity, tomato has been continuously bred to try and improve its productivity, fruit quality, and resistance to biotic and abiotic stresses. And unlike other model plants, like Arabidopsis, the tomato has been widely used not only as research material but also as a food resource. Also, tomatoes have agronomically important traits that cannot be studied using other model plants, such as rice, since rice does not produce a fleshy fruit, it is not useful to study fleshy fruit related characteristics.

Tomato, *Solanum lycopersicum*, belongs to the Solanaceae family. The genus *Solanum* is one of the largest genera of angiosperms. The Solanaceae family comprises many commercially significant crops that are used as food sources, like potato, eggplant, bell peppers; as recreational drugs, like, tobacco, *Nicotiana tabacum*, and as ornamental plants, such as the *Petunia hybrida*. Many economically important plants in the Solanaceae family make tomato pivotal as a model plant species. Knowledge obtained from research on tomato can be translated and applied to these other plants and to many other fleshy fruits that are not that easy to study in a lab environment. So, because of these particularities, the tomato serves as a model organism for the family Solanaceae and, also, for other plants that produce fleshy fruits (Kimura & Sinha, 2008).

Many plants have been studied in terms of development, maturation, ripening, and other associated quality and yield characteristics, and because of this, several species have emerged as model systems. And most of the available information regarding the molecular regulation of development and ripening has derived from studies in these plant species. These species include *Arabidopsis*, tomato and strawberry. Each of these models represents specific fruit development and maturation programs, and each has traits that make it useful as a model system. These three plants can be used for gene function assessment, but due to their importance as an agriculture crop, the tomato has served as the primary model for climacteric fruit ripening. Compared with other model plants, like rice and *Arabidopsis,* the tomato has many commercially interesting features that other model plants do not have (J. Giovannoni, 2001).

Tomato's agronomic and economic importance combined with its diploid inheritance, self-pollination, ease of seed and clonal propagation, efficient sexual hybridization, a short generation period, and year-round growth potential inside greenhouses makes tomato one of the plants of choice for fruit ripening research. Also, progress on the tomato genome sequencing project has generated useful information to help in the study of tomato. In 2012, the tomato reference genome sequence was published, and it helped unveil the functional diversity of tomato genes. Also, what makes tomato such a great model is that from the perspective of genetic and molecular investigations, the tomato has a small genome for which many molecular markers have been identified (J. Giovannoni, 2001). The resulting genetic map has been able to identify and localize many quantitative trait loci (QTLs) that influence numerous fruit development and ripening characteristics. This QTL analysis has allowed the identification of different loci that control many different fruit traits, such as fruit size, shape, pigment accumulation and ripening time (Gascuel et al., 2017; J. Giovannoni, 2001).

The evolution of crops brought about by domestication has caused a decrease in crop genetic diversity, cause by the loss of several alleles that control important quality traits in favor of other traits related do productivity (Khan et al., 2020). The rapid evolution of marker technology and marker-based breeding methods have long surpassed the conventional breeding approaches that depend on phenotyping-based selection. So, with the use of technologies such as and genomic engineering and genomics-assisted breeding, it is possible to use genomic tools and information in breeding programs in order to develop better lines with enhanced characteristics (Khan et al., 2020). However, to use genetic engineering or genomics-assisted breeding to produce better varieties it is necessary to identify markers, loci and genes associated with traits of interest, and, due to the development of sequencing technologies, such as next generation sequencing (NGS) the crop improvement with genomic resources has been boosted, by allowing to have reference genomes for many crops, serving as a base for crop improvement. Crop varieties lack in genetic biodiversity especially compared with crop wild relatives which have very diverse genetic material making them a great source of diversity when creating new crop varieties. And while using traditional methods make this mission more taxing new technologies like CRISPR-Cas9 can help overcome issues like linkage drag that greatly delay breeding programs (Khan et al.,

2020). With the evolution of new breeding technologies, bioinformatics, and broader genomics new efficient resources are available to identify genetic diversity. The concept of pangenomics has been introduced has a way to represent a complete genetic repository of an entire species. When using a single reference genome, a lot of information is lost,and highly polymorphic regions are not represented. So with pangenome analysis the identification of genes that are missing in reference genomes is much more efficient and makes genomics-assisted breeding programs more effective. (Khan et al., 2020)

This information about the variable part of the genome that is encompassed within the pangenome highlights the highly polymorphic regions in certain wild varieties and combined with new genetic engineering technologies makes the creation of new varieties easier and less time and resource consuming, using the resources that were previously left unexplored in order to create genetic variety (Khan et al., 2020). For example, in *De novo domestication* programs certain agronomically desirable traits present in wild varieties of tomato can be inserted into crop varieties with genome engineering strategies like, CRISPR-Cas9 (Zsögön et al., 2018), and with the information gathered by the pangenome the genes responsible for these traits in wild varieties can be targeted and exploit the genetic diversity present in wild plants making molecular breeding programs more accessible.

There are also many T-DNA lines created that have the potential to be used for loss of function by knock-down our complete knock-out studies. In tomato there are a few mutant collections are publicly available, which greatly facilitate functional analysis studies (Jacobs et al., 2017; Saito et al., 2011) . Almost all of these collections are generated by agrotransformation of a T-DNA at random sites in the tomato genome. In (Gidoni et al., 2002.) 405 individual tomato lines were developed containing a characterized copy of a multifunctional T-DNA/modified *Ds* transposon element construct to be used as a tool for molecular analysis.

Recently more tools have been developed, such as TomExpress, which combines RNA-seq data in order to analyze a multitude of genes in different stages of the tomato plant and fruit development (Zouine et al., 2017).

Because of the use of the tomato has a plant model, there are many protocols already developed for tomato, from protocols to grow, cross and transform tomato plants, and most of the more common molecular techniques, such as genomic DNA isolation, RNA isolation, and protein extraction. Also, most of the protocols used for Arabidopsis can be applied to tomato. Also, tomatoes can be stably transformed using tissue culture methods to make overexpression of specific genes, insert RNA interference (RNAi) or analysis using reporter genes such as the promoter-β-glucuronidase (GUS), and other assays to functionally characterize a gene of interest (Kimura & Sinha, 2008).

All this information gathered from tomato studies can be easily applied to other plants, making tomatoes an important plant material for research. All the genetic research on tomato has resulted in linkage maps, molecular markers, mutants, and introgression lines.

In recent years the area of the ‘-omics’ has seen great development and recent advances in the area have enabled the identification of epigenetic marks and how these marks influence plant development and response to environmental stimuli. Epigenetic alterations occur to better adapt momentarily or permanently to stresses and occur due to alterations in the chromatin states in plant cells. These changes vary to allow the cells of specific organs to tweak their transcriptional profiles. Epigenetic modifications involve a wide range of alterations that result in alterations in the chromatin states, and these epigenetic modifications are brough about by post-transcriptional histone modifications, histone variants, DNA methylation, and non-coding RNAs, which can determine specific transcriptional turnouts.

In *Solanum lycopersicum* one of the most notable epimutation is the Colorless nonripening (Cnr) mutation. This mutation is considered a landmark for plant epigenetics, because is related to an important agronomical trait, ripening. Due to this epimutation present in tomato, this crop has become a model for studying the epigenetic basis of agronomical and economic important phenotypes, specially related to fruit ripening (Nogueira, 2014).
Because of all these excellent resources, the tomato serves as a model organism not only for the Solanaceae but also in a broader spectra for other fleshy-fruited plants.

1.3 - Fleshy fruit set and ripening

1.3.1 - Plant and fruit development

During development, plants go through distinctive phases, from vegetative growth, followed by a reproductive phase and ultimately seed set and senescence. After seed germination, plants go through the vegetative stage, where plants increase their photosynthetic capacity and consequently their size and mass. This vegetative growth mode can be divided into two phases, a juvenile, and an adult vegetative phase. When the plant reaches the adult vegetative phase and the right environmental cues are sensed, the plant transitions to another phase, the reproductive phase. In this transition, plants switch from vegetative growth to reproductive growth. To do this, the vegetative shoot apical meristem (SAM) takes on an floral meristem (FM) identity and starts producing flowers (Huijser & Schmid, 2011).
With the transition to the adult vegetative phase, the plant can respond to floral inductive signals, and the plant officially enters the reproductive phase. After the flower becomes fully developed germination occurs followed by the embryo, seed, and fruit set.
Tomato flowers have at least five or more stamen that are fused to form a hollow cone around the pistil. This cone consists of sterile extensions of the anthers, and it surrounds the pistil allowing for mature pollen to be released and fall directly onto the stigma where it might germinate. When the flower reaches anthesis, the petals get fully turned backwards, and the pollen grains fall from the anthers directly to the stigma. Pollen must adhere to the stigmatic surface in order to achieve fertilization (Picken, 1984).
Fruit set marks the transition from flower to fruit and corresponds to a significant developmental shift that transforms the ovary into fruit. The transition from flower to fruit is regulated by a complex network of signalling pathways that are tightly programmed by specific genes and respond to endogenous and exogenous stimuli (Takei et al., 2019).
Upon fertilization, fruit tissues start to develop, and ovary growth and maturation evolve in a synchronized way with seed development (Seymour et al., 2013). The fruit's growth and development involve cell division and expansion of the ovary tissues until these tissues reach a certain size, and the fruit starts to ripen. In certain tomato varieties such as the miniature tomato, cv. Micro-Tom, cell division begins at two-days post-anthesis (DPA), when the ovary is one mm in diameter with ten cell layers. These cell divisions occur both in a periclinal and anticlinal plane in the pericarp collenchyma cells. Cell division usually stops ten to thirteen DPA, and cell expansion progresses at a fast rate until approximately thirty DPA when the fruit reaches a diameter of 1.5–2 cm. Cell expansion is the process that causes fleshy fruit to increase its size. In fruit growth, the cells enlarge and accumulate reserves, and when it reaches maturity, a series of changes occur, the ripening process.
The ripening of fruit organs is the last stage of development and the stage where the fruit prepares to release matured seeds (J. Giovannoni, 2004). The ripening of fruits is initiated in a coordinated

manner triggering an alteration in colour, flavour, texture, and aroma. The ripening process turns a hard, unappealing fruit into a nutritious product that appeals to consumption and to seed dispersal aid (J. Giovannoni, 2004).

1.3.2 - Cell Wall Metabolism and Softening

The variation of cell turgor and cell wall structure causes textural modifications, resulting in the fruit's softening. Softening results from cell wall disassembly and decreased cell adhesion (Vicente et al., 2007), accompanied by the solubilization of pectin and depolymerization of hemicellulosic polysaccharides. The sequenced tomato genome has revealed that more than 50 structural genes encoding known, or putative, cell-wall-modifying proteins are expressed in developing and ripening fruit (The Tomato Genome Consortium, 2012) (Uluisik et al., 2016). Out of these genes, polygalacturonase (PG), pectin methyl esterase, β-galactanase and expansin were the most significant during ripening, and all these genes have been investigated as candidates for promoting cell wall modifications that lead to softening. Also, due to these alterations, the fruits also get more susceptible to opportunistic pathogens (Gapper et al., 2013). One of the aspects of ripening is tissue softening because of cell wall–degrading activities. Cell wall softening is related to polygalacturonases (PGs). There are three PGs identified in tomato and appear to be functionally redundant. When analyzing PG mRNA expression, it can only be detected in ripening fruit and cannot be found in unripe tomatoes, leaves, or roots. Therefore the expression of this gene is developmentally regulated and must be related to fruit ripening (Bird et al., 1988). The *Pectate lyase* (*PL*) gene also appears to have a fundamental role in cell wall modifications that result in fruit softening (Uluisik et al., 2016). Uluisik et al. (2016) found that silencing five *PL* genes can alter texture of the fruit without affecting other aspects of ripening.

1.3.3 – Volatile metabolism and colour change

Fruit development and ripening involve several processes in which the biochemistry, physiology, and structure of the organ are developmentally altered to influence the appearance, texture, flavour, and aroma (Seymour et al., 2013)

The accumulation of sugars, acids, and volatile profiles affect nutritional quality, flavour, and aroma, which determine flavour. The smell of the ripe fruit is attributed to the production of a complex combination of volatile compounds such as hexanal, myrcene, and ocimene, and the degradation of bitter principles, like, tannins and flavonoids (Prasanna et al., 2007). The majority of plant volatiles originate from fatty acids (Schwab et al., 2008). The taste development is due to an increase in sweetness, which results from increased glucose accumulation, hydrolysis of polysaccharides, mainly starch, and decreased acidity (Prasanna et al., 2007).

The modification of colour occurs due to the alteration of chlorophyll, carotenoid, and flavonoid accumulation. Colour change from green to red is a significant indicator of tomato ripening. Ripening in tomato causes a change in fruit colour due to a transition from chloroplasts to chromoplasts, which is brought about by chlorophyll degradation and carotenoid synthesis (Park et al., 2018). This colour change is related to chlorophylls' degradation and the shift of the carotenoid composition from xanthophylls to carotenes (lycopene and β-carotene) (Fraser et al., 2000).

The work of (Giuliano, 2014) elucidates the diversity of genes involved in the carotenoid biosynthesis pathway that work in a coordinated way controlling carotenoid synthesis during the

ripening of tomato fruit.The carotenoid biosynthetic pathway starts with the condensation of two geranylgeranyl diphosphate molecules (*GGPP*), by the enzyme phytoene synthases (*PSY*) that forms 15-cis-phytoene. This 15-cis-phytoene is then desaturated and isomerized to all-trans-lycopene. The lycopene ε-cyclase (ε-*LCY*) and β-cyclases form δ-carotene and γ-carotene from lycopene, and then the orange α - carotene and β-carotene are synthesized by β- cyclases. Lastly, these carotenes are transformed into lutein and zeaxanthin. Zeaxanthin epoxidase (ZEP) then converts zeaxanthin to violaxanthin and further to neoxanthin. Finally, these last xanthophylls are cleaved by 9-cis-epoxycarotenoid dioxygenase (*NCED*), which is also a key enzyme in the biosynthesis of ABA(Ji et al., 2014).
In the initial steps of the carotenoid biosynthetic pathway, the key rate-limiting steps are catalyzed by the enzymes like *PSY1*, and the expression of the genes that code to the enzymes is directly regulated by the ripening inhibitor (*RIN*) protein (Martel et al., 2011).

1.3.4 - Hormonal control of climacteric ripening – the central role of ethylene

Fruits are conventionally classified into climacteric fruits, like the tomato and non-climacteric, like grape, strawberry, and others. The process of ripening is a very well-orchestrated physiological process, and because of that is under tight hormonal and epigenetic regulation, which are finely regulated by environmental stimuli. Several signalling molecules, known as phytohormones, have been associated with the control of fruit ripening. Some of these hormones are very well documented, like ethylene, Gibberellic acid and abscisic acid (ABA), with ethylene being the most studied. The particularity of climacteric fruits such as tomato shows a characteristic rise in respiration and a distinct rise in ethylene production at the beginning of ripening (Li et al., 2020). In climacteric fruits, ripening can also be induced by exposure to exogenous ethylene. In non-climacteric fruits, there is no respiratory increase, and ethylene does not seem to be critical for the ripening process (Seymour et al., 2013).
Two systems in climacteric plants regulate ethylene regulation. The first, System 1, functions during the vegetative growth, is ethylene auto-inhibitory and is responsible for producing basal ethylene levels that are detected in all tissues, including those of non-climacteric fruit. The second, System 2, operates during the ripening of climacteric fruit (Alexander, 2002).
The autocatalytic ethylene biosynthesis of the system-2 only is active during ripening, and it involves specific forms of *ACO* and *ACS* that produce much higher levels of ethylene that are not autoinhibited. The ethylene produced in this stage of ripening is then perceived by a family of receptors, the ETHYLENE RESPONSE, specifically *ETR4* and *ETR6* (Seymour et al., 2013).
Abscisic acid has been studied as a promoter of ripening in tomato. When the gene *SlNCED1*, which encodes for 9-*cis*-epoxycarotenoid dioxygenase and catalyzes a crucial step in ABA biosynthesis, is suppressed, it is possible to see that several ripening related factors are affected and that retardation of ripening is apparent in some fruits (Seymour et al., 2013).

1.4 – Molecular control of fruit ripening

1.4.1 - Role of transcription factors in ripening

In plants, organogenesis is the process of growing new organs, which occurs continuously throughout the plant life. The development of new organs is controlled by distinct genetic pathways. This development is established by cell differentiation and elongation. In recent years, it has become evident that to determine cell fate this process must be precisely coordinated both

in time and space. The ripening of the tomato fruit starts at the distal end of the fruit (Blanco-Ulate et al., 2016). Complex positive and negative regulatory mechanisms emerge at the start of maturation and impact on the ripening progress. The reprograming of fruit development to initiate ripening is controlled by complex pathways that are triggered by several transcription factors while hormones and other molecules function downstream to activate various ripening pathways) (Klee & Giovannoni, 2011).

There are a few tomato ripening mutants that have been identified, and these mutants´ physiological characterization suggests that they may have defects in ripening regulatory systems. These mutations are the *ripening-inhibitor* (*rin*), *nonripening* (*nor*), and *Colorless nonripening* (*Cnr*) mutations. These mutations are a result of spontaneous alleles that share common physiological characteristics. All three mutants develop to the mature green stage, where the fruit reaches full size and the seeds mature, yet the subsequent development stages do not progress, and the fruits do not advance to ripening. They fail to produce ripening-associated ethylene, meaning they do not get through the climacteric rise in respiration. And lastly, these mutants do not ripe in response to exogenous ethylene (Li et al., 2020).

All three genes encode transcription factors, and altogether these characteristics suggest that the *rin*, *nor* and *Cnr* mutants are impacted in the central ripening phenomena. It is hypothesized that such genes might represent conserved regulators affecting ripening even in non-climacteric fruits (Klee & Giovannoni, 2011).

In previous studies, *RIN* mutants presented softening rates similar to wild-type fruits, although detailed analysis showed that the expression of some cell wall-modifying enzymes was delayed (Li et al., 2020). *RIN*-deficient fruit also never completely ripened, even when supplied with exogenous ethylene. Also, *RIN* expression is enhanced by ethylene and that RIN is required for the autocatalytic system-2 of ethylene production and consequent full ripening (J. Giovannoni et al., 2017).

The *RIN* gene is very well-studied and has shown not to be directly involved in the ripening pathways but works as a trigger for these pathways to start and induce ripening in fruits. Even though *RIN* plays a crucial role in the full completion of fruit ripening, its expression might regulate downstream ripening controllers. A study on the RIN gene showed that a specific gene from the *GRAS* family (*SlGRAS38*) is a direct target of *RIN* (Fujisawa et al., 2012). This gene has a putative binding site for *RIN* and exhibit *RIN*-dependent regulation during fruit ripening, suggesting a piece of important evidence to clarify the complex transcriptional cascade for fruit ripening (Fujisawa et al., 2013).

1.4.2 - The GRAS gene family

The *GRAS* gene family is a plant-specific family of transcription factors with key roles in the regulation of plant organ development. The *GRAS* plant gene family was defined based on nuclear localization, DNA binding and transcriptional activation features (Grimplet et al., 2016). The name *GRAS* derives from the first three identified members in *A.thaliana*: gibberellic acid-sensitive (*GAI*), repressor of GA1(*RGA*) and scarecrow (*SCR*). A genome-wide analysis revealed that this gene family is widely distributed throughout the plant kingdom (Grimplet et al., 2016). *GRAS* proteins are typically 400-700 amino acids in length and share highly conserved motifs in the C-terminus, whereas the N-terminal part is highly variant both in sequence and length. The variable N-terminal a.a sequence may be responsible for the specificity of regulatory functions. The N-terminal sequence also establishes the family groups in which the *GRAS* family is divided: *DELLA, HAM, LISCL, PAT1, LAS, SCR, SHR* and *SCL3* (Grimplet et al., 2016).

The *GRAS* transcription factor family has been characterized in several plant species (Grimplet et al., 2016; Huang et al., 2015; Kumari et al., 2021) and has shown to participate in regulation of organ development. The analysis of this transcription factor family expression in different plant tissues indicated that the organogenesis processes are under the regulation of *GRAS* transcription factors. Despite the series of *GRAS* gene expression patterns that have been studied, the comprehensive understanding of such large families demands the characterization of their pathways, such as downstream targets, post-translational modifications, and protein localization. Not only is the *SlGRAS38* gene, a member of the tomato *GRAS* family, a direct RIN target, but this gene family has also been characterized in other fleshy fruits, such as grapevine and has shown to have an essential role in plant growth and development. Additionally, in *Arabidopsis*, following fertilization, gibberellic acid promotes fruit growth by promoting the degradation of growth-repressing proteins, such as DELLA proteins, a group from the GRAS gene family (Seymour et al., 2013).

By comparing the information available for tomato and grapevine GRAS genes, Grimplet et al. (2016) identified candidate genes that might constitute conserved transcriptional regulators of both climacteric and non-climacteric fruit ripening and that deserve further functional analysis (Grimplet et al., 2016). This study also has shown that the expression pattern of *VviPAT6* an orthologous gene of *SIGRAS10*, varies during ripening in the fruit. In strawberry, a gene from the GRAS family, *FaSCL8*, was shown to play a role in the expression of genes related to anthocyanin production, ripened fruit receptacles, and the flavonoid pathway (Pillet et al., 2015).

A study by Huang et al. (2015) showed *SlGRAS9* and *SlGRAS10* had conserved expression patterns, implying genetic redundancy retention (Huang et al., 2015). Therefore, it is suggested that *SlGRAS9* and *SlGRAS10* might compensate for each other in a functional redundancy and neofunctionalization way. In recent work, the knock-down of SlGRAS10 alone appear to have a slight impact on ripening and, unexpectedly, an effect on the number of seeds, which decreased.

1.4.3 - New Genomic techniques and CRISPR-cas9

Tomato is an economically important crop worldwide. Its demand has also created a need to produce more high-yielding varieties with better nutritional quality and allow distribution reducing significant losses.

In recent years, plant genetic modification techniques, like gene editing, have evolved quickly, allowing faster and more precise results than conventional plant-breeding methods. These gene-editing techniques are promising to the agriculture industry, offering great potential to generate new varieties (Seymour et al., 2013)(Purnhagen et al., 2018)

With the advance of high-throughput sequencing technologies, genetic and epigenetic variation has been revealed on a genome-wide scale and consequently open to new insights into the evolution of genomes and trait variation. With sequencing becoming more accessible, more genomes have been sequenced in recent years, making breeding fruit crops easier. With the genomes sequenced marker-assisted selection is facilitated. This allows for an easy selection of genes and efficient cloning of those genes that encode for quantitative trait loci, and it also increases prospects to optimize functional gene studies. Genome-wide information also allows rapid analyses of gene structure, binding-site motifs, and gene regulatory regions (Seymour et al., 2013).

According to EU legislation, GMOs must be identifiable using detection methods, nevertheless the plants grown using many of these new methods can hardly be distinguished from conventionally bred plants since no foreign DNA is kept in their genome after segregation.

Consequently, it is often difficult to understand whether the mutation was natural or triggered by one of these new breeding techniques (Purnhagen et al., 2018).
Traditional breeding techniques can undoubtedly bring many benefits and should not be overlooked. However, they tend to be complicated and very time-consuming (Ahmar et al., 2020). Thus, it is undoubtedly imperative to develop and improve faster and more effective plant improvement methods that can respond to the ever-growing population.
The recent progress of sequencing techniques and advances in bioinformatics have generated enormous amounts of genetic resources that, through gene editing, can be used to study the function of genes and to identify biological mechanisms that can be explored in the context of plant improvement.
The term New Plant Breeding Techniques has been used frequently in recent years. New Plant Breeding Techniques does not refer to a single technique, and the techniques encompassed in this term do not cause major genomic changes.
New Plant Breeding Techniques is a general term for plant breeding techniques that do not introduce foreign DNA in the genome. Techniques such as genome editing, epigenome editing, cisgenesis, grafting on genetically modified rootstock are included in the New Plant genomic Techniques category.
Genome editing allows specific insertions, deletions, and DNA sequence sequences modifications in specific genome regions. The first technique to be described was the Zinc finger nucleases (Urnov et al., 2010) followed by the Transcription activator-like effector nuclease, TALEN (Joung & Sander, 2013) and more recently, the clustered regularly interspaced short palindromic repeats (CRISPR) associated with the endonuclease Cas9. This last gene-editing technology that has been developed in recent years, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - Cas9 system, is a versatile, simple, fast and low economic cost technique that allows precise mutations to be generated without the need of permanent integration of the foreign DNA in the plant (Cui et al., 2018).
Due to years of breeding crops for productivity has led to a loss of diversity and characteristics related to disease and environment resistant but also to quality characteristics, like flavour and smell. Many domestication traits follow the simple rules of Mendelian inheritance and mutations of loss of function and gain of function are enough to generate a new phenotype. This information means that it is possible to restore these traits with genetic engineering, using technology like CRISPR-Cas9. Consequently, genome editing could be used to domesticate wild plants and regain lost but desirable traits, including nutritional qualities, productivity, stress resistance, and other agronomically valuable characteristics (Zsögön et al., 2018). Zsögön et al. set out to create a new crop derivative from the ancestral progenitor of S. lycopersicum, the S. pimpinellifolium. To do this, a set of genes was targeted using a multiplex CRISPR–Cas9 approach to create loss-of-function alleles, in loci that had previously been proposed to have a role in the domestication process. This targeted reverse genetic engineering of wild plants has showed that it is possible to create new crops quickly and also that this technique can also be used in many crops and be applied broadly across all cultivated plants (Zsögön et al., 2018).

1.5 - Scope and research goals

Tomato is one of the most relevant fruit crops in the world and acts as a crop model for plant biology and genomics in fleshy fruits. Fresh and processed tomato products play a crucial part in the human diet, providing essential minerals, vitamins and health promoting phytochemicals.
Tomato is important for worldwide economy and Portugal being the third biggest producer in Europe as of 2020. However, post-harvest losses may account for a total loss of 39.3% from the producer until it reaches the consumer (Abera et al., 2020). Also, tomato production involves a considerable use of pesticides which impose health risks and environment pollution.
Gene editing allows to manipulate the biological processes that regulate the pathways responsible for ripening and senescence, the characteristics that lead to produce postharvest loss and waste. Out of the most common gene editing tools, CRISPR is relatively cheap and easy, with minimal impact on the genome yet producing very significant results, allowing breeders to feasibly engineer postharvest traits and have that new germplasm be commercially viable. CRISPR allows to remove or insert nucleotides causing mutations and promote traits seen as desirable. Various CRISPR techniques and approaches have been developed and can be used to introduce nuanced changes in the expression of single and multiple genes, which proves a real asset to dissect the biological networks that regulate ripening and senescence (Shipman et al., 2021).
The sequencing of the tomato genome and genome wide analysis of tomato gene families, such as the *GRAS* gene family has revealed that the development and ripening of fruits could be under control of *GRAS* genes, since the expression of many of them is modulated during this process. In the study by (Grimplet et al., 2016)) some genes were chosen as robust candidates for further functional analysis. A functional analysis of these genes may contribute to the improvement of fruit quality as well as resistance to biotic and abiotic stresses by the development of breeding program.
There is a lot of phenotypic diversity in tomato that can be explained by many domestication traits accumulated in the crop variety *Solanum lycopersicum*. These traits can cause new phenotypes with mutations of loss of function and gain of function on genes that play an important role in those traits.
In tomato, the most economical essential traits relate to fruit quality. Since fruits start to develop their organoleptic quality increases with maturity, but also their vulnerability to infection by fungi and loss of quality by over-maturing. Therefore, to unveil the master regulators of fruit ripening it is possible through breeding programs to understand and control ripening increasing fruit shelf-life while retaining fruit quality (Fig.1.5.1).

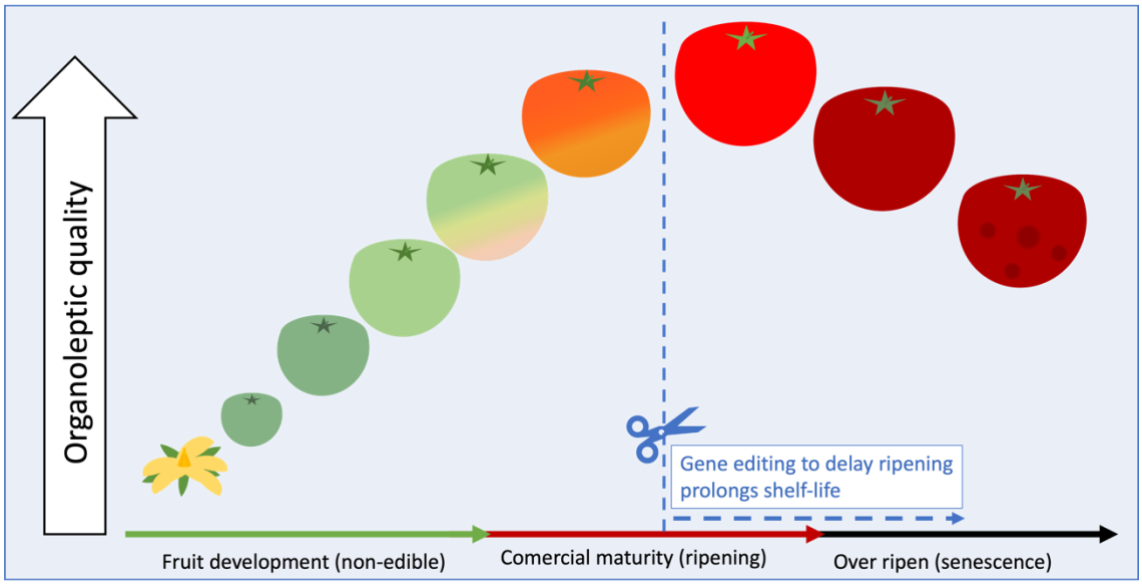

Figure 1.5.1 - Effects of ripening on tomato fruit quality

A few studies based on genome wide analysis of the *GRAS* gene family have revealed that some genes belonging to this gene family might have a role in fruit ripening along with other roles in plant development (Grimplet et al., 2016; Huang et al., 2015). Nevertheless, fruit ripening is an extremely complex process that is highly coordinated, and the triggers that elicit this process are still not very well known. Therefore, the study of genes like *SlGRAS9* and *SlGRAS10* will contribute to establish a more complete molecular model of tomato fruit ripening.

The goal of this Master Thesis is to contribute for the improvement of tomato production by increasing shelf life. In this context, we aim at controlling fruit ripening and to study whether the SlGRAS*10* gene causes changes in the phenotype when mutated and if they affect the expression of other genes directly linked to ripening. The primary goal is to study the functional role that *SlGRAS*10 has in tomato fruit ripening and how that role can impact shelf-life. So, this project will evaluate CRISPR-Cas9 genetically edited tomato lines with a knockout on the *SlGRAS*10 gene for their potential to impact agronomic and quality traits and to generate data for molecular characterization of these lines, and also start analysis on lines with a knock-out with *SlGRAS9* and *SlGRAS*10 knocked-out.

2 – MATERIALS AND METHODS

2.1 - Plant material, plant transformation and acclimatation

Plants of the tomato *MoneyMaker* variety were used in this study.
Previous to this work, within the scope of Flávio Soares' PhD thesis, *In vitro* grown seedlings were used as the sources of initial explants. In order to generate the transformed lines cotyledons from the initial seedlings grown from seed were used to induce calli. Tomato cotyledons were co-cultured with *Agrobacterium tumefaciens* containing the vectors for 48h at 23ºC in the dark. The explants were transformed, through CRISPR- Cas9 mediated transformation with *Agrobacterium tumefaciens*. The gRNAs constructs were developed previously using the adaptation of the RNA-guided Cas9 system to GoldenBraid as described in (Vazquez-Vilar et al., 2016). After co-cultivation, the explants were transferred to the shoot induction medium I. After 10 days had passed the explants were transferred into the shoot induction medium II. If the *calli* were underdeveloped the *calli* were transferred into new shoot induction medium II when 21 days had passed. When *calli* were well developed, the buds were separated and were transferred to root induction medium. Once a fully regenerate seedling with leaves and roots had formed *in vitro* these seedings were transferred to soil and put in the climatic chambers (Figure S1.4).

These mutations gave rise to three work topics, as listed in Figure 2.1.1, one related to fruit ripening on the single SlGRAS10 mutants, another related to seed development on SlGRAS10 mutants and fruit ripening on the double mutants. The SlGRAS10 lines were edited using different sgRNAs.

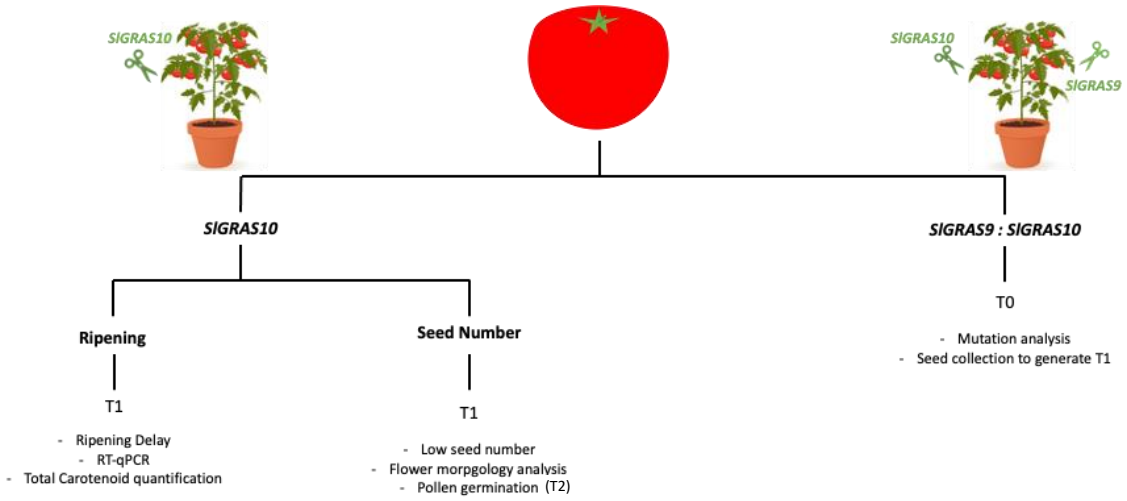

Figure 2.1.1 – Schematic representation of the different topics studied in this thesis covering fruit fertilization and ripening.

The subsequent generations were obtained by germinating seeds of mutated plants *in vitro*. The seeds were submerged in a solution of 30% bleach and then in autoclaved ultra-pure water for 30 minutes. Then the seeds were sequentially washed in new autoclaved ultra-pure water for 5 minutes, 10 minutes and lastly for 15 minutes. The sterilized seeds were then germinated on half-strength Murashige and Skoog (MS) medium (Murashige & Skoog 1962) with vitamins in MES buffer. The medium contained 1,5% (w/v) sucrose, 1% phyto-agar at pH between 5.6 to 5.8

These seeds were grown in climatic chambers under 15 h light/1h adaptation/7 h/1h adaptation dark conditions with 70% of fluorescent light at 300 μmol m^{-2} s^{-1} and a temperature of 21 °C in light conditions and 18 °C in adaptation and dark conditions. After a few weeks the seedlings were transplanted to soil and kept in the same conditions in a ARALAB walk-in chamber.
When the plants had grown enough, they were transferred to a climatic chamber with 15 h light/1h adaptation/7 h dark conditions with 100% of fluorescent light at 300 μmol m^{-2} s^{-1} and a temperature of 25 °C in light conditions and 22°C in adaptation and 20°C in dark conditions.

2.2 - Phenotypic analysis

All mutant and wild-type control plants lines were grown together in the climatic chambers. All plants were accessed during their development for morphological alterations, such as the number of branches until the first flower bud appeared, the number of days until anthesis, and when the fruit transitioned from mature green to ripe (Breaker) (data not shown). All fruits were marked when the flowers were at anthesis, and the tagged fruits were annotated when they reached the Breaker stage (transition from mature green to ripe). When collected, the fruits were measured (diameter and height), weighted, and the number of seeds was counted. After collection, to collect the seeds, the fruits were cut transversely, and pictures were taken (data not shown).
The seeds from each fruit were collected and treated in a solution of distilled water and hydrochloric acid overnight, then the seeds were sieved and dried out and finally stored in a cool, dry, room temperature conditions.
To analyze flower morphology, fresh flowers were collected in anthesis and further stages of development and kept in a humid chamber until the moment of observation. The flowers and different organs were observed under a Zeiss SteREO Lumar V.12 stereoscope. The taken images were then analyzed using the Fiji image processing package.

2.3 - DNA extraction and genotyping

DNA was extracted from the tissue of young leaves that were collected from all the plants. Approximately 100 mg of plant material was macerated in liquid nitrogen. To extract the DNA a DNA extraction buffer (1,4 M NaCl, 20mM EDTA, 100mM Tris-HCL at pH 8.0, CTAB 2% and ß-mercaptoethanol 2%) was prepared. The extraction buffer was previously heated to 65ºC. To extract the DNA 600 µL extraction buffer was added to each sample, and the samples were thoroughly mixed in the vortex and incubated for 45 minutes at 65ºC. After incubation 600 µL pf chloroform: isoamyl alcohol 24:1 (v/v) were added, and the mixture was centrifuged for 15 minutes at 13.000rpm. The supernatant was collected to a new tube (approximately 500 µL). To eliminate RNA contamination, 1 µL of RNase (100µg/µL) was added to each sample, and the samples were incubated at 37ºC for 30 minutes. To precipitate the DNA 1 volume of isopropanol was added and the samples precipitated in ice for 20 minutes. Another centrifugation at 13.000rpm for 10 minutes at 4ºC was performed in order to separate de DNA pellet. The supernatant was discarded, and 1 volume of cold ethanol 80% was added and mixed by vortexing. The sample was centrifuged again for 10 minutes at 13.000rpm at 4ºC. The supernatant was discarded again, and the samples were incubated at room temperature in order for the ethanol to

evaporate. After the pellet was resuspended in 50µL Mili Q water. After extraction, the DNA quality was verified in a 1% agarose gel. The DNA purity and concentration were measured using a Nanodrop spectrophotometer. The DNA purity was determined by the 260/280 and 260/230 ratios.

2.3.1 - PCR for Cas9 detection

To confirm if the plants had been correctly infected with the vector containing the CRISPR-Cas9 construct a PCR was performed, in order to amplify the Cas9 sequence, if present in the plant tissues. To do this, the DNA was extracted from approximately 100 mg of young leaf's tissue that was collected from all the plants. The experience used a negative control, which used the DNA from a wild-type plants, a positive control, with DNA that has Cas9, a blank control with just the PCR Master Mix and water, to compare with the samples. After the DNA was extracted, a PCR was performed in order to detect the presence of Cas9. The cas9 PCR primers (Table S2.1) were specific for the Cas9 sequence. The program used in these essays started with a step at 95ºC for 5 minutes, followed by a series of 25 cycles that consisted of a denaturation step at 95ºC for 30 seconds, an Annealing step at 56ºC for 30 seconds and an extension step at 72ºC for 60 seconds. After the 25 cycles, another extension step was taken at 72ºC for 10 minutes and a hold step at 4ºC until the samples were retrieved from the thermocycler.
The PCR products were then used to perform a gel electrophoresis in order to confirm if the transformed plants had Cas9 present, indicating that they were correctly transformed, in the case of T0 plants. Only the samples that had Cas9 present were sequenced.

2.3.2 - Sequencing of transformed plants

To verify if the plants had been transformed, sequencing of the transformed plants followed by a TIDE analysis (Tracking of Indels by Decomposition) was performed. To do this, the DNA was extracted from approximately 100 mg of young leaf's tissue that was collected from all the plants. After the DNA was extracted, a PCR was performed in order to amplify the region of DNA that contains the mutation target area. The PCR primers (Table S2.1) flanked the region of the RNAguides used to transform the plants. The program used in these essays started with a step at 95ºC for 5 minutes, followed by a series of 25 cycles that consisted of a denaturation step at 95ºC for 30 seconds, an Annealing step at 57,5ºC for 30 seconds and an extension step at 72ºC for 60 seconds. After the 25 cycles, another extension step was taken at 72ºC for 10 minutes and a hold step at 4ºC until the samples were retrieved from the thermocycler.
The PCR products were then sent to be sequenced and analyzed using the TIDE software (Brinkman et al., 2014).

2.4. – RT-qPCR

2.4.1 - RNA extraction and precipitation

In order to analyze gene expression, RNA was extracted from the pericarp of fruits previously macerated in liquid nitrogen. To extract the total RNA from the samples, 350mg of sample were weighted to use the Macherey-Nagel NucleoSpin RNA Plant, Mini kit for RNA from plant. The RNA concentration was not enough for cDNA synthesis, so a precipitation step was required. To do so, for each sample 0.1 volumes of 3M NaOAc and 2.7 volumes of 100% cold ethanol were added and mixed gently After the precipitation step, the samples were centrifuged at 10,000× g for 20 minutes at 4 ºC, the supernatant was discarded, and 150 µL of 70% cold ethanol was added to the samples. Then the samples were centrifuged again at 10,000×g for 8 minutes at 4 ºC. And once again the supernatant was discarded, and the pellet was left to dry in the fume chamber for 15 minutes. After that, the pellet was solubilized in 20 µL DEPC- water by pipetting. After extraction, the RNA quality was verified in a 1% agarose gel stained with GreenSafe Premium (NZY Tech). The RNA purity and concentration were measured using a nanodrop spectrophotometer. The RNA purity was determined by the 260nm/280nm and 260nm/230nm ratios.

2.4.2 - Synthesis of cDNA

First-strand cDNA was synthesized from 1µg of total RNA extracted from tomato fruit pericarp. To synthesize cDNA, the Thermo Scientific RevertAid H minus Reverse Transcriptase protocol was followed. For each sample, 11,5µL of DEPC treated water and extracted RNA were added to a sterile microtube with 1µL of oligo dT primers. The tubes were mixed gently and incubated at 65ºC for 5 minutes, after which they were centrifuged briefly (spin-down) and put on ice. After that, 4 µL of reaction buffer (5x), 0,5 µL of RNase inhibitor, 2 µL of a dNTP mix, and 1 µL of RevertAid H Minus reverse transcriptase were added to each sample, in this order. The samples were centrifuged briefly again to spin down the contents and incubated for 60 minutes at 42ºC. The final cDNA was stored at -20ºC.

2.4.3 - Gene expression analysis - qPCR

The qPCR assays were conducted using the StepOne Real-Time PCR System (Applied Biosystems). The cDNA from the samples that were to be analyzed were diluted in a proportion of 1:40. The primer pairs were designed using the DNASTAR Primer Select software.
A calibration curve was also prepared using a mixture of all the samples. The calibration curve followed the next dilutions ratios - 1:5, 1:20, 1:100, 1:500 and 1:2500. A calibration curve allows to perform an absolute quantification permits to directly determine the samples C_Ts to the curve.

In each qPCR reaction plates with 48 wells were used where the first wells were used for the dilution series in which each sample had replicates. The remaining wells were used for the samples, with three biological replicates for each sample and one well for the negative control.
For each plate, a master-mix was prepared for a total of 49 reactions to avoid pipetting errors. To each well 9 µL of Sybr Green, 0,63 µL of each primer (forward and reverse, diluted to 10µM from a stock solution of 100 µM), 3,74 µL of ultra-pure water, and 4 µL of the sample cDNA were added, in this order. The master-mix was prepared and distributed in the wells inside a UV chamber.
The program used in these essays consisted of 42 cycles with a starting step at 95ºC for 10 minutes followed by the denaturation step at 95ºC for 15 seconds, an annealing step for 40 seconds whose temperature was based on the calculated melting temperature of the primers being used (Table S2.4).
The melt curve was performed to assess the dissociation characteristics of double-stranded DNA and followed the following steps. A denaturation step at 95ºC for 15 seconds followed by an elongation step at 60ºC for 1 minute and then an extension step at 95ºC for 15 seconds.
The method $2^{-\Delta\Delta}C_t$ was used to determine the relative expression of the target genes, in the end, since the calibration curve was not linear and sensitive enough to get good results from it. When treating the Ct values, the data were normalized using the actin gene expression (*Solyc04g011500*) and Clathrin adaptor complex medium subunit (*AT5G46630*), which are the most stable genes during the ripening phenomenon, according to (Expósito-Rodríguez et al., 2008)). Relative quantification depicts the variation in the expression of the target gene in contrast with the reference genes.
The C_t values from the target genes were linearised using the least-squares method by LinRegPCR ((11.0) version 7.4). And were imported into a Microsoft Excel spreadsheet and there the $^{\Delta\Delta}C_t$ and $2^{-\Delta\Delta}C_t$ values were determined. In Table S2.5 are the $^{\Delta\Delta}C_t$ and $2^{-\Delta\Delta}C_t$ results values from each gene tested. The fold change between the control and the mutated samples represents the relative gene expression levels due to the mutation in the different lines.

2.5 - Carotenoid extraction and quantification

Carotenoids were extracted from the tomato fruit pericarp for quantification using the protocol described in Zhang et al., 2018. To extract the carotenoids approximately 50 mg pericarp previously ground in liquid nitrogen, then 800 µL of hexane: acetone (6:4, v/v) were added to the microtube. The samples were then mixed by vortexing for 5 minutes. After vortexing, the samples were centrifuged at 5000 rpm for 5 minutes, where the samples were separated into two phases. The supernatant was collected and transferred to a new microtube. After the first extraction, the steps were repeated as often as necessary to the supernatant become colourless. Using a spectrophotometer and the hexane: acetone (6:4, v/v) as a blank, the supernatant's absorbance was then measured at 450 nm. The volume of collected supernatant was measured by pipetting. Lastly, the total carotenoid content was quantified using the adapted equation $total\ carotenoids(mg\ ml–1) = 4\times(OD450)\times V_{supernatant}(mL)/sample\ weight(g)$ to calculate total carotenoids (Zhang et al., 2018).

2.6 – Pollen germination Assay

Medium for pollen germination (0,1% Boric Acid, 10mM MgSO4|7H2O, 10mM CaCl2|7H2O, 50mM KCl and 10% sucrose) was prepared based on (Boavida & McCormick, 2007) protocol. The medium was prepared in a stock solution, the pH was adjusted to 7.5 pH and the solution was aliquoted and stored at -20ºC. Each germination assays the aliquots were thawed and 1,5% of agarose was added.

Pollen was extracted from fresh flowers that were collected in anthesis and kept in a humid chamber for a maximum of three hours until the pollen germination assay was performed. To extract pollen, the anthers were carefully removed from de rest of the flower and cut open to shake the pollen out into the medium on the slide with medium spread evenly. The pollen was then germinated on a humid chamber for two hours at 25ºC. After the two hours the pollen was observed under a Zeiss SteREO Lumar V.12 stereoscope. The taken images were then analyzed using the Fiji image processing package.

2.7 - Statistical analysis

All the results are expressed as mean ± standard error of the mean (SEM). The data was examined by the t-test using the GraphPad software, with a statistical significance of $p \leq 0.05$.

3 – RESULTS

3.1 – Line generation and in vitro germination

Before starting this work, mutated lines were generated through CRISPR-Cas9 mediated knock-out mutations delivered via *Agrobacterium tumefaciens.* Two different lines were generated, one with a single mutation on the *SlGRAS10* gene and another line with both *SlGRAS10* and *SlGRAS9* knocked out. In the *SlGRAS10* knock-out lines, two separate target sites were selected to construct individual vectors using GoldenBraid 2.0 CRISPR domesticator vectors (Vazquez-Vilar et al., 2016). Tomato cotiledons were co-cultured with *A. tumefaciens* containing the vectors for 48h at 23ºc in the dark. After co-cultivation, the explants were transferred to the shoot induction medium I (Figure 3.1.1.(a)). After 10 days had passed (Figure 3.1.1 (b)) the explants were transferred into the shoot induction medium II (Figure 3.1.1 (c)). If the calli were underdeveloped the calli were transferred into new shoot induction medium II when 21 days had passed. When calli were well developed, the buds were separated and were transferred to root induction medium. Once a fully regenerate seedling with leaves and roots had formed in vitro (Figure 3.1.1 (d)) these seedings were transferred to soil and put in the climatic chambers. Mutations in the targets were detected by Sanger sequencing.

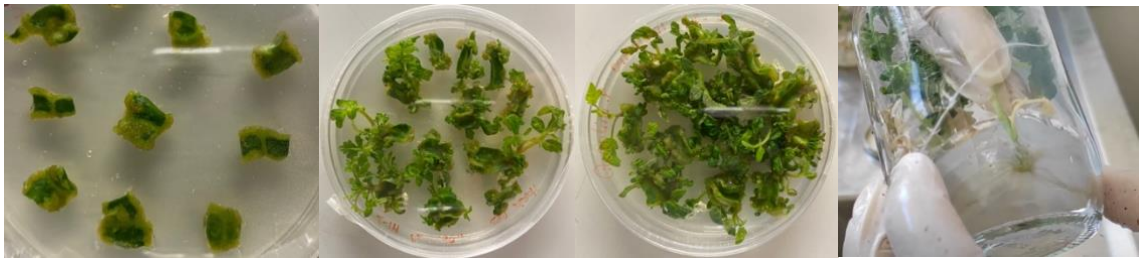

Figure 3.1.1 - Regeneration of tomato (S. lycopersicum cv. MoneyMaker) explants by using a) cotyledon as explants.; b) regenerated shoots from cotyledon; (c) developed shoot buds; (d) regenerated fully rooted plantlet ready to be potted.

These mutations gave rise to the three work topics, the first where fruit ripening dynamics and seed development were analyzed, the single SlGRAS10 mutants and the other topic where the stabilized double mutant lines were grown to generate the T1 generation where the ripening dynamics are going to be further investigated.

3.2 Assessment of plant genotype

To identify mutations in the mutant tomato plants DNA was extracted from young leaves and a PCR was performed to detect the presence or absence of Cas9 in the plants (Figure S1.2). To determine the type and mutation rates another PCR was performed to amplify the region surrounding the mutation site. This PCR products were then sent to be sequenced by Sanger sequencing. The data from the Sanger sequencing was then analyzed using the web tool TIDE.
The lines that targeted the *SlGRAS10* gene were generated using two different sgRNAs. The first one targeted the 37th nucleotide on the *SlGRAS10* gene, the second guide targeted the 909th position. The plant lines that were edited using these sgRNAs were already stable and had high frequencies of mutation. After sequencing, it was possible to determine the InDels and how these InDels affected the transcription factor functions. All the insertions and deletions gave rise to a

premature STOP codon that will lead to a truncated protein, except the deletion of three nucleotides which caused the deletion of an amino acid which should form a non-functional protein (Figure 3.2.1).

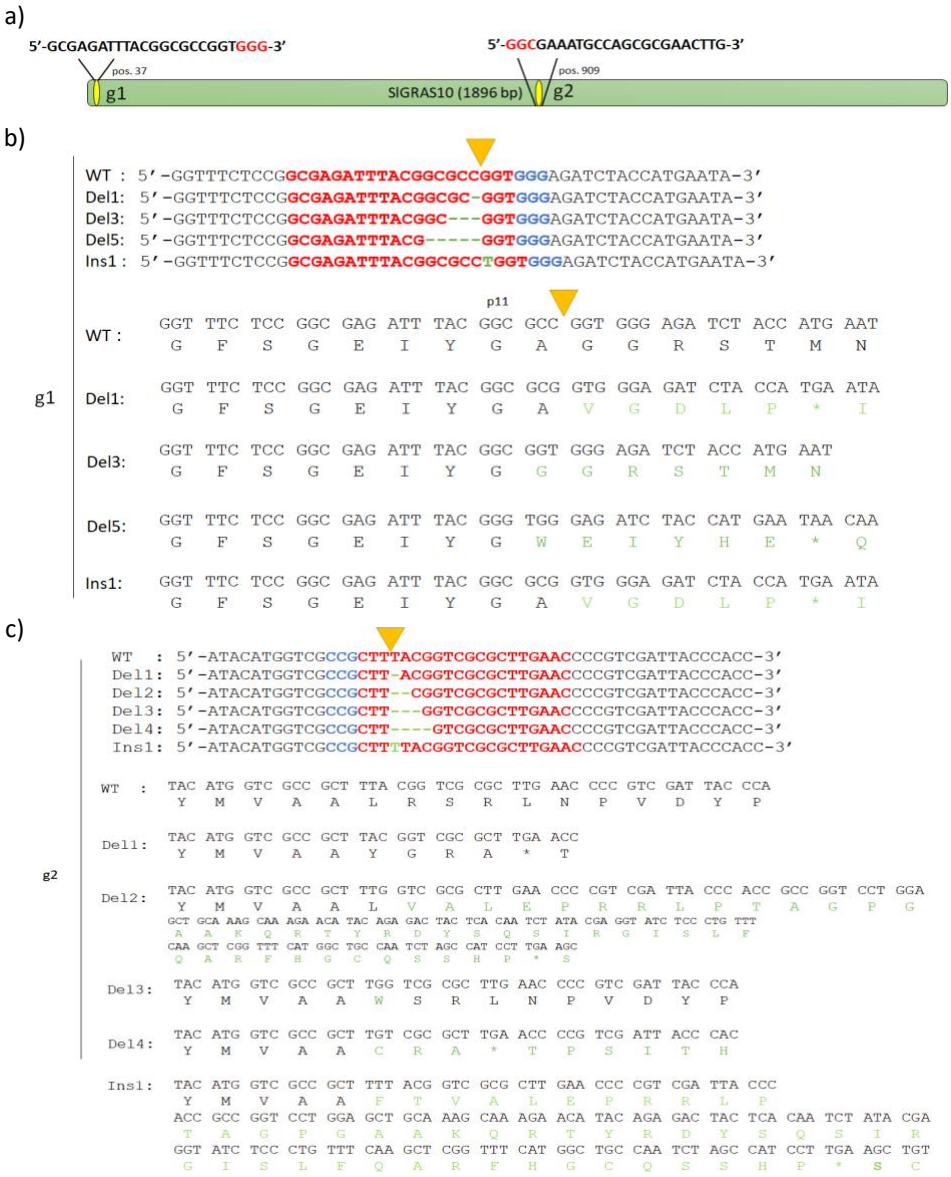

Figure 3.2.1 – CRISPR-Cas9 induced SlGRAS10 mutations in SlGRAS10 mutant T1 tomato plants. a) Schematic representation of the SlGRAS10 gene and guideRNA sequences. b) Mutation sequences in edited tomato plants transformed with the guideRNA designated g1 and respective amino-acid sequence. c) Mutation sequences in edited tomato plants transformed with the guideRNA designated g2 and respective amino-acid sequence. In both b) and c) the Wt is shown first with a yellow triangle representing the PAM break-zone. Green dashes indicate number of deleted nucleotides and green letter indicate the inserted nucleotide. The asterisk indicates a premature STOP codon.

The plants analyzed were grown i*n vitro* from seeds collected from the previous T0 line. This originated the T1 *SlGRAS10* plants. After the seedlings were transferred to soil and the first leaves started to develop a few young leaves were collected to analyze. These leaves were used to extract DNA and to test for Cas9 presence. All the stabilized lines (no longer had Cas9 present) were sent to be sequenced. The 2B5 and 2B4 lines are the ones that have the highest degree of mutation and are homozygotic. The other lines have a slight lower percentage of mutation, but still very high,

the 2A3, 2B1 and 2B2 lines and are biallelic, as can be seen in Table 3.2.1. These lines were selected for further molecular analysis regarding fruit ripening.

Table 3.2.1 –Estimates of the spectrum and frequency of small insertions and deletions (indels) generated in a pool of cells edited with CRISPR/Cas9-induced SlGRAS10 mutation in transgenic tomato plants from T1 generation.

Plant ID	Zygosity	Genotype	Mutation (%)	Cas9
1A1	Heterozygote	wt, d5	46	+
1A2	Homozygote	d5	95	+
1A3	Heterozygote	wt, d5	45	+
1A4	Heterozygote	wt, d5	47	+
1A5	Heterozygote	wt, d5	48	+
1A6	Heterozygote	wt, d5	43	+
1B1	Biallele	d3, i1	86	+
1B2	Homozygote	d1	96	+
1B3	Homozygote	d1	95	+
1B4	Biallele	d3, i1	84	-
2A1	Biallele	d4, d3	90	+
2A2	Biallele	d4, d2	82	+
2A3	Biallele	d4, d3	89	-
2A4	Homozygote	d3	89	+
2A5	Homozygote	d4	89	+
2B1	Biallele	d3, d2	87	-
2B2	Biallele	d3, d2	89	-
2B3	Homozygote	d3	88	+
2B4	Homozygote	d2	90	-
2B5	Homozygote	d3	94	-
2B6	Homozygote	d3	96	-
2B7	Chimera	d3, d2, d1, i1	87	+
2B8	Chimera	d9, d3, d1	92	+

In grey, samples used for RNA extraction and RT-qPCR – Ripening phenotype

3.3 Assessment of molecular and metabolic parameters of fruit ripening in SlGRAS10 mutants

3.3.1 - Optimization of RNA extraction protocol

To analyze the ripening at a molecular level in the *SlGRAS10* mutated plants, fruits at a Breaker +7 days (Br+7) and Breaker +10 days (Br+10) were used to do an analysis by RT-qPCR and to study carotenoids, an important metabolite related to ripening.

Prior to RT-qPCR analysis RNA was extracted from tomato fruit pericarp using the NucleoSpin RNA Plant, Mini kit for RNA from plant protocol and due to the nature of the plant material the yield was not enough for the reverse transcriptase reaction. Therefore, an additional step of precipitation was performed in order to get higher concentrations. Two separate RNA extractions (Table 3.3.1.1) were performed and diluted in the volume of UP-RNase-free water indicated in the kit protocol. Then, the two samples were precipitated together and diluted again as one sample. This additional step did not cause RNA degradation, nor did it contaminate the RNA samples with DNA, the samples had high quality and integrity with a high yield allowing for being converted to complementary DNA (cDNA) in order to perform an analysis by RT-qPCR.

Table 3.3.1.1 – RNA quantification on the Nanodrop prior to precipitation and after precipitation step. The RNA was obtained from tomato fruit pericarp of wild-type plants and SlGRAS10 knock-out plants at two different ripening time points, Br+7 and Br+10.

Plant ID	ID Fruit	Stage	RNA (ng/uL) before precipitation and purity ratios						precipitated RNA (ng/uL)		
			1st extraction	260nm/ 280nm	260nm/ 230nm	2nd extraction	260nm/ 280nm	260nm/ 230nm	1st + 2nd	260nm/ 280nm	260nm/ 230nm
Pool Wt1	Wt1_3 Wt1_4 Wt1_5	Br+10 Br+10 Br+10	26,4	1,76	1,81	30,7	1,75	1,81	208,3	1,91	2,28
Pool Wt4	Wt4 B Wt4_F	Br+10 Br+10	34,7	1,79	1,94	25,7	1,78	1,81	226,0	1,96	2,29
Pool Wt5	Wt5_A Wt5_G Wt5 I	Br+10 Br+10 Br+10	29,3	1,92	1,86	22,6	1,55	1,63	190,2	1,92	2,27
Pool Wt8	WT8 A WT8 B WT8 C	Br+10 Br+10 Br+10	38,7	2,02	1,95	53,5	2,03	2,15	349,7	1,97	2,37
2B1	g2_8A_1_2	Br+10	34,3	1,91	1,71	42,3	2,02	2,05	287,1	1,94	2,31
	g2_8A_1_A	Br+10	45,4	2,12	2,16	44,2	2,01	2,04	324,4	1,97	2,34
	g2_8A_1_5	Br+10	55,4	2,1	2,06	54,3	2,04	2,07	396,0	1,98	2,29
2B2	g2_8A_2A	Br+7	46,6	2,01	1,38	-	-	-	197,9	1,89	2,41
	g2_8A_2C	Br+7	69,5	2,05	2,01	-	-	-	284,0	1,92	2,44
	g2_8A_2_1	Br+7	84	2,07	2,29	-	-	-	299,1	1,9	2,51
	g2_8A2_G	Br+10	33,7	1,94	1,9	46,3	1,96	1,89	244,4	1,95	2,23
2B4	g2_8A_4_B	Br+7	52,1	2,03	2,05	-	-	-	247,9	1,82	2,47
	g2_8A_4_1	Br+10	74,9	2,05	1,99	103,3	2,06	2,2	677,7	2,04	2,36
	g2_8A_4_2	Br+10	37,2	1,82	1,76	31,5	1,81	1,88	264,0	1,95	2,33
2B5	g2 8A5 A	Br+10	30,7	1,8	1,97	47	2,04	1,94	308,2	2,0	2,38

3.3.2 - Gene expression analysis – qPCR

Fruit ripening is a complex and extremely regulated process that is controlled by many transcriptions factors such as the *MADS-box transcription factor RIN*. The ripening process is also very dependent on hormone production, such as ethylene and abscisic acid. Finally, one of the most remarkable changes in tomato fruit ripening is carotenoid accumulation which cause a dramatic change in color or ripe fruits. To understand the function of *SlGRAS10* on fruit ripening knock-out lines were studied in order to comprehend if the transcription factor has any influence on other genes that are known by the literature to be pivotal to fruit ripening. Additionally, a simple STRING analysis (Szklarczyk et al., 2019) was performed and the gene *E2F*, related to cell cycle and DNA replication (Vandepoele et al., 2005) appeared has a potential target for the *SlGRAS10* gene. The expression patterns of genes related involved in the complex regulation of tomato fruit ripening were studied by real-time quantitative RT-PCR.

As shown in Figure 3.3.2.1, the expression of many genes related to ripening is significantly different compared to the wild-type in particular in the later time point, Br+10. Since the *SlGRAS10* was knocked-out its expression pattern was analyzed and the results appear to suggest that in general the *SlGRAS10* relative expression is lower in the mutated lines, likely due to non-sense mRNA decay. Since the *SlGRAS9* resulted from a duplication event we also evaluated its expression, and even though it is not significant there appears to be an overexpression of *SlGRAS9* which might compensate for the mutation in the *SlGRAS10*. The relative expression of other genes which have been reported in the literature as ripening related were also measured. The RIN relative expression appears significantly lower in most lines at the Br+10 stage. ACS2 appears to be significantly overexpressed in two lines, which might indicate that these lines have a slight delay in the production of ethylene.

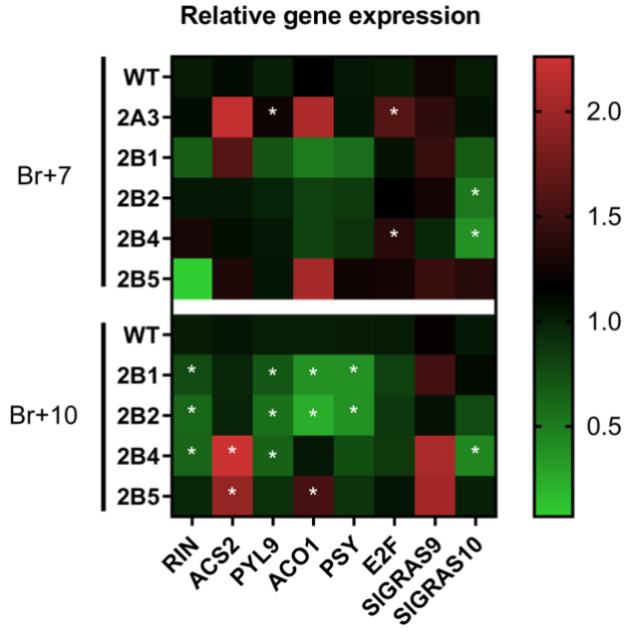

Figure 3.3.2.1 - Relative expression profiles of ripening related genes: RIN (Ripening Inhibitor – MADS-bos), ACS2 (1-aminocyclopropane-1-carboxylate synthase), PYL9 (Abscisic acid receptor PYL9), ACO1 (1-aminocyclopropane-1-carboxylateoxydase1), PSY(Phytoene synthase 1), E2F(E2F_TDP domain-containing protein), SlGRAS9 and SlGRAS10, in the pericarp tomato fruits of WT and SlGRAS10 transgenic tomato fruits at Br+7 and Br+10 stage. The expression data of the fruits was normalized to a value of 1. Each value represents the mean of three biological replicates pooled in one sample and three technical replicates. Asterisks indicate significant differences (P-value<0,05) between mutant lines and the wild-type.

3.3.3 - Carotenoid quantification

From the onset up until the peak fruit ripeness the tomato pericarp progressively changes colour reflecting the accumulation of carotenoids. The tomato characteristic red colour is a result of accumulation of carotenoids in the pericarp (Quinet et al., 2019). During the ripening process tomato carotenoid concentration increases as can be seen in the Figure 3.3.3.1. The quantitative determination of the carotenoid content in the wild-type tomato fruits revealed that carotenoid values range from 5,9 mg mL^{-1} at the green stage to 52,7 mg mL^{-1} at the Br+10 stage. The most substantial changes in carotenoid accumulation appear to occur between 2 days after the breaker stage up until 7 days after the breaker stage.

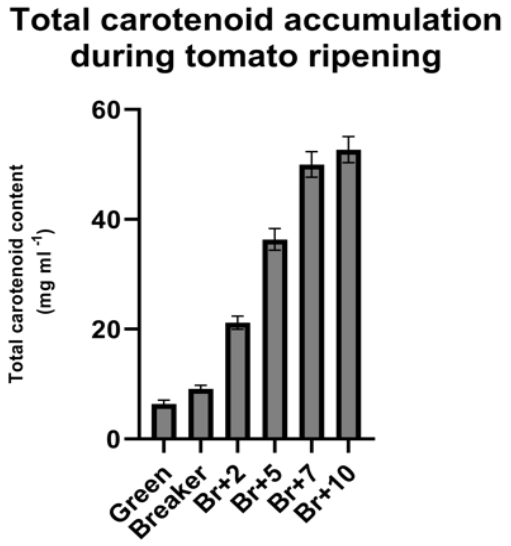

Figure 3.3.3.1 -Total carotenoids content in wild type tomato fruits in different stages of ripening. Br+X stands for number of days past the breaker stage of ripening. Each value represents the mean ± SEM of three biological replicates pooled in one sample and three technical replicates.

When analyzing the mutant lines content in total carotenoids it can be seen that the most significant differences occur at the Br+10 time point. At the Br+7 time point only the 2A3 line is significantly different from the wild type (Figure 3.3.3.2) but at the Br+10 stage all the lines except 2B1 have a significant lower content in total carotenoids compared with the wild type, as can be seen in Figure 3.3.3.2. The wild-type ranged between 52,7 mg mL^{-1} and fruits at Br+10 from the mutant lines had a lower content in carotenoids which was significant especially at a more advanced stage of ripening, Br+10. Specifically at the Br+10 stage the total carotenoid content ranged from 38,1 mg mL^{-1} to 40,2 mg mL^{-1}.
Carotenoids are a metabolite related to ripening and mark an important characteristic in ripening, the color change. The mutant plants showed to have a lower carotenoid content in further development stages which indicates that the mutant lines appear to have a delay in ripening.

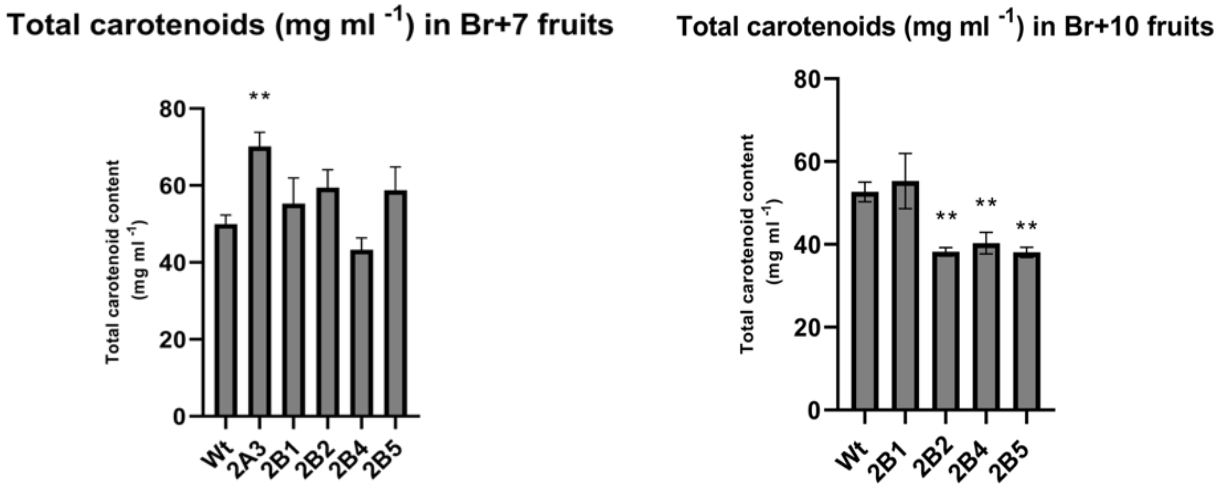

Figure 3.3.3.2 – Total carotenoids content in tomato fruits at (A) Br+7 and (B) Br+10 stages. Wt – Wild Type and name of different mutated lines. Each value represents the mean ± SEM of three biological replicates pooled in one sample and three independent technical replicates. Asterisks indicate significant differences (P-value<0,05; ** P-value<0,01) between mutant lines and the wild-type.*

3.4 Assessment of plant genotype and generation of T1 for the SlGRAS10 and SlGRAS9 Mutants

The *SlGRAS9* gene exists due to a duplication event (Huang et al., 2015) and therefore it may have a compensatory role on a mutation in *SlGRAS10*, so another mutant line was developed (previously to this work) with knock-out in both genes in order to have a more evident phenotype, as the lines for the GRAS10 knock out had a phenotype not visible to the naked eye, but marked and significant in terms of gene expression and carotenoid accumulation.

3.4.1 - Mutation analysis – sequencing

Using the CRIPR-Cas9 system plants were edited with a knock-out in both *SlGRAS10* and *SlGRAS9* genes. The DNA of the mutated plants was extracted and sent to sequence. The sequencing results are shown in Table 3.4.1.1.

Table 3.4.1.1 - Estimates of the spectrum and frequency of small insertions and deletions (indels) generated in a pool of cells edited with CRISPR/Cas9-induced SlGRAS10 and SlGRAS9 mutation in transgenic T0 generation tomato plants.

Plant ID	Genotype – T0			
	g2GRAS10	Mutation (%) GRAS10	g3GRAS9	Mutation (%) GRAS9
WT_	-			
10|9_A	Wt, d4, d3	8.2	Wt, d1, i1 (A, C)	5,3
10|9_B	Wt, d3, d1	23.8	Wt, d6, d1	28,6
10|9_C	d3	96.4	Wt, d1	2
10|9_D	d6, d3, d1	80,3	Wt, d2	16
10|9_E	Wt, d7, d3, d1	77,1	Wt, d4, d3, d1	56,6
10|9_F	Wt, d3, d1	42,5	Wt, d3, d1	7,1
10|9_G	Wt, d3, d1, i1(T)	29,4	Wt, d1	1,8
10|9_H	- (Cas9 not present)			
10|9_O				
10|9_N				
10|9_J				

In early stages of plant editing the mutation frequencies are usually low, the SlGRAS10 guide was optimized but the *SlGRAS9* guide was being used for the first time, so the mutation frequencies are lower. The plant with the highest mutation percentages overall is 10/9 E with a 77,1% mutation on the *SlGRAS10* gene and 56,6% mutation on the *SlGRAS9* gene, nevertheless with segregation InDel frequencies will rise in future generations of these plants.

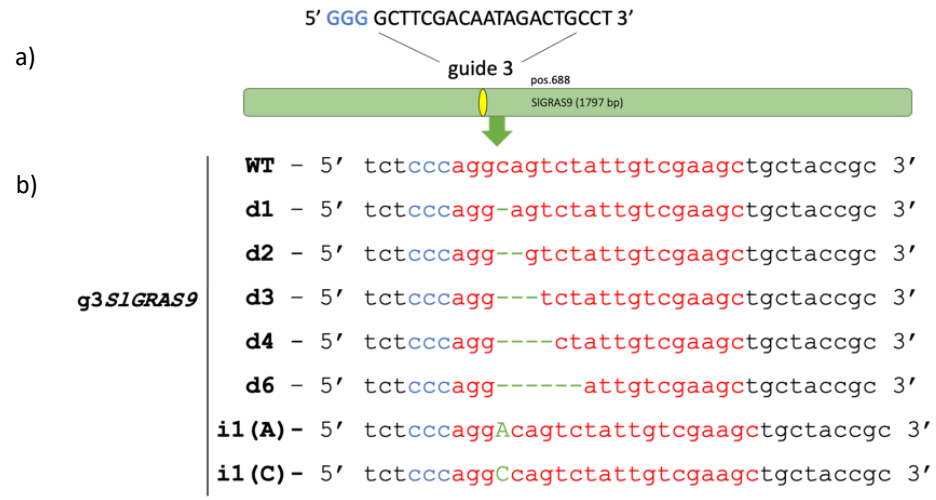

Figure 3.4.1.1 - CRISPR-Cas9 induced SlGRAS10 and SlGRAS9 mutations in transgenic tomato plants. a) Schematic representation of the SlGRAS9 gene and guideRNA sequence. b) Mutation sequences in edited tomato plants transformed with the guideRNA designated g3. The WT is shown first with a green arrow representing the PAM break-zone. Green dashes indicate number of deleted nucleotides and green letter indicate the inserted nucleotide.

After using TIDE to analyze the sequencing results and determine the type and frequency of the mutations the putative protein generated was analyzed. The lines generated with the sgRNA that targeted the *SlGRAS9* gene targeted the 688th position of the *SlGRAS9* gene. These mutations were either deletions or insertions of nucleotides that caused premature STOP codons or frameshift mutations. For example, a Deletion of one nucleotide causes a premature STOP codon and will result in a truncated protein that should not have a function. But, on the other hand, we also get deletion of three nucleotides, which only cause the deletion of an amino acid as can be seen in Figure 3.4.1.1.
These plants were grown, and its fruits were collected in order to produce a new generation, T1. Preliminary analysis of offspring from the T0 double mutants has showed that mutation rates have indeed increased. Some lines still have low mutation rates but particular the lines 10:9_D2, 10:9_F2 and 10:9_E1 are completely mutant, as can be seen in the Table 3.4.1.2.

Table 3.4.1.2 - Estimates of the spectrum and frequency of small insertions and deletions (indels) generated in a pool of cells edited with CRISPR/Cas9-induced SlGRAS10 and SlGRAS9 mutation in T1 generation transgenic tomato plants.

Plant ID	Cas9	Mutation on T1 generation of Double Mutants			
		g2GRAS10	Mutation (%) GRAS10	g3GRAS9	Mutation (%) GRAS9
10|9_B1	+	Wt, d3, d1	32,8	Wt, i1, d1	9,1
10|9_B2	+	Wt, i1, d1	28,8	Wt, i1	1,2
10|9_D1	+	d3	80,9	Wt, i2, d3, d1	43,1
10|9_D2	--	d3, d1	61,9	d3, d2	98,7
10|9_E1	+	d3	81,1	d3, d2, d1	72,5
10|9_E2	+	d5, d3	58	Wt, i1, d3, d2	29,9
10|9_F1	+	Wt, i1	35,5	Wt, i1, i2	41,2
10|9_F2	+	d5, d4, d3, d2	38,8	d3, d2, d1	69,1

Another approach that we followed is if it is possible to edit plants again after the plants have been edited and stabilized. Therefore, some plants that were already mutated for the *SlGRAS10* gene and were stabilized in the T1 generation were mutated again with the same *SlGRAS9* construct has the double mutants. When the sequencing results came, a TIDE analysis was performed, and the mutation percentages (Table 3.4.1.3) were similar to the mutation frequencies in the plants that were only edited once (Table 3.4.1.2). This proves a very interesting fact that, in fact, it is possible to edit more than once, which was not expected that frequencies of mutation in twice edited plants would be the same as in plants that have been edited only once.

Table 3.4.1.3 - Estimates of the spectrum and frequency of small insertions and deletions (indels) generated in a pool of cells edited with CRISPR/Cas9-induced SlGRAS9 mutation in T0 generation transgenic tomato plants.

Plant ID	Cas9	Mutation on T0 generation of SlGRAS9 mutants	
		g3GRAS9	Mutation (%) GRAS9
10:9_1	+	Wt	0
10:9_3	+	Wt, d1	4,9
10:9_5	+	Wt	0
10:9_6	+	Wt, d1	26,8
10:9_9	-		
10:9_10	+	Wt, d4	6,1
10:9_12	+	Wt, i1(A,G,T)	2,8
10:9_13	+	Wt, d4	4
10:9_14	+	Wt, d1	27,7
10:9_15	-		
10:9_17	-		
10:9_18	+	Wt, d1	1,3

3.5 — Assessment of low seed phenotype in SlGRAS10 mutants

Fruit set marks the transition from flower to fruit and corresponds to a significant developmental shift that transforms the ovary into a fruit. The transition from flower to fruit is regulated by a complex network of signaling pathways that are tightly programmed by specific genes and respond to endogenous and exogenous stimuli (Takei et al., 2019).
When fertilization does not occur properly, there is no fruit set or a seedless fruit form, and this process forms parthenocarpic fruit. Usually, when there is inefficient fertilization but the ovary transitions to fruit, it is due to male sterility, meaning poor pollen development (Takei et al., 2019).
The genetic mechanisms that control parthenocarpy in fruits have been more explored in recent years because of the prospects in plant breeding programs (Takei et al., 2019). Producing fruits with fewer seeds has commercial importance and understanding the genetic pathways that are involved in early fruit development has the potential to create a desirable product if the fruits maintain their desirable traits, such as flavour, nutritious value and typical size and shape (Martinelli et al., 2009).

3.5.1 Fruit weight and seed number

In the analysis of the fruits of the *SlGRAS10* mutated plants, an unexpected phenotype of a lower number of seeds emerged which was then further analyzed to see if it is related to the mutation in the *SlGRAS10* gene (Figure 3.5.1.1). The lines with the most significant phenotype were the plants that were edited using the first guide, guide 1 for the *SlGRAS10* gene, as can be seen in Figure 3.5.1.2(a). In particular, the lines 1A2 and 1B2, as well as 2A3, 1B1 and 2B5 showed to have the lowest significant number of seeds compared to the wild type. Some of the lines that had a lower number of seeds also had smaller fruits, measured in weight, as can be seen in Figure 3.5.1.2(b). The lines, 1A2, 1B2 and 2B5 have both a low seed phenotype as well has lighter fruits (Figure 3.5.1.2).
The lines with a low seed phenotype were selected for further analysis of flower morphology and pollen germinations assays.

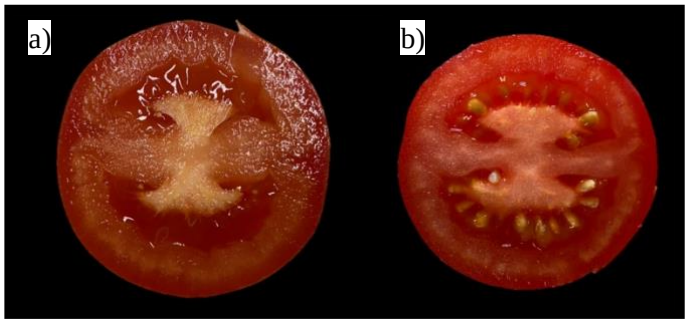

Figure 3.5.1.1 - a) Tomato from the 1A2 line with only a few undeveloped seeds. b) Wt tomato with regular amount of normal seeds

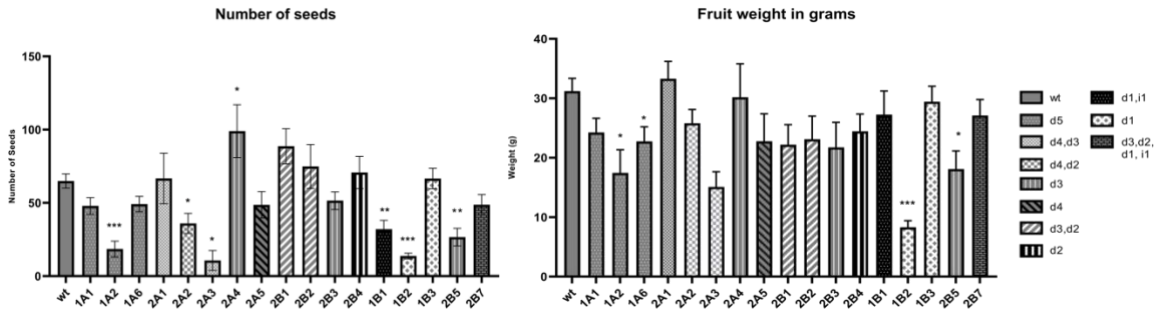

Figure 3.5.1.2 – a) Number of seeds in tomato fruits at ripe stage. Wt – Wild Type and name of different mutated lines with type of mutation, d – deletions of x number of nucleotides and i - insertion of x number of nucleotides. Each value represents the mean ± SEM of at least three biological replicates. Asterisks indicate significant differences (P-value<0,05; ** P-value<0,01) between mutant lines and the wild-type. b) Fruit weight at ripe stage. Wt – Wild Type and name of different mutated lines with type of mutation, d – deletions of x number of nucleotides and i - insertion of x number of nucleotides. Each value represents the mean ± SEM of at least three biological replicates. Asterisks indicate significant differences (* P-value<0,05; ** P-value<0,01; *** P-value<0,001) between mutant lines and the wild-type.*

3.5.2 – Pollen germination assay

In some cases of parthenocarpy the low seed phenotype is due to abnormal ovule formation, which causes low seed set, but the most reported situations are due to male sterility, either by malformations in the male part of the flower or due to poor pollen germination (Takei et al., 2019). Due to the low seed phenotype additional assays were performed, first the morphology of the flower was examined, and there were no differences detected (data not shown), then with the intent to further investigate the cause of low seed number the pollen germination was analyzed (Figure 3.5.2.1). The lines with the more significant lower number of seeds were selected to analyze pollen germination (Figure 3.5.2.2). As can be seen in Figure 3.5.2.1 the 1A2 plant in the T1 generation had the lowest mean number of seeds and in the pollen, germination assay the 1A2 T2 generation plants had significantly lower germination compared with the wild-type. The *SlGRAS10* knock-out mutation appears to interfere with the plant reproductive parts, in particular, with pollen which might lead to the reduced number of seeds in the fruits.

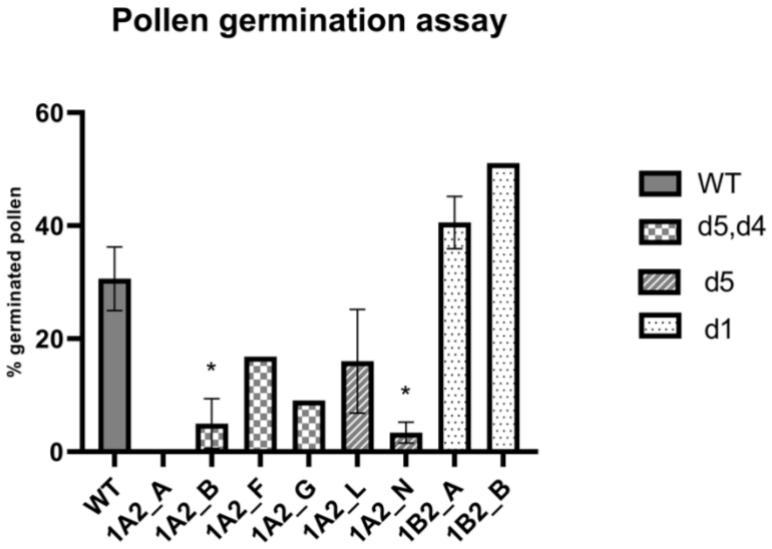

Figure 3.5.2.1 - Percentage of pollen germination from pollen collected from flowers at anthesis. Wt – Wild Type and name of different mutated lines with type of mutation, d – deletions of x number of nucleotides and i - insertion of x number of nucleotides. Each value represents the mean ± SEM of at least three biological replicates. Absence of SEM in Lines 1A2_A, 1A2_F, 1A2_G and 1B2_ B is due to only having one replicate. Asterisks indicate significant differences (P-value<0,05) between mutant lines and the wild-type.*

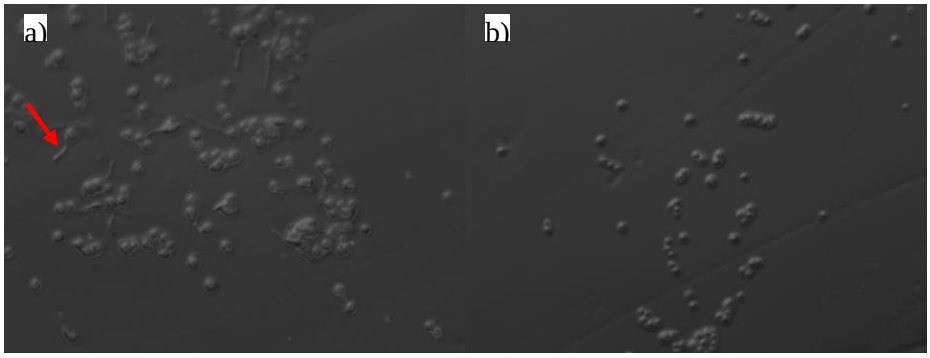

Figure 3.5.2.2 - Success of pollen germination in a) Wt indicated by red arrow and absence of germination in b) Line 1A2_A.

4 – DISCUSSION

4.1 – Involvement of SlGRAS10 transcription factor in the expression of ripening related genes and carotenoid accumulation

CRISPR is a natural mechanism that occurs in bacteria and can be engineered to remove and insert nucleotides or even alter them to promote traits seen as desirable in the organism of interest. The use of the CRISPR technology allows to remove or insert nucleotides causing mutations and promote traits seen as desirable and therefore allows do manipulate the biological processes and regulate the pathways responsible for ripening and senescence. For editing using CRISPR, a synthetic guide RNA (gRNA) is designed to an identified protospacer adjacent motif (PAM), this designed gRNA will target a specific region of the organism's genome. This gRNA along with the Cas9 protein sequence is then inserted into a cell through various mechanisms and are processed using the cell's transcription apparatus. Once synthesized within the cell the gRNA directs the Cas9 protein to the specific region and the protein induces a double- stranded break at the nucleotides identified by the gRNA (Shipman et al., 2021). Initially the first gRNA designed (previous to this work, during the work developed in Flávio Soares' PhD Thesis) to generate a knock-out on the *SlGRAS10* gene modest mutation rates. Subsequently then another gRNA (during the work developed in Flávio Soares' PhD Thesis) was designed, a more efficient one that yielded much higher mutation rates. The fruits collected from the lines which were edited with this second gRNA were then used for further analysis of the ripening phenomenon.

Since, the cell's repair mechanism after the Cas9 double- stranded break is random, arbitrary insertions and deletions occur at the break site, causing polymorphisms that results in a gene knock-out. In our results, the indels ranged from insertions of one single nucleotide to deletions of one to five nucleotides. Apart from the plants in which there was a deletion of three nucleotides, all the other mutations gave rise to a premature STOP codon, which results in a truncated non-functional protein. The lines which had a deletion of three nucleotides only had a deletion of an amino acid, with no premature STOP codon, which is less desirable when trying to create a gene knock-out, nevertheless, these lines still yielded promising results. We hypothesized that the gene expression of ripening related genes is not uniform along the lines due to the different mutations that gave rise to those specific lines, but also could be due to increased variability caused by the stress in the climatic chambers since plants have lesser space available. These phenotypes will now be assessed in plants grown in the greenhouse.

During development the tomato plant goes through diverse stages of development, from vegetative growth to flower development, fruit development and fruit ripening. All these processes are very complex and require extreme coordination within the cell to synchronize gene expression (J. Giovannoni, 2001). Based on the structural and functional analysis of the GRAS gene family by (Grimplet et al., 2016) this work hypothesized that the *SlGRAS10* and *SlGRAS9* genes, both transcription factors, are responsible for the coordination of these processes.

The *MADS-RIN* (*RIN*, *RIPENING INHIBITOR*) is also a transcription factor and its role as a ripening regulator has been extensively studied. RIN has showed to affect the accumulation of many gene transcripts (Giovannoni, 2004). Fujisawa et al., 2012 found that a specific gene from the *GRAS* family is a direct target of RIN. This gene has a putative binding site for *RIN* and exhibit *RIN*-dependent regulation during fruit ripening, suggesting a piece of important evidence to clarify the complex transcriptional cascade for fruit ripening. (Fujisawa et al., 2013). Based on this information *RIN* was selected to evaluate its expression pattern on the SlGRAS10 mutants. Our results suggest that at the Br+10 stage RIN is significantly less expressed in the 2B1, 2B2

and 2B4 lines. Since both *RIN* and *SlGRAS10* are transcription factors these results might suggest that these two transcription factors might be part of the same ripening inducing pathway, since some *GRAS* genes have been described as putative targets of *RIN* (Fujisawa et al., 2012).

We also analyzed the relative expression of *ACS2* (1-aminocyclopropane-1-carboxylate synthase) which is an enzyme that catalyzes the conversion of S-adenosyl-L-methionine (SAM) into 1-aminocyclopropane-1-carboxylate (ACC), a direct precursor of ethylene. Tomato fruit, which is a climacteric fruit is regulated by two systems that regulate the production of ethylene. System 1 operates during vegetative growth and the system 2 operates during the ripening of climacteric fruit. (Zhong et al., 2013). During the system 2 of ripening, ethylene is critical for the induction of ripening in climacteric fruit and the genes *ACO1* and *ACS2*, which encode ACC oxidase (ACO) and ACC synthase (ACS), respectively, are the major enzymes involved in ethylene biosynthesis during this phase of fruit development. Particularly these enzymes produce much higher levels of ethylene and are not subjected to autoinhibition (Gapper et al., 2013).

Based on the Tomato Expression Atlas Houben & Van de Poel, 2019 reported that *ACO1* starts being expressed shortly after anthesis, and that expression levels increase moderately during fruit ripening on what is known as the system I of ethylene production. At the onset of ripening, when the fruit transitions to the System II of ethylene production, *ACO1* expression increases significantly. ACO is particularly less expressed during fruit development and its expression considerably increases on the onset of ripening with a gradual decrease in activity when the fruit reaches peak ripeness and through postharvest storage (Van de Poel et al., 2014). ACO is particularly more expressed in pericarp tissue and ACS in the placenta, but ACS is still produced in the pericarp with the same pattern of expression has in the placenta. ACO is particularly active from the Breaker stage up until the orange stage decreasing when the fruit turns pink which is the equivalent of between Br+3 and Br+5 (Van de Poel et al., 2014). ACS increases activity in the pericarp predominantly at the light orange and light orange stages which again is the equivalent of Br+3 (Van de Poel et al., 2014).

Our data shows that in the mutant lines 2B4 and 2B5 the gene for ACS2 at the Br+10 stage is being over expressed. The ACO1 gene is only significantly over expressed in the 2B5 Br+10 line. Seeing that ACS and ACO1 are predominately active during earlier ripening stages it appears that these lines are seeing some delay in the expression of these ripening related genes associated with the production of ethylene.

The SlPYL9 is involved in ABA signaling, thereby playing a role in the regulation of flower abscission and fruit ripening in tomato. *SlPYL9* is expressed in fruits during development and ripening, and even though expression level of *SlPYL9* is not very high, it can still cause the response of downstream ripening-related genes. *SlPYL9* appears to have a pivotal role in the transition from Mature Green fruit to breaker fruit. The expression levels of *SlPYL9* increases several times, and its expression pattern is in parallel with the ABA accumulation and *SlNCED1* expression (Kai et al., 2019). In the mesocarp of Micro-Tom tomato the expression pattern of SlPYL9 starts increasing at the breaker phase and progressively increases its expression peaking at 10 days post breaker (Kai et al., 2019).

In our results at the later stage of development, Br+10, the expression of *SlPYL9* is significantly lower than in the wild-type, suggesting that *SlGRAS10*, might have an influence in abscisic acid synthesis and signaling accumulation and therefore a knock-out in this gene can delay ripening in tomato fruit.

Color change marks an important event in tomato fruit ripening and this color change is brough about by the accumulation of carotenoids. The carotenoid biosynthetic pathway that is responsible for the chlorophyll degradation and carotenoid accumulation has been well studied and described (Su et al., 2015). The first step of this pathway is the condensation of two molecules of

geranylgeranyl diphosphate (GGPP) that form the colorless form of carotene 15-cis-phytoene. This first reaction is catalyzed by phytoene synthases (*PSY*) (Su et al., 2015).
Numerous genes in the carotenoid pathway, like the PSY are regulated by the *RIN* transcription factor, which might also be influenced by the *SlGRAS10* (Su et al., 2015). Our data shows that the *SlGRAS10* mutants appear to have a similar phenotype to the RIN mutants, in which the knock-out *SlGRAS10* mutants have a significantly lower expression of phytoene synthases. These results also are consistent with further analysis on the total carotenoid content.
All through the years breeding programs have put an effort on improving yield, productivity and fruit size (Yuste-Lisbona et al., 2020) putting nutritional and taste quality on hold. Tomato is a main staple in diets all over the world and carotenoids content make an important part of this fruit quality, and also determine the nutritional value of this fruit.
Carotenoids act as antioxidants and have been linked to have health benefits. Tomato is one of the main sources of carotenoids such as lycopene, *b*-carotene and vitamins (Park et al., 2018).
Carotenoids are the major contributors to tomato fruit pigmentation during ripening. These pigments are responsible for the red coloration of the tomato fruit pericarp tissue (Park et al., 2018). This change in fruit color during fruit ripening is due to a transition from chloroplasts to chromoplasts, which is brought about by chlorophyll degradation and carotenoid synthesis. Carotenoid synthesis is part of a complex biosynthetic pathway which is highly regulated by transcription factors that determine the exact time-point at which precursor is being synthetized. The RT-qPCR data showed that the *SlGRAS10* appear to have an effect on PSY, a phytoene synthase, whose relative gene expression was lowered compared with the wild-type. This data is consistent with the data on the total carotenoid content on the mutant lines, which had a significantly lower content in carotenoids at the later ripening stage, Br+10. These results suggest that the mutant lines have a slight delay on ripening. Thus, this delay in ripening is significantly associated with the knock-out mutation in the *SlGRAS10* transcription factor.

4.2 – Assessment of mutation evolution in double mutant tomato plants

The *GRAS* transcription factor family acts in many plants species as a participant in several growth regulatory and environmental signals. Huang et al., (2015) have identified 53 GRAS genes, comparing these results to Arabidopsis there are more GRAS proteins in tomato, which suggests more gene duplications events or higher frequency of the retaining copies after duplication in tomato. And that the duplication events are the most common phenomenon that has contributed to the expansion of GRAS gene family in different species (Huang et al., 2015). These duplication events might be the reason for the abundancy of intronless genes in the GRAS gene family. The GRAS gene family is probably of prokaryotic origin and followed by extensive duplication events in the evolutionary history has been retained in many plants genomes (Huang et al., 2015).
The results in the work of Huang et al., (2015) propose that the *GRAS* genes may undergo neo-functionalization or sub-functionalization in many higher plant species. Nonetheless, some *SlGRAS* genes with extremely high sequence identity, such as the *SlGRAS9* and *SlGRAS10* genes, showed a conserved expression pattern. These results suggest a retention of these genes by genetic redundancy, and that the knock-out of one of these genes has little or no effect on the phenotype. Since these transcription factors appear to have redundant functions the single mutant lines *SlGRAS10* phenotype might be attenuated by a compensation in the increased expression of the duplicated *SlGRAS9*. This compensation is not substantial, but the RT-qPCR data showed that there was an increased expression of *SlGRAS9* in the *SlGRAS10* mutant lines, what might suggest that the phenotype noticed in the *SlGRAS10* mutants possibly is being concealed by the over-

expression of *SlGRAS9* the *SlGRAS10* duplicate. Therefore, to further analyze the function of these genes in tomato fruit ripening a new line was created to be studied, the double mutants. The plants were transformed using the same construct to cause the knock-out mutation on the *SlGRAS10* and the results were similar to previous experiments with the single mutants. The mutation rates were high with deletions of one to four nucleotides in general with the occasional but much less frequent insertion of a few nucleotides. The new *SlGRAS9* construct was not optimized so the mutation rates for the knock-out in the *SlGRAS9* were slightly lower however the kind of mutations caused were similar to the mutations caused by the *SlGRAS10* construct. Therefore, almost all the double mutants had some degree of mutation, and their fruits were collected to generate the T1 generation in which we will likely see an increase in mutation rate. Early analysis in T1 plants has showed that some plants saw a noteworthy increase in the mutation rates, which was expected since Cas9 is still present and still being expressed and inducing mutations at the target site.

4.3 – Mutation in SlGRAS10 affects seed content in tomato fruit and in pollen germination

Fruit ripening represents a terminal phase in plant development, which ultimately leads to disintegration of the fruit and seed dispersal (Griffiths et al., 1999). All through time fleshy fruits have evolved in a way that has made them more appetizing to the organisms that ingest them and disperse their seeds by influencing the ripening pathways. The ripening process favors pigment concentration, sugar and acids accumulation and increased aroma volatiles in order to make the fruits more appealing. All these changes are also accompanied by tissue softening which allows for easier seed release (Quinet et al., 2019).

Successful pollination and the fertilization of the ovules marks the start of fruit development. After pollination the ovary starts to grow, and the seeds develop concomitantly. These processes are marked by a decrease in ABA and ethylene concentrations in the fruits (Quinet et al., 2019).

At this stage the tomato floral meristem is highly regulated by the CLAVATA-WUSCHEL (CLV-WUS) feedback loop. This control of the floral meristem regulates the number of carpels in the flower and therefore, the seed locules in the fruit (Quinet et al., 2019).

The low seed number in the *SlGRAS10* mutated lines indicates that the knock-out of this transcription factor has an influence in the tomato fruit fertilization and development. During fruit development the number of seeds might be influenced by the number of locules in the fruit but also by the ability of the pollen to fertilize the ovules. Due to the results of low seed number in specific lines a further preliminary analysis was done to determine if pollen germination was altered in the mutant lines. The pollen germination results appear consistent with the seed number data, that is, the lines in which the seed number was significantly lower the pollen germination was also significantly lower compared with the wild-type. Our results confirm that *SlGRAS10* mutant plants not only have lower seed number but also less pollen germination, which is indicative of the *SlGRAS10* role in fruit development and ripening. This results also suggest that *SlGRAS10* might influence the pollen ability of reaching the ovules instead of interfering with the floral meristem forming fruit locules.

Parthenocarpy is a desired trait in tomatoes because it can overcome problems with fruit setting under unfavourable environmental conditions; if the ovary can transition without being fertilized, the fruit set is less dependable on the sensitive process that is pollen maturation (Takisawa et al., 2018). The genetic mechanisms that control parthenocarpy in fruits have been more explored in recent years because of the prospects in plant breeding programs (Takei et al., 2019). Producing fruits with fewer seeds has commercial importance, and understanding the genetic pathways that

are involved in early fruit development has the potential to create a desirable product if the fruits maintain their desirable traits, such as flavour, nutritious value and typical size and shape (Martinelli et al., 2009). There are natural parthenocarpic fruits that usually occur due to ploidy disturbances, such as trisomy, aneuploidy and translocation heterozygotes. However, there are diploid homozygous cultivars that present a parthenocarpic phenotype, and this is due to the effect of a mutation on a particular gene that is involved in the complex pathways since fertilization to fruit set (Lukyanenko, 1991). An example of a natural tomato with genetic parthenocarpy are the *pat*, *pat-2*, and *Pat-k/SlAGL6* mutants (Lukyanenko, 1991). The flowers of these plants usually have characteristically short anthers that cause partial male sterility since pollen does not fall directly on the stigma, and the fruits are also smaller (Lukyanenko, 1991). The mutant designated *sds* (seed development suppressor) is also a spontaneous mutant that has a small number of seeds compared with the average fruit. Compared to the *pat* mutants, the *sds* fruits are superior in quality, but the mutant has several undesirable pleiotropic effects, such as reduced weight (Lukyanenko, 1991).

The increased knowledge about these natural mutants has triggered an interest in understanding the processes that originate parthenocarpic fruits and the developing breeding programs. Ueta et al., 2017 and Kim et al., 2019 have developed a rapid breeding program studying the *SlIAA9* gene, which had been previously marked as a putative controller of seed development, and therefore parthenocarpy, and got similar results, the regenerated mutants exhibited morphological changes but more important, seedless fruits.

Martinelli et al., 2009 also produced parthenocarpic transgenic tomato plants by regulating the expression of genes for auxin synthesis or responsiveness driven by an Arabidopsis thaliana promoter and got parthenocarpic tomato fruits. The development of parthenocarpic fruits through the modulation of ethylene- and IAA- associated genes not only produces seedless fruits but can also have effects on fruit shelf life and softening (Martinelli et al., 2009).

Most parthenocarpic fruits have been caused by abnormalities that lead to male sterility, but in Takisawa et al., 2018, the down-regulation of Pat-k/SlAGL6 caused abnormal ovule formation, which led to a reduced number of seeds. The Pat-k/SlAGL6 mutant ovules had abnormal micropyles, which indicates that Pat-k/SlAGL6 might be involved with ovule formation and that its downregulation causes abnormal ovule formation, which causes low seed set (Takisawa et al., 2018).

In our study the low seed number appears to be associated to male sterility, specifically, to the low rates of pollen germination. Also, parthenocarpy is a very interesting trait in fruits but is often linked a trade-off, smaller fruits. Our results suggest that some of the lines with a lower number of seeds maintain regular weight which poses a very interesting phenotype for future commercialization.

5 – CONCLUSIONS AND PERSPECTIVES

One of the main objectives of plant breeding, apart from increasing yield is to improve fruit quality. Towards guaranteeing this quality improvement breeders have undertaken studying how to improve flavour, increasing nutraceutical compounds on top of the desired traits of improved productivity, efficiency, and also pest and disease resistance. The enhancement of fruit quality relies on the knowledge of the ripening phenomenon, and fruit ripening and senescence comprise complex and highly coordinated molecular and biochemical processes. This highly regulated process involves ripening-associated genes, transcription factors, enzymes, repressors, signaling molecules, and metabolic pathways in both climacteric and non-climacteric fruits. Nevertheless, further analysis of this crosstalk between transcription factors, such as the *SlGRAS10* and *SlGRAS9*, and ripening-associated genes is needed in order to be validated and to formulate a representative model of the ripening pathways.

The study of fruit ripening is also exceptionally critical for commercial implications since post-harvest losses are due to the decrease in fruit quality during post-harvest. As the fruits ripen senescence occurs concomitantly leading to fruit to become more susceptible to infection resulting in fruit deterioration. Consequently, most strategies include pre-harvest and post-harvest management techniques to hold up senescence and preserve fruit quality.

The translation of Genome Wide Association studies and functional analysis of big gene families into the application of this knowledge to breeding programs remains slow, so in this work we developed two lines of work in which two mutant lines that were previously generated were studied. The first with a knock-out mutation on the *SlGRAS10* gene, and the second with a knock-out mutation on both the *SlGRAS10* and the *SlGRAS9* genes.

When analyzing the relative gene expression of ripening related genes on the *SlGRAS10* mutant lines the results showed that the mutant lines appeared to have a slight delay, especially at the Br+10 stage. This analysis is concordant with the quantification of total carotenoid in tomato fruits, the Br+10 fruits show a significant lower content in total carotenoid which is similar to wild-type fruits approximately three-day earlier in fruit ripening process. Further analysis will involve a more in-depth RNAseq analysis of the fruits from the double mutant lines, and also a metabolomics approach (GC-MS) to understand how the double knock-out affects gene expression and metabolite in the plant and fruits.

Additionally, the SlGRAS10 mutants exhibited a low seed phenotype. Some of the mutant lines that exhibited this phenotype of low seeds did not have a lighter fruit phenotype which is extremality important for parthenocarpic phenotypes. To unveil where in the flower the fertilization was impaired a preliminary pollen germination assay was performed, and it appears that the seedless fruits are a result of poor pollen germination. In future work more analysis is going to be done, using the flowers to collect RNA, from anthers and pistils, separately, and perform a RNAseq analysis. Additionally, crosses are going to be performed to evaluate whether indeed the female part of the mutant flower is functional.

Finally, the output of our study are clues related to the ripening programming in tomato fruits. We were able to increase our knowledge in the mechanisms responsible to unlock ripening mechanisms. Over the last years the new plant breeding techniques have been introduced has a breakthrough in the field of crop improvement and have often praised for being able to solve the problem of reassuring food security. This work would need further in-depth analysis and if the initial results outcome in a phenotype with a longer shelf-life the tomato mutant would be defined as a new variety and could be introduced in the market pending on European laws regarding new breeding techniques.

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7. ANEXES

Appendices I – Supplementary Figures

Figure S1.1 - Fruits in climacteric chamber at Breaker stage indicated by red arrow.

Figure S1.2- PCR results visualized using 1% agarose gel electrophoresis stained with GreenSafe Premium (NZY Tech). After DNA was extracted from T0 mutant plants a PCR was performed to detect the presence of Cas9. The amplicon has 1549 base pairs. The samples WtA, WtB along with the negative control serve as negative controls, with no presence of Cas9 as indicated by the absence of a line. The positive control is a sample with known cas9 present with a line present same as all the samples except for 10:9H.

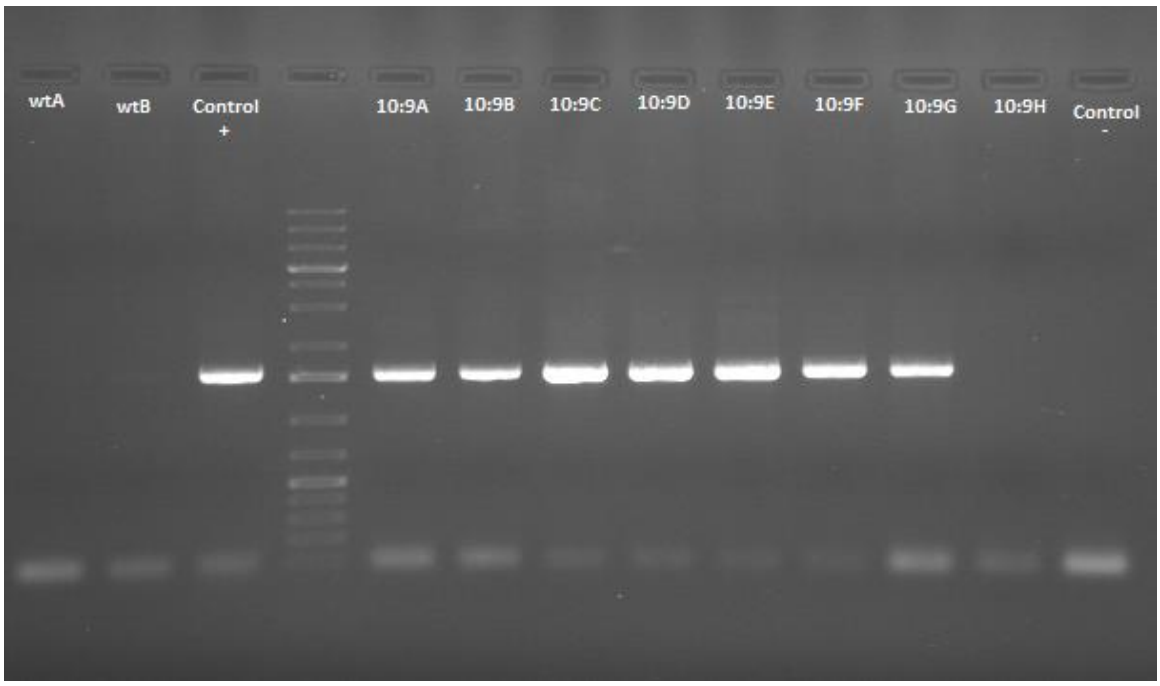


Figure S1.3- RNA integrity with optimized extraction protocol adapted from kit. RNA was extracted from wild-type and mutant tomato fruits. Two time points were analyzed - Br+7 and Br+10 and visualized using 1% agarose gel electrophoresis stained with GreenSafe Premium (NZY Tech).

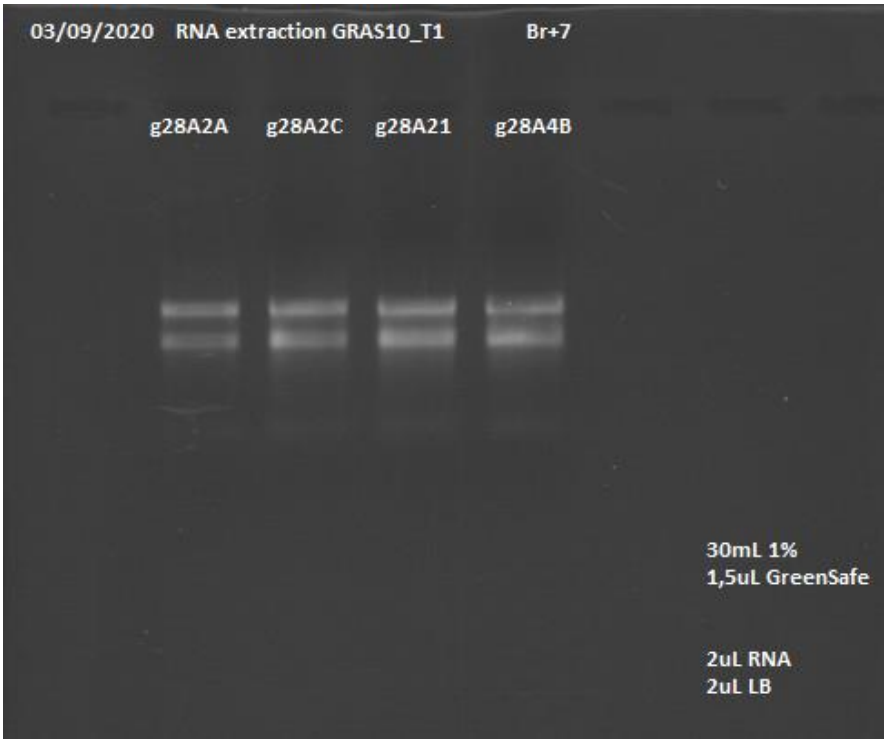
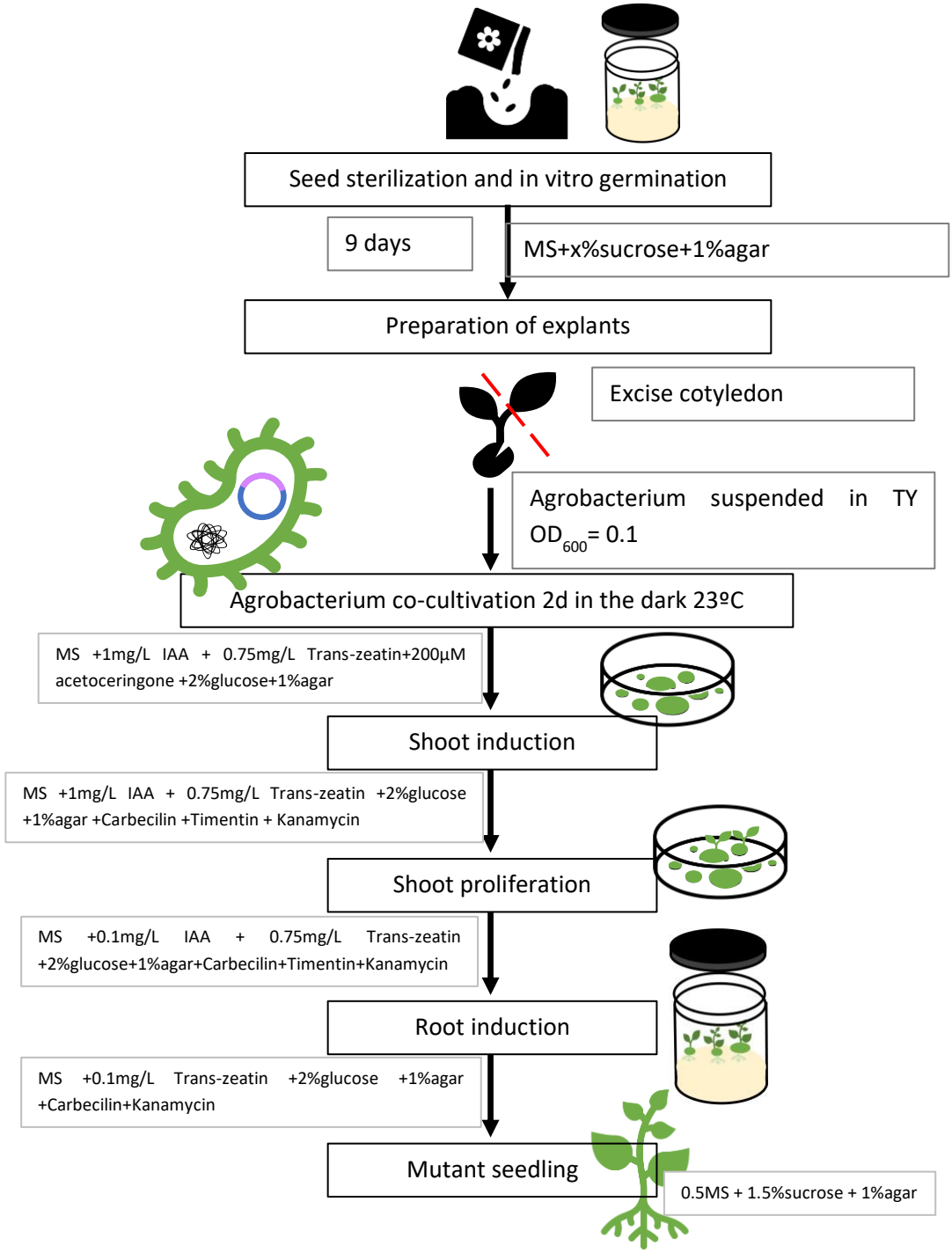


Figure S1.4- Regeneration of tomato (S. lycopersicum cv. MoneyMaker) explants using cotyledon as explants



Appendices II – Supplementary Tables

Table S2.1 - Primer Pairs for sequencing

		Primers 5' → 3'
Cas9	PFw	GAA AGT TAC CGT GAA ACA GC
	Prev	CAC GAT TTC TCC TGT TTC TC
gRNA1_SlGRAS10	PFw	GAG AAT AGC ACC AAA CAC AAG G
	Prev	TGA ATA CCC GGA GAT AAA AAC C
gRNA2_SlGRAS10	PFw	CCC CTC TCA GAA CCA AAA CC
	Prev	ACG GCG GGG TTG TTT GAT T
gRNA3_SlGRAS9	PFw	GCG AAC ACG AAC GGG GTT TTA T
	Prev	TCA GCT CTG ACC GTG AAC TCT G

Table S2.2 – Guide RNA sequence

	GuideRNA sequence 5'→ 3'	PAM	strand	Position
gRNA1_SlGRAS10	GCGAGATTTACGGCGCCGGT	GGG	(+)	37 bp
gRNA2_SlGRAS10	GTTCAAGCGCGACCGTAAAG	CGG	(-)	909 bp
gRNA3_SlGRAS9	GCTTCGACAATAGACTGCCT	GGG	(-)	688 bp

Table S2.3 – Concentration of extracted DNA from tomato young leaves

Plant ID	gDNA (ng/µL)	260nm/280nm	260nm/230nm
WT_C	724,5	2,19	2,17
WT_D	941,0	2,19	2,16
WT_G	672,8	2,19	2,06
WT_H	711,8	2,17	2,18
10|9_B	1096,9	2,18	2,21
10|9_D	546,5	2,11	2,12
10|9_F	807,1	2,18	2,1
10|9_H	709,2	2,16	2,12

Table S2.4 - Primer Pairs for Real Time - qPCR

Primer Name	Annotation	ID gene	Primer sequence		Tm (ºC)
PYL9	Abscisic acid receptor PYL9 (AHRD V1 **-- PYL9_ARATH)	Solyc12g055990	PF	GGAGCGTACATGGATTTTGATT	62
			PR	TGCCACAGTAGGGTAGGATA	60
PSY1	Phytoene synthase 1	Solyc03g031860	PF	CAACTCCTTTGAGAGAACTTTC	62
			PR	TCATGCCCACAGTAAAATTCTG	62
ACO1	1-aminocyclopropane-1-carboxylateoxydase1	Solyc07g049530	PF	ACAAACAGACGGGACACGAA	60
			PR	GCTCTTTGGCTTGAAACTTGA	60
E2F	E2F_TDP domain-containing protein	Solyc01g007760.3.1	PF	GGCTCAGGACGGTTTACTTAC	64
			PR	CCCATACTTTCAACTCAATCTG	62
RIN	Ripening Inhibitor (MADS-box)	Solyc05g012020	PF	GGAACCCAAACTTCATCAGA	58
			PR	TTGTCCCAAATCCTCACCTA	58
Nr-ETR3	never ripe mutant (ethylene receptor3)	Solyc09g075440	PF	AGGTAACATTTGGATTGAGAGTGAGG	74
			PR	CTCCAGTACTCACTTTACGAGCATTG	76
ACS2	1-aminocyclopropane-1-carboxylate synthase	Solyc01g095080	PF	GAAAGAGTTGTTATGGCTGGT	64
			PR	GCTGGGTAGTATGGTGAAGGT	64
SlDML2	DEMETER-like DNA demethylases 2	Solyc10g083630	PF	AGTACTCATGCCAAAGCCAAA	60
			PR	CCTATCTTCTTTTTACCGACTGGA	68
SlGRAS9	GRAS9	Solyc06g036170	PF	ACCGGAGACCTATGTTCATCG	64
			PR	ATCCCAAACCTTGTTGCTGC	60
SlGRAS10	GRAS10	Solyc03g025170	PF	AAACGGAGCAACCATCGGAGT	66
			PR	CACTCGTTGTTGGTGATGACCG	68
CAC	CLATHRIN ADAPTOR COMPLEXES SUBUNIT	Solyc08g006960	PF	CCTCCGTTGTGATGTAACTGG	64
			PR	ATTGGTGGAAAGTAACATCATCG	64
Actin	Actin	Solyc03g078400	PF	CAGCAGATGTGGATCTCAAA	58
			PR	CTGTGGACAATGGAAGGAC	58
NCED	9-cis-epoxycarotenoid dioxygenase	Solyc07g056570	PF	CATGAACTTGAACACCCTTTG	64
			PR	CGTTTCGAACGTAAACGCCT	60
εlcy	lycopene epsilon cyclase	Solyc12g008980	PF	CTTACCAGTTCAAGTATCCCGAG	68
			PR	GCAATATCAGAGCCAGTCCA	60

Table S2.5 – $2^{-\Delta\Delta C_T}$ values after linearization using the LinReg software for all the genes which relative expression was analyzed through RT-qPCR.

	HouseKeeping Gene			GRAS10	GRAS9	RIN	NCED	ACS2	SlDML2	PYL9	ACO1	PSY	E2F
	Actin	CAC	REF										
Sample	Cт	Cт	Cт[ref]	2^-ΔΔCт	2^-ΔΔCт	2^-ΔΔCт	2^-ΔΔCт	2^-ΔΔCт	2^-ΔΔCт	2^-ΔΔCт	2^-ΔΔCт	2^-ΔΔCт	2^-ΔΔCт
Wt_Br7	20,36	24,47	22,41	0,83615936	0,48753297	1,05403708	0,28927147	1,00877566	0,88882786	1,02934322	0,76132785	0,86107985	1,04276032
Wt_Br7	20,33	24,09	22,21	0,92256935	0,84149519	0,82972887	0,68007012	0,58391724	0,84445457	0,94556342	0,62420618	0,78845831	0,84367845
Wt_Br7	20,26	23,50	21,88	1,29631911	2,43749856	1,14342559	5,08324095	1,69767327	1,33231235	1,02742264	2,10426397	1,47291552	1,13668087
Wt_Br10	20,49	25,45	22,97	0,88566339	0,8333944	1,05429659	0,79428557	1,57597405	0,94259481	0,95518574	0,96085637	0,97048099	1,18200249
Wt_Br10	20,46	25,15	22,80	1,63057059	0,69958214	1,09086409	1,09628769	1,00867463	1,15055429	1,11154773	1,02119057	1,09264452	1,14760798
Wt_Br10	20,06	23,63	21,85	0,60045013	2,6242534	0,79032403	1,7356961	0,64429794	0,67671669	0,87384327	0,88690982	0,85498557	0,70799363
Wt_Br10	20,98	25,17	23,08	1,15322689	0,65358931	1,10017397	0,6616451	0,97636701	1,36257668	1,0778306	1,14909323	1,10299944	1,04125883
2A3_Br7	19,98	23,68	21,83	0,61670782	2,05330697	1,18851226	2,97754535	3,08259521	1,04038085	1,2441569	1,99769267	0,99740277	1,97197736
2A3_Br7	20,50	24,47	22,49	0,98593538	1,00375999	1,08916873	0,48085739	2,15851761	1,16270856	1,13909512	1,91380727	1,0208508	1,42702893
2A3_Br7	20,29	23,86	22,07	1,38919787	1,16207877	1,02374598	5,27028611	1,37410098	0,90461295	1,29480292	2,34281487	1,13148804	1,44490216
2B1_Br7	20,19	23,53	21,86	0,16522114	1,68638622	0,73591063	5,69054531	1,34871516	0,86093865	0,92748044	0,66187012	0,65692977	1,12702586
2B1_Br7	17,91	24,11	21,01	0,05309514	1,0725517	0,30301148	1,18978573	0,71043675	0,39028219	0,3963297	0,27518271	0,35740725	0,57285463
2B1_Br7	19,82	23,51	21,66	0,63543568	1,57832975	0,94494175	6,37374012	2,79752563	0,52012394	0,81459971	0,55559209	0,7359309	1,47760205
2B1_Br10	20,15	24,41	22,28	1,51680768	1,30717557	0,78172853	0,62599269	0,64692812	0,6668681	0,83705727	0,51831281	0,40533923	0,84995634
2B1_Br10	19,73	23,81	21,77	0,38615939	1,27457054	0,76617321	1,03190431	1,49062907	0,58384104	0,62077131	0,45062464	0,36226289	0,78935271
2B1_Br10	19,87	24,39	22,13	1,84798175	1,98418332	0,72263778	1,02413611	0,75334341	0,4632121	0,60939962	0,21562805	0,41636567	0,82542956
2B2_Br7	20,04	23,85	21,95	0,52059262	1,52744357	1,07358923	4,53359484	1,06171459	1,16853433	0,88807517	0,85883694	0,72915218	1,28198748
2B2_Br7	19,68	23,85	21,76	0,41202971	1,29980838	1,08654253	3,43088958	1,15972061	1,00246487	0,92080928	0,99338906	0,9057031	1,08546865
2B2_Br7	20,67	24,57	22,62	0,74564928	0,97954688	0,93878739	1,29136748	0,86393732	1,13523549	1,09823355	0,61338826	0,92886598	1,14742266
2B2_Br10	19,76	24,14	21,95	0,72768811	0,97029472	0,59785507	1,07046457	0,91487875	0,76132539	0,57650602	0,24703618	0,39743471	0,89111897
2B2_Br10	19,92	24,47	22,20	1,14959119	1,17327064	0,64175297	1,22904619	1,02787915	0,82929143	0,54626957	0,22469041	0,39206615	0,85382574
2B4_Br7	20,30	24,35	22,32	0,47769033	0,67287956	1,14556111	0,26138333	0,86641466	1,22715289	0,97138697	0,6255168	0,70165263	1,28883122
2B4_Br7	20,04	23,75	21,89	0,46865614	1,53648478	1,41477798	5,00061382	1,3578139	1,40604129	1,02555669	0,8498867	0,98329474	1,449241
2B4_Br7	21,33	25,19	23,26	0,29515231	0,63486324	1,33526254	0,9204732	1,01701645	1,42748503	1,11210021	0,94458462	0,98646167	1,38918143
2B4_Br10	20,42	24,30	22,36	0,73588602	2,04963316	0,59498133	7,41273821	2,69356548	1,05488856	0,65441825	1,91891129	1,07250583	0,79758595

2B4_Br10	19,49	23,42	21,46	0,34560251	2,15128545	0,73652687	2,09359714	1,81727321	0,75166612	0,52633241	0,26305939	0,42883438	0,88506973
2B4_Br10	20,39	24,39	22,39	0,76787479	2,018315	0,55763076	3,71380727	2,25726896	1,00459067	0,73306794	0,92722336	0,74384257	0,87215882
2B5_Br7	20,69	24,30	22,49	1,95512315	1,67008433	1,0688584	9,91948602	1,84832073	1,32061931	0,983467	1,7652055	1,15350092	1,509283
2B5_Br7	20,54	24,53	22,53	1,74392092	1,23200932	0,84756579	4,30645776	0,86066939	0,87533965	0,94670528	1,30752478	1,00118496	1,10789175
2B5_Br7	20,46	24,16	22,31	1,42149253	1,48345052	0,85353069	4,22208348	1,25346021	0,99614646	1,23069318	3,08536227	1,60836437	1,1956827
2B5_Br10	20,54	24,13	22,34	1,1841371	2,00874265	0,90998404	2,01406958	1,97989391	1,3036459	0,88969737	1,47775009	0,87722711	1,13745994
2B5_Br10	20,69	24,20	22,45	1,05709446	2,07821324	1,01983492	1,84445884	1,93091267	1,23603655	0,9192587	1,61780881	0,91180932	0,95971989