Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Generation of *Arabidopsis thaliana* mutant lines using the CRISPR-Cas technology for reverse genetics studies of a sugar-responsive LRR-RLK1 (At1g74360) gene.

Master's thesis in Master of Science in Biology
Supervisor: Atle M. Bones
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Kunnskap for en bedre verden

Abstract

Plants are sessile organisms that are exposed to environmental changes and therefore require highly regulated pathways and mechanisms that allow them to adapt to such changes. Sugars have a central role in coordinating metabolic fluxes in response to the changing environment and in providing cells and tissues with the necessary energy for continued growth and survival. Internal sugar sensory mechanisms are mostly related to metabolite control and regulation. These mechanisms have been broadly studied, while until recently, the role of sugars towards extracellular sugar sensing mechanisms has remained a mystery. Therefore, work remains to be done in order to gather comprehensive knowledge and information about these extracellular signaling events.

A recently discovered gene, At1g74360, encodes an LRR-RLK that has shown properties that relate it to extracellular sugar detection and connecting sugar availability to growth. In this study, the previously mentioned Sugar Responsive RLK 1 (SRR1) has been shown to respond towards sucrose and different hormones treatment.

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"A doctor can save maybe a few hundred lives in a lifetime. A researcher can save the whole world." Craig Venter

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Abbreviations

A	Adenine
ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
Agro	Agrobacterium
Amp	Ampicilin
AtHXK1	*Arabidopsis thaliana* hexokinase1
BiFC	Bimolecular Fluorescence Complementation
bZIP11	basic leucine zipper11
C	Cytosine
Cas	CRISPR-associated
cDNA	Complimentary DNA
CKs	Cytokinins
cm	Centimeter
Col-0	Columbia-0
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNAs	CRISPR RNAs
ddH_2O	Double-distilled H2O
DNA	Deoxyribonucleic acid
DSB	Double strand break
EB	Ethidium bromide
ein2	Ethylene insenstitive 2 mutant plants
g	Gram
G	Guanine
Gen	Gentamicin
gin2	Glucose insensitive
goi	Genes of interest
gRNA	Guide RNA
HDR	Homology-directed repair
Hyg	Hygromycin
Hz	Hertz
IAA	Indole-3-acetic acid
Kan	Kanamycin
kb	kilo base
KO	Knockout

kV	Kilo-volt
LA	LB-agar
LB	Lysogeny broth or Luria-Bertani medium
LRR-RLKs	Leucine-rich repeat receptor like kinases
MeJA	Methyl Jasmonate
mg	Miligram
mM	Milimolar
MS	Murashige-Skoog
ng	nano gram
NHEJ	Non-homologous end joining
nm	nano meter
NS	No sucrose/without sucrose
ON	Over-night
OX	Overexpression
Pal	Palatinose
PAM	Protospacer Adjacent Motif
PCR	Polymerase chain reaction
PPI	Protein-protein interaction
PTGS	Post-transcriptional gene silencing
qPCR	Quantitative polymerase chain reaction
racrRNAs	Trans activating CRISPR RNAs
Rif	Rifampicin
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
RVC	Reverse complement
SA	Salicylic acid
sgRNA	Single guide RNA
SnRK1	Sucrose Non-fermenting Related Kinase1
Spec	Spectinomycin
Suc	Sucrose
T	Thymine
T-DNA	Transfer-DNA
TAE	Tris-acetate-EDTA
TFs	Transcription factors

Ti plasmid	Tumor-inducing plasmid
Tn	Number of generation
TOR	Target of Rapamycin
V	Volt
Vir-plasmid	Virulence plasmid
WT	Wild-type
YFP	Yellow fluorescent protein
Zt	Zeatin
µL	micro-Liter
µM	micro-Molar
µF	micro-Farad

Introduction

1.1 Photosynthesis, from light to carbohydrates

Unlike animals or other heterotrophic organisms, plants are autotrophs that obtain their energy from sunlight by fixating CO_2 and producing glucose. This biochemical process of carbon fixation is known as photosynthesis. This process is of vital importance not just for plants, since is the primary source of energy in the trophic chain and therefore provides support to nearly all life on Earth. Additionally, photosynthesis is also the main source of oxygen liberated to the atmosphere. [1,2]

The autotrophic growth of plants depends on their photosynthetic tissues (source), mainly leaves, and their ability to produce sugars. Leaves act as solar collectors full of photosynthetic cells, known as chloroplasts, and capture CO2 when this enters through structures known as stomata that allow gas to enter inside the leave. [3]

Moreover, the excess amounts of photo-assimilates, in the form of sucrose, are exported source to non-photosynthetic tissues such as roots and storage organs (sink) to sustain the plant life cycle. The production of carbohydrates via photosynthesis and their mobilization to sink organs is tightly regulated and its coordination involves both metabolite and sugar-specific signaling pathways [4].

It has also been shown in the past decade that sugars also have a major role as signaling molecules beside their classic function as energy and structure sources [5].

1.2 Sugars in plants, more than an energy source

Plants, as sessile organisms, are influenced by changes in their environment. Therefore, they need to adapt to these alterations having efficient and highly regulated signaling mechanisms. Moreover, these signaling pathways should be able to transmit external stimuli inside the cells. [6]

Sugars have a central role in coordinating metabolic fluxes in response to the changing environment and in providing cells and tissues with the necessary energy for continued growth and survival. For this reason, plants have developed a broad variety of mechanisms to sense and respond to different sugar signals. [5,7]

In *Arabidopsis thaliana,* the hexokinase1 (AtHXK1) has been isolated in a mutant screen as glucose insensitive (*gin2*) and functionally characterized. Similar to its yeast homolog, AtHXK1 is the core component in plant sugar sensing and signaling inside the plant cell [8,9]. Glucose signaling has been highly examined. Different studies have revealed the

roles of different components in glucose sensing and signaling pathways and their interactions with phytohormones in regulating plant growth and development under different conditions [10–14]. On the contrary, even though sucrose is the major transport form of sugar, not much is known about its signaling role. Only few sucrose-specific signaling pathways have been identified in plants including anthocyanin biosynthesis in *Arabidopsis* seedlings [15,16], *Impaired Sucrose Induction 1* (*ISI1)* [17], and *bZIP11/ATB2* [18–20]. bZIP11, a transcription factor, acts in a significant form connecting sugar signaling and free amino acid level in a sucrose-dependent mode [21]. However, stress factors often interrupt the optimum photosynthesis rate in source tissues resulting in imbalances between source-sink tissues. The common consequence of this stresses is a process known as low energy syndrome (LES), where the energy is depleted [22,23]. This decrease in energy sources has a significant impact on plant developmental and produces a reprogramming event in the metabolic and gene expression patterns of plants. These changes include repression of biosynthetic pathways and activation of catabolic processes, together with nutrient remobilization to maintain the metabolism [24–26].

1.3 Intracellular sugar sensing, the main focus towards sugar signaling so far

Internal sugar sensory mechanisms are mostly related to metabolite control and regulation. The metabolite status sensory system is composed of diverse regulatory molecules focused on the conserved TOR (Target of Rapamycin) and SnRK1 (Sucrose Non-fermenting Related Kinase1) [12]. These two master regulators function in an opposite manner and are thought to have an antagonistic effect on each other [27]. TOR-kinase senses nutrient abundance and promotes biosynthetic pathways, that convey in growth and development processes. On the other hand, SnRK1 is activated upon starvation conditions and triggers catabolic pathways and growth arrest [22,28]. Plants can sense accessible nutrients through a mechanism that is able to sense or detect sugars such as sucrose, glucose and fructose.

1.4 Extracellular sugar perception, the forgotten role of sugars

The extracellular sugar sensing mechanism is not well understood in plants. Such mechanism is thought to be composed of one or more receptors which are able to sense the status of apoplastic sugar and activate or repress downstream components inside the plant cells. Leucine-rich repeat receptor-like kinases (LRR-RLKs) meet all mentioned

criteria. LRR-RLKs belong to the superfamily of RLKs consisting of about 600 members in the Arabidopsis genome [29]. RLKs function as antennae to constitutively monitor internal and external changes and to control fine-tuned physiological responses of plants to stimuli. Despite the large number of identified LRR-RLKs, biological functions have been assigned for only about 30 members of this family, reviewed in De Smet et al. (2009) [30].

1.5 Background of the study

Previous work at Atle Bones lab has revealed the role of an LRR-RLK, which labeled as Sugar Responsive RLK 1 (SRR1), in the sensing and signaling of extracellular sugars and connecting sugar availability to growth. Constitutive expression of *SRR1* leads to a sugar insensitive phenotype in transgenic plants. The observed phenotype resembles gin phenotype. *SRR1* expression was revealed to be induced by exogenous sugars and was predominantly localized in vascular tissue. Analysis of major carbohydrates showed that the production but not the consumption of starch is deficient in *srr1* plants. Taken together existing data suggest that SRR1 plays a role in extracellular sugar sensing and connects carbohydrate availability to the regulation of gene expression and plant growth.

1.6 Aim of the study

1. Creation of genetically modified plants using CRISPR/Cas genome editing technology for SRR1 gene in different domains (extracellular LRR domain, transmembrane domain and cytoplasmic kinase domain).
2. Verification of the phenotype of new knock-out and over-expression lines and comparison to the wild-type and available T-DNA lines.
3. Establishment of sugar and ABA-insensitive phenotype of over-expression and knock-out lines of SRR1.

Material and methods

The experiments mentioned below were performed using *Arabidopsis thaliana* accession Columbia-0 (Col-0) plants and transgenic and mutant lines based on the Col-0 background. With the exception of watering, double-distilled H_2O (ddH_2O) was used during the experiments.

1.7 Seeds sterilization

All the used seeds where sterilized following two different procedures depending on their origin. The seeds obtained after *Agrobacterium tumefaciens* transformation, T_0 seeds, were sterilized following a 70% ethanol and 50% bleach:0.1% Triton-X wash, based on the protocol described by Lindsey et al., 2017, and then immersed in 0.1% agarose. The first and successive generations of mutant seeds (T_1, T_2 and T_3 seeds) and wild-type (WT) seeds were exposed to chlorine gas (100 mL of bleach and 3 mL of HCl) in a "gas box" for 3 hours and later embedded in 0.1% agarose or directly planted in soil depending on their purpose. [31]

1.8 Growth conditions

The plants were either grown in autoclaved soil or *in vitro* both in plastic Petri dishes and in 6-, 12- or 24-well plates in solid and liquid media respectively.

On one hand, previously sterilized seeds were sowed in pots containing soil (5:1 mix of Såjord, Hasselfors Garden AB, SE-695 84, Hasselfors and perlite from LOG) and set in a growth room under controlled conditions.

When grown *in vitro,* after sterilization the seeds were placed in plastic plates containing half-strength Murashige-Skoog (½ MS) (M5524, Sigma-Aldrich; pH 5.7), 1% sucrose and 0.6% phytoagar (P1003.5000, Duchefa Biochemie B.V.). To measure the root growth of the different lines, the seedlings were grown in vertical position in ½ MS square plates with 0.8% phytoagar. Moreover, depending on the experiment, the ½ MS plates also contained different sugars (Sucrose or Palatinose, standard in vitro conditions of 25 mM concentrations [32]) or 1-Aminocyclopropane-1-carboxylic acid (ACC). Additionally, seedlings were also grown in liquid ½ MS supplemented with different phytohormones or sugars.

In all the above-mentioned cases, the seeds were stratified for 2 days at 4ºC to ensure homogenous germination. Then, all plants were placed in a growth room under 16h/8h light (70 $\mu mol\ m^{-2}\ s^{-1}$)/dark photoperiod at 22ºC with 20-25% relative humidity.

It's important to mention that to avoid contamination of the samples while preserving good aeriation or oxygenation conditions, the plates were sealed using Micropore™ tape (3M)

1.9 Mutant plants obtention

Using the CRISPR-Cas9 technology and floral dip *A. tumefaciens* mediated transformation, plants with knocked-out (KO) *SRR1* (At1g74360) gene where generated. Three different regions of the protein coded by the *SRR1* gene, the island, transmembrane and kinase domains, were targeted to generate 3 mutant lines, named ISL-, TRA- and KIN-KO respectively.

1.9.1 Vectors generation

The CRISPR-Cas9 gene editing technology relays in the use of specific synthetic Oligonucleotides (Oligos) that have a Spacer sequence (~20 nucleotides) that is complementary to the genomic region or gene of interest targeted for modification. These Oligos are known as guide RNAs (gRNAs) and can be designed using different tools such as CRISPOR [33] and Cas-Designer [34]. For this study, CRISPOR was used to generate 2 sets of Oligos, sense- and antisense-gRNAs, for each of the 3 domains of interest (Appendix 2).

To generate a transformation vector, these sets of gRNAs were introduced in the pKI1.1R plasmid from (Tetsuya Higashiyama, Addgene plasmid # 85808) (Appendix 1) via digestion and ligation. This plasmid, based in a pFAST-R vector, contains the Cas9 gene expressed under the RPS5A (Ribosomal Protein S5 A) promoter, a gRNA cassette under the AtU6.26 promoter, a red fluorescent protein (*OLE1*–TagRFP) cassette and the hygromycin resistance gene (Hyg^R). These last two features allow the selection of transformed seeds. [35]

1.9.1.1 Guide RNAs (gRNAs) annealing

Before integration of the set of 2 gRNAs designed for every target domain inside the pKI1.1R plasmid, a ligation of this 2 Oligos was performed so both of them were present in the gRNA scaffold of the vector. The annealing was performed by adjusting the concentration of the 2 gRNAs, sense and antisense, to a concentration of 100 µM (adding ddH_2O to the vials that contained the freeze-dried Oligos) and mixing 5 µL of sense and antisense in a 1.5 mL tube. Then, 50 µL of T4 ligase buffer was added to each mix, to avoid non-specific binding of the oligos and create a better environment for the annealing.

The mix was incubated for 10 minutes at 85ºC in a heating block and left on the bench to cool down at room temperature (RT, 25ºC) and stored at -20ºC. [36,37] A total of 3 gRNA mixes were prepared, one for each of the regions of interest of the SRR1 protein.

1.9.1.2 Insertion of the gRNAs, Digestion and Ligation

The pKI1.1R plasmid presents a region between the gRNA scaffold and the AtU6.26 promoter that contains *AarI* (restriction enzyme) recognition sites. The digestion with *AarI* generates overhangs in this zone (5'-TAA and GTTT-5') that allows the insertion of oligos presenting complementary overhangs (5'-ATT and CAAA-5'). The used gRNAs were designed to have these overhangs to ensure their ligation in this specific region (Appendix 2).

A modified protocol from the one described by Cong and Zhang (2015); Vad-Nielsen et al. (2016) [36,37], was used to generate the transformation vectors.

First, a 1:100 dilution of each gRNA mix (495 µL ddH_2O + 5 µL of gRNA mix) was prepared to obtain a 0.1 µM concentration of each mix. The diluted gRNA mix was added to a PCR tube containing 18 µL of Master mix (Appendix 3) and incubated in a thermal cycler following a specific program (Appendix 3) to generate 3 pKI1.1R plasmids with the desired gRNAs (pKI1.1R-gRNA) (pKI1.1R-ISL, pKI1.1R-TRA and pKI1.1R-KIN).

1.9.1.3 Cloning of pKI1.1R-gRNA, Heat-shock transformation of *Escherichia coli*

The generated vectors containing a specific set of gRNAs (pKI1.1R-ISL, pKI1.1R-TRA and pKI1.1R-KIN) were cloned in *E. coli* chemically competent cells for their amplification. A volume of 2 µL of the pKI1.1R-gRNA vectors was added to *E. coli* aliquots that were transformed using a heat-shock procedure (42ºC for 45 seconds) and incubated at 37ºC and 220 rpm during 1 hour [38]. Later, 50 and 100 µL of the transformed cells were plated in 2 LA (LB agar) plates containing Spectinomycin (Spec, 100 µg/mL) as a selective antibiotic.

1.9.1.4 Colony PCR and Gel electrophoresis

The Polymerase chain reaction (PCR) is a commonly used method in molecular biology to generate copies of a DNA sequence [39]. This method can be used to amplify a determined DNA region by using specific primers, and later on the length or size of the PCR product can be checked and observed by running it in an Agarose gel electrophoresis [40]. To confirm the presence of the desired vector (pKI1.1R-gRNA) in the transformed and selectively grown *E. coli* cells, colonies obtained after incubating them for 24 hours at 37ºC in LA-Spec plates were used as templates for a Colony PCR reaction (Appendix

3). The sense gRNAs of each domain or region of interest (Island gRNA sense, Transm gRNA sense and Kinase gRNA sense) were used as forward (FW) primers and the pKIseq primer (5µM), specifically designed to target a region downstream of the gRNA scaffold in the pKI1.1R plasmid (Appendix 1), was the reverse primer (RV) of the reaction. This set of primers should generate a PCR product of ~450 bases (bp). The FW primers were diluted 20 times (1:20) to obtain a 5 µM concentration (20 µL of primer + 380 µL of ddH_2O). As a negative control for the reaction, the empty pKI1.1R plasmid was also used as a template for the Colony PCR. The PCR templates were obtained after touching an *E. coli* colony with a sterile pipette tip and immersing the tip in a PCR tube containing 5 µL of ddH_2O. Then, 15 µL of the appropriate Master mix (with the correct FW primer) depending on the different vectors used for the bacterial transformation (pKI1.1R-ISL, pKI1.1R-TRA and pKI1.1R-KIN) were added to these PCR tubes. The PCR products were then separated by size (number of kb) by running a 1% agarose gel (1g of agarose, 100 mL of TAE buffer) using 1xTAE (242 g of Tris base, 57.1 mL of Acetic acid, 100 mL of 0.5 M EDTA) as the running buffer and GelRed® (Biotium, Inc.) to dye the gel. This dye acts as an intercalating agent between nucleic acids in a similar way to ethidium bromide (EB) but is safer and less toxic [41]. Since the *Taq* buffer used for the PCR master mix already had a loading dye (Appendix 3), 20 µL of the samples were directly charged in the gel wells. In addition, 5 µL of GeneRuler™ 1kb Pus DNA Ladder (Thermo Fisher Scientific) (Appendix 6) were also run in the agarose gel as a DNA standard.

The gel was run at 66 Volts (V) for 45 minutes and later on the length of the DNA bands was checked under UV light using a Gel Doc™ 200 (BioRad).

1.9.1.5 Plasmid DNA isolation

To further check the presence or not of the desired Oligos inside the pKI1.1R plasmid, liquid cultures of the colonies analyzed by Colony PCR were prepared. The same pipette tips used to obtain the Colony PCR templates were placed in 13 mL plastic tubes with 5 mL of LB-Spec (100 µg/mL) medium and incubated over-night (ON) at 37ºC with 220 rpm shaking conditions.

The E.Z.N.A.® Plasmid Mini Kit I (Omega) was used, following the protocol specified by the manufacturer, to obtain 30 µL of plasmid DNA eluted in ddH2O. The concentration (ng/µL) and quality of the isolated plasmids was determined by

NanoDrop™ spectrophotometry (Thermo Fisher Scientific) by loading 1.5 µL of the isolated plasmids in a NanoDrop-100 spectrophotometer (Thermo Fisher).

1.9.1.6 DNA sequencing

The isolated plasmids were prepared for external Sanger DNA sequencing (GATC Biotech AG, Cologne, Germany) depending on their concentration. For that, 5 µL of the pKIseq primer (5µM) and a maximum volume of 5µL of the isolated plasmid (80-100 ng/µL) were mixed in a 1.5 mL tube to a final volume of 10 µL.

The sequencing results were aligned to the pKI1.1R plasmid sequence using the alignment tool from the SnapGene software **(**from GSL Biotech; available at snapgene.com) looking for mismatches in the plasmid region up-stream of the gRNA scaffold where the *Aar1* restriction enzyme generated a double strand break (dsb) were the gRNA oligos were inserted.

1.9.2 Electroporation of Agrobacterium tumefaciens C58

As previously mentioned, to generate mutant plants a binary vector transformation assay was performed. This method relies in the presence of a helping vector or virulence plasmid (Vir-plasmid) in the *Agrobacterium tumefaciens* C58 strain that allows the transfection of the T-DNA region, flanked by a right and left border, of a binary plasmid [42]. This agro (*Agrobacterium*) strain only has a Vir-plasmid and therefore is possible to introduce, thanks to electroporation, and replicate the pKI1.1R plasmid in these cells, so that acts as the binary plasmid for the posterior binary transformation of *A. thaliana*. The helping vector, or Vir-plasmid, gives Rifampicin (Rif) and Ampicilin (Amp) resistance to the *A. tumefaciens* C58 cells, while the pKI1.1R plasmid confers them with Spec resistance.

For the agro transformation, 2µL of diluted plasmid (1:10) (8 µL of ddH_2O + 2 µL of plasmid) was added to tubes containing *A. tumefaciens* C58 and these were incubated on ice for 2 minutes. Then the content of the tubes was added to a pre-chilled electroporation cuvette (Gene Pulser® II cuvette, Bio-Rad) and a voltage of 2.5 kV (25 µF of capacitance) was applied during 5 seconds to each cuvette (3 cuvettes, one per each pKI1.1R-gRNA vector) with the Gene Pulser® II Electroporation System (Bio-Rad). Later, 400 µL of LB medium were added to the cuvettes and the transformed cells were returned into its previous tubes. These were incubated at 28ºC for 2 hours with 220 rpm of shacking conditions. [43]

After the incubation period, 80 and 100 µL of the transformed *Agrobacterium* were plated on LA + Rif (25 µg/mL) Amp (100 µg/mL) Spec (100 µg/mL) plates and incubated for 48 hours at 28ºC.

1.9.3 Transformation of *A. thaliana* by floral dip

Plants destined to be transformed were grown in pots for 4-5 weeks until they developed inflorescences. These were cut, using scissors, to induce the formation of higher number of inflorescences that could be transformed by floral dip. Thus, increasing the number of seeds that could be later harvested for screening.

Before transforming *A. thaliana* plants, a Colony PCR of the grown *Agrobacterium* colonies was performed (see "Colony PCR" section and Appendix 3) to check that the desired binary plasmid, pKI1.1R-gRNA, was present in these agro cells. Those colonies that showed the desired bands after running a 1% agarose gel, were grown in plastic tubes with 3 mL LB + Rif (25 µg/mL) Amp (100 µg/mL) Spec (100 µg/mL) over-night (ON) at 28ºC and 220 rpm, to select cells with both the Vir-vector and the binary plasmid. Later, 100 µL of those cultures were added to Erlenmeyers with 50 mL LB + Rif Amp Spec and grown ON at 28ºC and 220 rpm, to generate cultures used for floral dip transformation as described by Clough and Bent (1998) [44]. In both cases, 3 and 50 mL, the cultures were incubated until reaching stationary phase of growth, that was checked by measuring the cell density at OD_{600} of the cultures (~2) [44] with a SmartSpec™ Plus Spectrophotometer (BioRad).

The 50 mL cultures were centrifuged 10 minutes at 13000 rpm and the supernatants were discarded. The pellets were resuspended in 250 mL of 5% sucrose freshly prepared solution, and Silwet L-77, to a 0.02% concentration, was added to the mix prior to the floral dip. Once dipped, the plants were placed in a plastic tray, containing damped paper, and covered to maintain high humidity conditions and protect them from excessive light, for 16-24 hours. After that period, the plants were returned to a growth room and grown under normal conditions (as described in the "Growth conditions" section). [44]

A glycerol stock of the agro lines used for transformation was prepared by mixing 250 µL of the 50 mL culture with 250 µL of glycerol in 1.5 mL tubes that were stored at -80ºC.

1.9.4 Mutant seeds screening and selection

After harvesting seeds from the transformed *A. thaliana* plants (T_0 seeds), these were screened to select the ones that would have possibly been mutated. For that, the seeds

were observed using a stereomicroscope (Zeiss Axio Zoom V16) using an RFP filter (excitation at 561 nm of wavelength). Thanks to the presence of the RFP protein under the *OLE1* (*OLEOSIN 1*) promoter, the modified seeds emit red fluorescence in the seed dormancy state that can be observed with the stereomicroscope [35]. These glowing seeds were stored in 2 mL tubes at 4ºC to prevent their germination.

To double check that the seeds were indeed mutated, a conventional selective growth screening was performed [44]. The seeds were sterilized (see "Seeds sterilization" section), immersed in 0.1% agarose and plated in ½ MS plates with Hyg (20 µg/mL) since the Hyg^R gene was also part of the pKI1.1R plasmid region that was transfected into the *A. thaliana* inflorescence cells [35].

The T_1 seeds (obtained from T_0 plants) were checked again for red fluorescence activity. Those seeds glowing under visualization using an RFP filter are discarded since this RFP activity is an indication of the presence of the Cas9 protein in the seeds. Cas9-free seeds are selected to have stable mutant plants without off-target mutations and free of undesirable mutations of a wild-type allele [35]. Then, these Cas9-free seeds are germinated and grown in selective ½ MS plates with Hygromycin to check if they present the expected 3:1 Mendelian segregation ratio ("3" being mutated and therefore "alive" seedlings and "1" being the "dead" non-mutant ones) calculated with a Chi-square test [45]. Both hetero and homozygous variants of the gene are englobed inside the "alive" plants.

1.10 Genotyping of mutant plants

The seedlings that were able to grow in ½ MS + Hyg plates were transplanted to pots after 2 weeks of *in-vitro* conditions. Two weeks later, tissue from those plants grown in soil was harvested and used to check if those plants were actual mutants, and to determine which type of mutation presented by first performing a High Resolution Melting (HRM) analysis and later on by sequencing the specific region of their DNA that was targeted for modification.

1.10.1 DNA extraction and quantification

To obtain DNA from the possibly transformed plants, a third of one of their rosette leaves was cut and deposited in 1.5 mL tubes with the help of sterile scissors and tweezers, that were washed with 70% ethanol before obtaining tissue from a different plant to avoid cross contamination. A modified version of the protocol described by Edwards et al. (1991) [46] was followed to extract DNA from the harvested tissue. The leaves were

crashed in the 1.5 mL tubes using sterile plastic grinders (Bel-art products: Scienceware, Pequannock, NJ, 07440 USA). Then, 200 µL of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) were added to the tubes, the tissue was grinded again and 200 µL of buffer were added to the tube again. The mix of 400 µL of extraction buffer and crashed tissue was vortexed for 5-10 seconds. Afterwards, the tubes were centrifuged for 3 minutes at 13000 rpm and 300 µL of the supernatant were transferred into a new tube. A volume of 300 µL of isopropanol was added to the tubes and left at RT (25ºC) for 2 minutes. The tubes were then centrifuged at 13000 rpm for 5 minutes, the supernatant was discarded, and the pellets were dried at 60ºC for 15-20 minutes. Finally, 100 µL of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) was added to each tube to dissolve the DNA pellets. The DNA quality and concentration were determined by NanoDrop spectrophotometry (see "Plasmid DNA isolation" section) and the samples were stored at 4ºC.

1.10.2 PCR for DNA amplification

A set of specific primers that targeted the different regions of interest of the *SRR1* gene were used (Appendix 2) to amplify the sections of the plants genome that should have been edited by the CRISPR-Cas9 technology. A modified Master mix from the one used for the Colony PCR was prepared. In this case, Ex-*Taq* was used instead of Dream*Taq* polymerase because it has a better efficiency and lower mutation rate than the latter (it incorporates a 3'-to-5' exonuclease with proofreading activity) (Takara). Due to this change, the PCR program was also adjusted to the conditions needed for the correct function of the Ex-*Taq* (Appendix 3). Moreover, the used buffer did not include loading dye since the PCR products were destined to be analyzed by HRM. In addition, WT genomic DNA was also amplified and used as a negative control. Later, the size of the amplified DNA was checked by running 10 µL of the PCR products in a 1% agarose gel and observing the bands under UV light. Since the PCR products did not have loading dye, 5 µl of a 6x concentrated DNA Gel Loading Dye (Thermo Fisher) was added to the samples before loading them in the gel wells.

1.10.3 High Resolution Melting (HRM) analysis

The remaining volume of those samples that presented the desired or expected band length, after observing the above-mentioned agarose gel under UV light, were used for an HRM analysis to determine if the DNA presented any mutations. This technique defines the analyzed PCR products based on their melting response when they transition

from double-stranded DNA (dsDNA) to single-stranded DNA (dssDNA)(Qiagen). Unlike gel electrophoresis, melting curve analysis can distinguish products of the same length but different GC/AT ratio thus allowing to identify single base pair changes such as insertions and deletions (indels) [47].

For this analysis, a set of 2 specific primers, HRM FW and RV, was designed for each region of interest of the *SRR1* gene (Appendix 2). These primers, FW and RV, were diluted to a concentration of 2 µM and 2 µL of each was added to the Master mix reactions made using the Type-it HRM PCR Kit (Qiagen) (Appendix 3). 15 µL of Master mix were loaded in a 96-wells plate containing 5 µL of PCR products, previously diluted 1:4x10^6 times with ddH_2O, to a total volume of 20 µL per well. Three technical replicates of each sample, plant, were loaded as control of possible deviations. The PCR plate was analyzed using a LightCycler®96 System (Roche) following a program specified in the "HRM analysis" section of the Appendix 3.

1.10.4 Purification of PCR product

The DNA of those plants that showed a melting peak shifted or different from the WT plants was amplified again following a modified version of the PCR procedure described in the "PCR for DNA amplification" section. In this case, 50 µL reactions were prepared for amplification in order to generate enough quantity of PCR product that could be purified for its use in TOPO-TA cloning. Part of the obtained product, 10µL, was used to check if the PCR was successful by running it in a 1% agarose gel electrophoresis and observing the number of kb under UV light.

The remaining 40 µL of PCR product were directly purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega), by following the instructions specified by the manufacturer and obtaining 50 µL of eluted DNA in Nuclease-Free water (Promega). The yield of the purification procedure was checked by NanoDrop spectrophotometry, measuring the concentration (ng/µL) and purity of the DNA samples.

1.10.5 TOPO-TA cloning of purified DNA

The purified PCR products were cloned using the TOPO TA cloning® Kit Dual Promoter (Invitrogen) to generate vectors containing the DNA regions of the *SRR1* gene that were targeted for modification. For the TOPO® reaction, 3.5 µL of purified DNA was mixed with 1 µL of Salt solution, 0.5 µL of pCR®2.1-TOPO®vector (Appendix 1) and 0.5 µL of H_2O to a total volume of 6 µL. The reaction was incubated at 22ºC for 30 minutes in a T100™ Thermal Cycler (BioRad). After the incubation step, the total volume of the

reaction, 6 µL, was used to transform *E. coli* DH5α chemically competent cells as described in the "Cloning of pKI1.1R-gRNA" section. Since the pCR®2.1-TOPO® vector contains the *Amp*^{R} gene (Appendix1), 100 µL of the transformed cells were plated in LB + Ampicilin (Amp) (100 µg/mL) plates and incubated ON at 37ºC. The obtained colonies were grown in plastics tubes containing 5 mL LB Kan to generate cultures that could be "mini prepped" for DNA isolation and used as templates for a Colony PCR analysis (see "Colony PCR and Gel electrophoresis" section). For this PCR reaction, the same plasmids used for the "PCR for DNA amplification" method were also part of the PCR master mix. The colonies whose PCR products showed the expected band after observing the 1% agarose gel under UV light, were processed as described in the "Plasmid DNA isolation" section in order to obtain DNA samples that were Sanger sequenced externally as mentioned in the "DNA sequencing" segment.

1.10.6 Multiple alignment of the DNA sequencing results

To compare the DNA from the transformed plants with the *SRR1* gene of WT plants, a multiple sequence alignment was performed between the Sanger sequencing results of the in theory mutated plants *SRR1* gene and the genomic sequence of the At1g74360 gene available at The Arabidopsis Information Resource (TAIR) (www.arabidopsis.org). Prior to the alignment, the sequences were submitted to the NCBI's BLASTn web tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to check that the obtained results corresponded to the *SRR1* gene and not just to the pCR®2.1-TOPO®vector. The sequencing chromatograms were also analyzed to determine the quality of the Sanger sequencing using the SnapGene (GSL Biotech) chromatogram viewer tool. Only those samples that had a clean chromatogram and that presented a high similarity score towards the At1g74360 gene (corresponding to the blastn analysis) were used for the multiple sequence alignment. For this alignment, the Clustal omega online tool [48] was used to generate the alignments and the GeneDoc software [49] was used to visualize and edit the obtained alignments.

1.11 Phenotypic analysis of mutant plants

To characterize the newly created mutant plants, their growth, specifically root growth, and response towards exogenous hormonal treatment, growth under ethylene presence, was studied and compared towards the behavior of WT plants in the same conditions. In addition, the CRISPR mutants (with a modified transmembrane region) were also compared with T-DNA knock-out (KO1) and overexpression (OX6) lines of the *SRR1*

gene used in a previous study (Javad Najafi's PhD thesis, data not published) to observe if the new mutant plants were indeed KO lines, as aimed, and how they responded to the different treatments in comparison with the overexpression and T-DNA KO lines. To gather information of how the two experiments affected the WT plants and the T-DNA KO1 and OX6 lines, pilot assays were done while obtaining CRISPR mutant plants.

1.11.1 Root length measurement

Previous experiments showed that the WT, the T-DNA KO1 and OX6 seedlings presented different phenotypes after exposing them to exogenous sugars once they had depleted their sucrose storage (data from the pilot experiment). For this reason, seedlings of the previously mentioned lines in addition to CRISPR mutant ones, were grown in 6-wells plates containing 2 mL (on each well) of ½ MS media without sucrose (No sugar treatment or NS). After being left at 4ºC for 2 days, the plates, containing ~30 seeds per well, were placed in a growth room for 3 days since that gives enough time to have germinated seedlings that are still not mature and growing. After that period of time, the media of the plates was removed and replaced by ½ MS with 25mM of sucrose (Suc treatment) or palatinose (Pal treatment). For the assay, 3 plates were used, one per treatment. Four of the 6 wells of each plate had seeds of one of the 4 different studied lines (WT, T-DNA KO, OX and CRISPR mutant). The plates were placed again in a growth room, allowing the seedlings to further develop for 1 week. After those 7 days, 15 to 20 seedlings from each line and treatment were plated in 1% agar plates with the help of tweezers. This transference allowed the obtention of picture of each line and treatment, that were analyzed using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Ins titutes of Health, Bethes da, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016) to calculate the root length of each of the plated seedlings. The mean of each line and treatment was subjected to analysis using a One-way ANOVA followed by a post-hoc Bonferroni's multiple-comparison test.

1.11.2 Ethylene response

Ethylene is a hormone involved in the germination an early growth of seedlings, among other processes [50]. It also interacts with sucrose in a signaling pathway that modulates the circadian clock of the plants [51]. Therefore, is possible that ethylene may interact with the *SRR1* gene or with the SRR1 protein. The fact that under ethanol treatment and dark conditions *A. thaliana* seedlings present a characteristic phenotype known as the "triple response" characterized by inhibition of root and hypocotyl elongation, an

exaggerated apical hook, and a thickening of the hypocotyls [52]; provides a positive control of the ethylene effect.

But since this hormone acts mainly as a gas, a precursor of ethylene was used for this experiment [53]. The 1-Aminocyclopropane-1-carboxylic acid (ACC) was added to a 10 μM concentration to vertical plates containing ½ MS media with and without sucrose (25 mM) and 0.8% agar. Wild-type seedlings, together with KO1, OX6 and CRISPR mutants were grown in vertical plates with sucrose (Suc), sucrose and ACC (Suc+ACC), without sucrose (NS) and ACC without sucrose (NS+ACC) for 5 days in dark (the plates were covered with aluminum foil to avoid light exposure). The "triple response" was compared with the absence of effect of *ein2* seedlings, that are mutants insensitive to ethylene exposure (negative control)[52].

Then, pictures of each treatment and line were analyzed with the help of the ImageJ software to obtain the hypocotyl length of the different lines and the mean of line was used for a One-way ANOVA analysis followed by a Bonferroni post-hoc multiple-comparison test.

1.12 Gene expression analysis, Starvation experiment

To gather information about how the *SRR1* gene reacts to the presence and absence of external sugars, in terms of growth and sugar signaling events. 5 genes were targeted for gene expression analysis: *bZIP11*, *DIN1*, *DIN6*, *PAP1* and *TPS9*. For this experiment, 12-wells plates were used to germinate and grow 4 different lines of *A. thaliana* plants. Wild-type (WT) plants, plants with a T-DNA insertion in the transmembrane domain (KO1), a line with an overexpression of the *SRR1* gene (OX6) and the transmembrane knock-out line obtained by CRISPR-Cas9 modification (TRA-KO). This last line was studied to corroborate that it behaved as its T-DNA KO homolog. The seeds of each line were sterilized and embedded in 0.1% agarose as described in the "Seeds sterilization" section. Then, 15-20 seeds were placed in each of the wells of three 12-wells plates containing ½ MS with 25 mM sucrose (Suc). There was one plate for each treatment: ½ MS with 25 mM Suc used as a control (CTR), ½ MS with 25 mM palatinose (Pal) and ½ MS without sucrose (NS, no sugar). On each plate or treatment there were 3 biological replicates, represented by the different wells (4 lines * 3 replicates = 12 wells). After placing the seeds at 4ºC for 2 days to ensure homogenous germination, the plates were moved to a growth room. The medium of all the plates or treatments was changed after 5 days, since that's the period of time that the seedlings need to germinate and to exhaust all their stored

sucrose. Therefore, an effect of exogenous sugars can be observed. Before replacing the medium of the Pal and NS plates, the seedlings were washed by adding 2 mL of ½ MS without sucrose to each well twice and waiting 5 minutes each time. This "washing" was done to eliminate the presence of sucrose in the wells that could interfere or mask the NS and Pal effect on the seedlings. After replacing the media, the NS and Pal plates were covered with aluminum foil to avoid light exposure and simulate an "Starvation" state in the seedlings of those treatments (lack of photosynthetic activity). All the plates were placed in the growth room again, and the dark conditions were kept for 6 hours. Once the 6 hours passed, the tissue (seedlings) of each well (from the 3 plates) was placed in a previously labeled 1.5 mL tube, that had inside a stainless-steel bead, and then snap-freezed in liquid nitrogen (N_2) to preserve the RNA stability of the samples. All the tubes were stored at -80ºC.

1.12.1 RNA isolation

For gene expression analysis, total mRNA is isolated because this molecule represents an estimation of the transcription state of genes and therefore how active they are (up or downregulated). To obtain RNA from the samples prepared as described in the previous section, the stored frozen tissue was grinded using a TissueLyser (Qiagen) with a frequency of 25 Hz (Hertz) for 2 minutes. After 1 minute, the recipient that contained the samples was flipped and grinded for the remaining 1 minute to ensure that the tissue was totally homogenized. Then, the SpectrumTM Plant Total RNA Kit (Sigma-Aldritch) was used following the protocol specified by the manufacturer to obtain total RNA from the samples. A slight modification was done to that protocol, since 80 µL of DNase I dissolved in DNase digestion buffer (RDD buffer) (Qiagen) was added to the samples, before the second wash with the Wash 1 solution, to digest the remaining DNA. The total RNA was eluted in 50 µL of elution buffer and quantified using a NanoDrop-One spectrophotometer (Thermo Fisher).

1.12.2 Complimentary DNA (cDNA) preparation

The fact that RNA molecules are more unstable than DNA and that nowadays there's no direct RNA sequencing technique, requires the conversion of RNA samples into complimentary DNA (cDNA), by reverse transcription PCR (RT-PCR), for gene expression analysis. This transcription was done with the QuantiTect Reverse Transcription kit (Qiagen). The cDNA was obtained by adding 2 µL of gDNA Wipeout buffer, to 1µg of RNA and water to a total volume of 14 µL depending on the RNA

concentration of each sample. 6 µL of the kit master mix (Appendix 3) was added to the 14 µL of diluted RNA, to obtain 20 µL reactions. These reactions were incubated at 42ºC for 15 minutes and then at 95ºC for 30 minutes in a T100™ Thermal Cycler (BioRad). Before being stored at -20ºC, the samples were diluted 5 times by adding 80 µL of water. To control the level of the DNA contamination in the samples, a negative reverse transcription (NRT) reaction was also prepared following the same procedure previously described.

1.12.3 Quantitative PCR (qPCR)

By performing a Quantitative PCR (qPCR) analysis, the mRNA amount of 6 different genes was quantified to determine their expression level. These genes were: *bZIP11*, *DIN1*, *DIN6*, *PAP1*, *TPS9* and *Tip41*-like that was used as a "Standard" for a posteriori analysis. The SYBR® Green I Master 2x was used as the fluorescent marker whose fluorescence is quantified when it binds to the amplified cDNA. This fluorescence intensity can be correlated with how amplified a sample is and therefore how quantitatively expressed a gene is [54].

For the quantification, a set of 2 specific primers (Forward and Reverse) was used for each gene at 5 µM concentration. 10 µL of the SYBR® Green I Master 2x dye were mixed with 2 µL of each primer, FW and RV, and 3 µL of water. The 15 µL master mix was added to 96-well plates containing 5 µL of cDNA. A negative control (NTC, no template control) that contained only 15 µL of the master mix, was also loaded for each gene (6 genes, 6 NTCs). For each treatment, 3 biological replicates of every line were loaded in the 96-wells plate to control possible deviations. The plate was run in a LightCycler® 96 System (Roche) following a program specified in the Appendix 3.

The fluorescence over cycle data generated by the LightCycler® 96 System (Roche) was first analyzed with the LinRegPCR software [55] to extract the Cq values of the different samples, and these were used to perform an ANOVA analysis and Multiple-comparison Test using the qbase+ 2.6.1 (Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com). For this last analysis, the WT CTR (½ MS Sucrose 25 mM treatment) was used as a reference group and the *Tip41*-like was the reference gene. The rest of the samples were compared (scaled), using the arithmetic mean of the Cq value of each treatment, and normalized towards the WT CTR treatment and the *Tip41*-like gene respectively.

1.13 GUS staining assays

The GUS reporter system is normally used to localize the tissues were a certain gene is expressed. For that, the *GUS* gene is expressed under the control of the promoter of the gene of interest. Two histochemical analysis using mutant plants that expressed the *GUS* reporter gene under the same promoter that controls the *SRR1* levels were used. These plants had been previously generated in the CMBG group and were tested under exposure to extracellular sugars (sucrose and palatinose) and phytohormones (ABA among others). These assays aimed to generate information that allowed to qualitatively analyse the expression of the *SRR1* gene. For the both experiments, the same staining procedure was used following a modified version of the protocol described by Scarpela et al (2009)[56]. The plant material was incubated in pre-chilled tubes containing 90% acetone for 1 hour at -20ºC. Then, the samples were washed twice with 100 mM phosphate buffer (Na-P-buffer pH7.7) for 5 minutes. This buffer was replaced with GUS rx-buffer (100 mM Na-P-buffer pH7.7, 10 mM Na-EDTA, 1mM Ferricyanide, 1mM Ferrocyanide, 1% Triton X-100 and 1mM x-Gluc) and the samples were vacuum infiltrated for 3 minutes. Then they were incubated for 3 hours at 37ºC in dark and after that the staining reaction was stopped replacing the GUS rx-buffer with Ethanol:Acetic acid (3:1) and leaving the samples at RT overnight.

The stained seedlings were mounted on glass slides with Ethanol (70%) and observed using a stereomicroscope (Zeiss Axio Zoom V16).

1.13.1 Sugar response

To observe how the *SRR1* gene is expressed in a tissue level in response to exogenous sugar treatments, mutant GUS-plants were grown in ½ MS liquid media without sucrose (No sugar or NS treatment) in 6-wells plates (15-20 seeds per well). After 5 days of growth, the media was replaced with ½ MS with 25mM of sucrose (Suc treatment) or palatinose (Pal treatment) or just changed for fresh media in the case of the NS treatment. 24 hours after the media were changed, the seedlings were stained as described above.

1.13.2 Hormonal treatment

There are different phytohormones that are involved in diverse metabolic events in plants, such as stress response and growth. Since the SRR1 gene seems to be involved in both growth and stress response signaling pathways, hormones that take part in both processes were used to treat GUS-plants together with and without sucrose. The aim of this assay was to determine if there was an interaction between the sugar and the hormones that

could modify the *SRR1* gene expression observed in the "Sugar response" experiment. For this experiment, 24-well plates containing 2 mL of ½ MS liquid media without sucrose (NS) were used to germinate seeds. Then, after 5 days of growth the media was changed with ½ MS liquid media with one hormone and with or without sucrose (ACC-Suc and ACC-NS for example). Six different hormones were selected due to their involvement in growth and/or stress responses. The hormones were: Abscisic acid (ABA, 10 μM), 1-Aminocyclopropane-1-carboxylic acid (ACC, 10 μM), Methyl Jasmonate (MeJA, 10 μM), Indole-3-acetic acid (IAA, 30 nM), Salicylic acid (SA, 500 μM) and Zeatin (Zt, 10 μM). One day after being treated, the seedlings were stained following the protocol described above ("GUS staining assays" section).

1.14 Protein-protein interaction experiments

The *SRR1* gene encodes a Receptor-like-kinase protein (RLK) that is believed to participate in extracellular sugar sensing events and in response to nematode infections, whose activity pathway is unknown. This protein is hypothesized to form a dimer after a ligand binding event in order to transmit a signal to the inside of the cell. This dimer can be formed with another SRR1 protein (homodimer) or with a different RLK protein (heterodimer). Since no clear dimer companion has been identified for the studied protein, two Bimolecular Fluorescence Complementation (BiFC) experiments were performed with the aim of gathering more information towards the signaling role of the SRR1 protein.

The two protein-protein interaction (PPI) experiments were done using 2 vectors, pUC-SPYNE and pUC-SPYCE, that allow the expression of proteins fused to the C- and N-terminal amino acids of fragments of a non-fluorescent Yellow fluorescent protein (YFP). When these proteins form a complex, between the protein encoded in pUC-SPYNE and the one encoded in pUC-SPYCE, the YFP protein can be excited for fluorescence emission under the right conditions. [57]

Before generating BiFC vectors containing the *SRR1* gene, and to test the efficiency of the 2 assays, the pUC-SPYNE-*bZIP63* and pUC-SPYCE- *bZIP63* vectors were used in the later described experiments. These plasmids express the bZIP63 transcription factor fused to a split YFP protein and allow the detection of yellow fluorescence, under confocal microscopy observation, in the nuclei of plant cells when the previously described complex is formed since bZIP63 forms homodimers [57,58].

The possible PPIs were observed using a CLSM Leica SP5 confocal microscope.

1.14.1 Protoplasts isolation and transformation

Protoplasts are cells whose cell wall has been destroyed by an enzymatic digestion. This makes them suitable for the observation of protein interactions events in different regions of the cell such as the cell membrane. [59]

Since the SRR1 protein is bound to the cell membrane, a BiFC assay using protoplast was considered as an adequate choice to study the dimer formation of this protein. A protocol modified from the one described by Wu et al. (2009)[60] was used to obtain protoplasts from *A. thaliana* leaves of 3-week old plants. Two different types of tape, Time tape (Time Med) and Magic tape (3 M), were used to peel the epidermal cells of the leaves. The leaves, 7-10 of them, were then placed in a Petri dish that contained 25 mL of enzyme solution (1% cellulase 'Onozuka' R10 (Yakult), 0.25% macerozyme 'Onozuka' R10 (Yakult), 0.4 M mannitol, 10 mM CaCl2, 20 mM KCl, 0.1% BSA and 20 mM MES, pH 5.7) and were left there in agitation (~50 rpm) for 1 hour. The enzyme solution containing released protoplasts was transferred to 50 mL tubes and centrifuged at 120x*g* for 3 minutes at 4ºC. The supernatant was decanted, and the protoplasts were washed twice with 25 mL of pre-chilled W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 2 mM MES, pH 5.7) and then incubated on ice for 30 minutes. After this incubation, the solution was centrifuged again at 120x*g* for 3 minutes and the protoplasts were resuspended in 5 mL of MMg solution (0.4 M mannitol, 15 mM MgCl2, and 4 mM MES, pH 5.7). A NovoCyte^{TM} benchtop Flow Cytometer (ACEA Biosciences, San Diego, CA) was then used to quantify the cell density of the solutions (cells or protoplasts per mL).

The transfection of the protoplasts was performed following a modified version of the protocols used by Wu et al. (2009) and Mitula et al. (2015) [60,61]. The previously obtained solution, containing protoplasts, was diluted to a cell density of $1x10^5$ cells/mL in 0.2 mL of MMg solution. The diluted solution, in a 2 mL tube, was mixed with 10 µL of pUC-SPYNE-*bZIP63* and 10µL pUC-SPYCE- *bZIP63* (5 µg of each plasmid). Then an equal volume (20 + n µL used for the dilution) of 40% (w/v) PEG (MW 4000) with 0.1 M CaCl2 and 0.2 M mannitol was added to the tube and incubated at RT for 5 minutes. Then, 450 µL of W1 solution was added to the mix and the protoplasts were pelleted by centrifugation at 300x*g* for 3 minutes. The supernatant was discarded, the protoplasts were resuspended in 300 µL of W1 solution (0.5 M mannitol, 20 mM KCl, 4 mM MES) and incubated in a 6-wells plate, coated with 1% BSA, at RT ON (overnight) with continuous light.

1.14.2 Infiltration of *Nicotiana benthamiana* leaves

The SRR1 protein is also present in *Nicotiana benthamiana*, that's why an *Agrobacterium* mediated infiltration experiment for transient expression analysis was performed. This method just requires having an agro strain containing a plasmid suitable for BiFC, making this assay simpler to perform than the one described in the previous section.

The pUC-SPYNE-*bZIP63* and pUC-SPYCE- *bZIP63* vectors were introduced inside *A. tumefaciens* GV3101 cells via electroporation as described in the "Electroporation of *Agrobacterium tumefaciens* C58" section. This strain, GV3101, was used instead of C58 because the vectors have a Carbenicillin (Car) resistance cassette. This antibiotic is a more stable analog of Ampicilin and therefore the C58 can't be used for the expression of the pUC-SPYNE and pUC-SPYCE vectors since it would not be possible to effectively select bacteria with both the helper and the binary plasmids. On the other hand, the GV3101 strain has a helper plasmid that gives Gentamicin (Gen) resistance to the cells.

Colonies containing one of these two vectors, pUC-SPYNE or pUC-SPYCE, were generated. The agro colonies were grown in LA + Rif Gen (30µg/mL) Car (50µg/ml) and used for infiltration of *N. benthamiana* leaves following a modified protocol from Bendahmane et al. (2000) [62]. Agro colonies were inoculated in tubes with 5 mL LB + Rif Gen Car ON at 28ºC and 220 rpm. 1 mL of this culture was added to 50 mL of LB + Rif Gen Car with 10 µL of acetosyringone (100 mM) and 500 µL of MES-KOH (pH 5.7, 1M). This culture was grown ON at 28ºC and 220 rpm. The same cultures were made to grow an agro strain containing a plasmid encoding the P19 protein, that acts as a suppressor of post-transcriptional gene silencing (PTGS) in *N. benthamiana* [63] and therefore facilitates the expression of the split YFP+bZIP63 proteins in the tobacco leaves. The 3 cultures were centrifuged at 13000 rpm for 15 minutes and the pellets were resuspended in 5 mL of infiltration buffer (10 mM $MgCl_2$, 10 mM MES-KOH and 150 µM acetosyringone). Then, the OD_{600} of each culture was measured and adjusted to a value of ~1.5 and incubated at RT on the lab bench for 2-4 hours. The 3 cultures were mixed later to a final OD_{600} of 0.5 for each strain and used to infiltrate leaves of 4-5 weeks old *N. benthamiana* plants using a 1 mL syringe loaded with the mixture of the 3 agro strains diluted in infiltration buffer. The plants were kept in the lab, and the treated leaves were observed after 4 days of the infiltration.

1.15 Statistical analysis

With the exception of the ANOVA and multiple comparison analysis performed for the qPCR data, done using the qbase+ software (Biogazelle), the rest of the generated information was analyzed by One-way ANOVA followed by Bonferroni's multiple comparisons test using GraphPad Prism version 7.00 for macOS, GraphPad Software, La Jolla California USA, www.graphpad.com. The graphs that represent the statistical analysis were also obtained with this software.

Results and Discussion

To create a more fluid and easier to follow text and reading experience, it was decided to include the discussion section of each experiment together with the results obtained for each assay.

1.1 Generation of Vectors for CRISPR-Cas9 mediated mutagenesis

At the beginning of the "Mutant plants obtention" section it was mentioned that the aim of this experiment was to design 3 different vectors that targeted 3 regions of the SRR1 protein (the Island, Transmembrane and Kinase domains).

From those 3 originally planned vectors, only the pKI1.1R-ISLgRNA, that targeted the Island domain of SRR1, was not successfully produced. Several attempts were done but the gRNA oligos (sense and antisense, Appendix 2) were not inserted inside the pKI1.1R plasmid after following the protocol described in the "Insertion of the gRNAs" section. This was confirmed by aligning the sequences of mini prepped and Sanger sequenced samples, where no mismatches towards the pKI1.1R plasmid were observed. This was done because the Colony PCR results can help determine which cells present the pKI1.1R plasmid [64,65], but this doesn't accurately and doubt free reflect the presence or not of the oligos inside the plasmid [65,66]. This could be due to the use of the gRNAs as FW primer, and not a specific one designed for this purpose. The decision of using of gRNA and pKIseq, a primer that as mentioned before targeted a region downstream of the gRNA Cassette of the pKI1.1R plasmid, was done trying to mimic the effect of Orientation-specific primers for Colony PCR detection of inserts [64,67].

The "Insertion" protocol was modified by increasing the duration of the "Ligation step" of the Incubation program from 10 to 20 minutes in an attempt to improve the chance of introducing the desired oligos in the pKI1.1R plasmid [68], but this modification did not help achieve the desired insertion. No conclusions towards why the insertion of the KINgRNA did not succeed were obtained, and it was set out that perhaps new gRNAs for the kinase domain of SRR1 should be designed to generate the desired vectors.

Since the other 2 vectors, pKI1.1R-KINgRNA and pKI1.1R-TRAgRNA, were successfully created following the previously described protocol, it was decided to stop trying to generate the pKI1.1R-ISLgRNA vector and to focus in obtaining transformed plants. This new method for obtaining the desired vectors seemed to be faster than the original ones that this protocol was based on [36,37,69]. This is based on the lack of a

phosphorylation step previous to the annealing of the gRNA oligos, the use of fewer reagents and because the reaction is PCR independent.

A snapshot from the multiple alignments generated with SnapGene (GSL Biotech) that confirmed the insertion of the gRNAs inside the pKI1.1R plasmid is presented below. In Figure 1 it can be observed the presence of the sense TRA (top) and KINgRNAs (bottom).

Figure 1 Snapshot of the multiple alignment of the pKI1.1R-gRNA vectors and the empty plasmid pKI1.1R generated with the multiple alignment tool of SnapGene (GSL Biotech). *Top) A mismatch in the region upstream of the AarI recognition site showed that the gRNA (highlighted in red) was inserted in the pKI1.1R-TRAgRNA vector. Bottom) The same mismatch was observed for the KINgRNA insertion in the pKI1.1R-KINgRNA vector. Figures generated with the multiple alignment tool of SnapGene (GSL Biotech).*

1.2 Characterization of transformed plants

It should be noted that in ideal conditions the characterization experiments of the mutant lines would have been done using T_3 or at least T_2 seeds instead of T_1. That's because in the event of a mutation taking place in a gene, this modification can be present in just one of the two alleles (in the T_0 generation). For this reason, the T_0 plants are grown and their seeds, T_1 or first generation, are harvested and analyzed to select homozygous mutants or to obtain this variation in the following generation (T_2) by crossing heterozygous mutants. Therefore, until the T_2 generation is reached, not all the population of harvested seeds have just homozygous variants of the targeted gene.

In this thesis, T_1 seeds were used to characterize the mutant lines due to time related restrictions, since the mutant T_0 plants were obtained after several transformation and screening attempts. This was in part caused by the floral dip transformation rate (1%, or 1 transformant for every 100 seeds harvested) [44] that implied screening a numerous

amount of T_0 seeds in order to identify transformant ones. In addition, as it's described in the following sections, not all the transformed seeds corresponded to mutant plants.

1.2.1 DNA analysis

To check the possible mutations of the plants that presented both red fluorescence (in seeds) (Figure 2) and Hyg resistance, tissue from 2-week-old plants grown in soil was collected and processed to isolate DNA from the targeted regions of the SRR1 protein using specific primers, Transm and Kinase PCR (see Appendix 2), as described in the "Genotyping of mutant plants" section). These primers generated ~300 and ~700 bp products respectively, that were directly used for HRM analysis, and purified for TOPO®TA Cloning®.

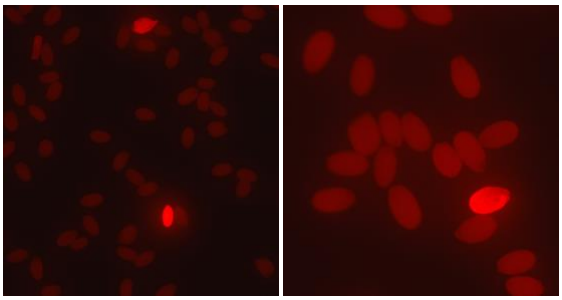

Figure 2 Pictures of glowing transformed T_0 seeds. *The transformed seeds presented red fluorescence when observed with an RFP filter. The images were obtained using a stereomicroscope (Zeiss Axio Zoom V16).*

1.2.1.1 HRM analysis

The PCR products of 8 transformed plants, together with a WT sample, were analyzed using a LightCycler® 96 (Roche) to detect possible melting behavior differences between the transformed and WT DNA PCR products when transitioning from dsDNA to ssDNA. For the TRA-gRNA transformed plants, a shift in their melting temperature (Tm) was observed for 3 of all the analyzed plants (Figure 3). Their melting curves (Figure 3A) and peaks (Figure 3B-D) were generated from the continuous monitoring of fluorescence during temperature changes using the LightCycler® 96 Software (Roche). These shifts are represented in the normalized melting peaks of Figure 3B-D and indicate that these plants had small indels (insertions or deletions) in the *SRR1* gene and therefore could be knock-out lines for the Transmembrane domain of the SRR1 protein. Since the difference in the Tm was not big (lower than 1ºC in all cases) the possible indels corresponded to small number of bases [70,71]. This low number of edited indels was expected since the CRISPR-Cas9 system is supposed to generate a low number of mutations in a controlled

manner, since the Cas9 nuclease just generates double-strand breaks (DSBs) 3 to 4 bp upstream from the PAM site recognized by the gRNA [72].

In Figure 3A, a decrease of ~0.1ºC respect to the Tm of the WT sample (purple peak) was observed, indicating not only that a small indel was present in the transformed plant but also that this edition could be a C/G or A/T change. On the other hand, the melting peaks from Figure 3B and C showed a Tm increase of ~0.2ºC and ~0.4ºC respectively. These Tm variations suggested that the mutations present in the transformed plants could correspond to C/G transversions. [73,74]

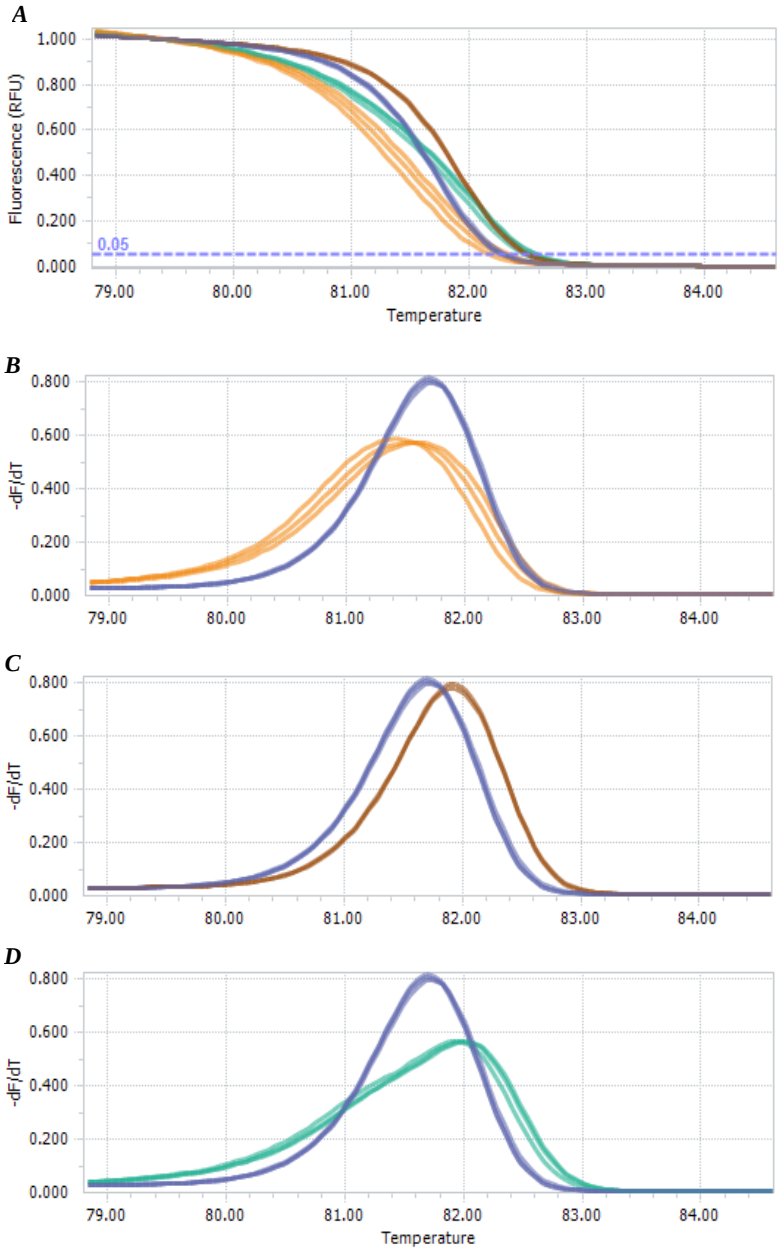

Figure 3 Normalized melting peaks of 3 different TRAgRNA edited plants compared to the melting peak of a WT sample. *A) Normalized melting curves of 3 transformed plants and a WT control (purple curve).* ***B)*** *Melting peaks showing a decrease in the melting temperature (Tm) of the transformed plant B-C) Melting peaks presenting a higher Tm in the transformed plants than the WT sample (purple curve). This were all indications that the plants presented mutation in the Transmembrane domain of SRR1.*

Additionally, the HRM analysis can also give clues relating to whether a mutation is present in one of the alleles of the gene (monoallelic), thus being an heterozygous variation, or in both alleles (biallelic) therefore producing homozygous organisms for this gene [75]. These two types of variation can be observed by the presence or not of a second melting peak in the transformed plants next to the expected one, thus indicating a monoallelic mutation [76]. In Figure 3B-D, only one melting peak is present for all the analyzed samples, therefore indicating that the mutation is probably biallelic and that the plants could have homozygous variations of the *SRR1* gene.

These biallelic mutations are desired when creating a knock-out line, because if only one of the alleles of a gene is mutated this modification could be compensated by the other copy of the gene. This genetic compensation can create knock-down versions of genes instead of knock-out ones [77]. However, since the HRM analysis revealed that the mutations of the studied plants were biallelic it could be possible that they are knock-outs of the *SRR1* gene.

On the other hand, for the KIN-gRNA transformed plants no differences between their melting curves and the WT sample was detected, indicating that the Kinase region of the SRR1 protein was not edited. This assumption was later on confirmed by performing a multiple alignment experiment.

1.2.1.2 Multiple alignment

To furtherly investigate the mutations of the TRA-gRNA transformed plants, the Sanger sequencing results of the 3 plants that were classified as non-wild type by the HRM analysis results, were aligned with the genomic sequence of the *SRR1* gene.

It is worth highlighting that, as previously mentioned in the Material and methods section, only the sequencing results that had a chromatogram profile with low background noise were used for the alignments. Moreover, from those samples just those that had a high score towards the At1g74360 gene when performing a Blastn (NCBI) analysis, were submitted to the Clustal Omega query (EMBL-EBI).

For each of the 3 plants identified as mutants by HRM analysis, 10 colonies obtained after TOPO® cloning® the purified PCR product from TRAgRNA transformed plants were mini prepped and Sanger sequenced. From those 30 colonies, 7 presented indels after being aligned with the genomic sequence of the At1g74360 gene. As shown in Figure 4, all the plants (with the exception of TRA7-4-RVC, bottom sequence in Figure 4) presented a Cytosine (C) insertion upstream from the 6 bp corresponding to the PAM site

of the Transmembrane domain (red box in the WT sequence). The fact that the same indel is present in 6 different colonies from 3 different plants, shows how specific the CRISPR-Cas9 editing is [35]. It should also be noted that for two of the plants, labeled as TRA6 and TRA7 in the picture shown below, additional indels were detected. In TRA6, one of the sequenced colonies presented an additional Adenine (A) insertion next to the previously mentioned C insertion. The chromatogram of the 2 sequences of the TRA6 plant were analyzed to elucidate if this additional indel corresponded to a sequencing error, but since no background noise was observed in that region, it was decided that this additional A insertion was caused by the Non-homologous end joining (NHEJ) repair mechanism that follows a DSB event caused by the Cas9 nuclease activity [78].

Figure 4 Multiple alignment of DNA from 3 different Transmembrane mutants and the genomic sequence of SRR1 (At1g74360). *The red box indicates the PAM sequence present in the WT gene, next to where the indels were detected.*

Moreover, 2 sequences from the TRA7 plant presented different indels that the one common between the 3 studied plants. One of the sequences, TRA7-1-RVC (RVC, reverse complement), had a second C insertion that corresponded to a transition event since the WT genome presents a Thymine in this same position (pyrimidine to pyrimidine change). This point mutations are more common than transversions, where a pyrimidine base is substituted by a purine and vice versa, but still not as common as the Cytosine to T transition [79]. This fact makes the T to C change not prone to be a Single nucleotide polymorphism (SNP) sign, reason why the chromatogram of the sequence was checked to see if the additional indel was the product of a sequencing error. Since no background noise was observed in this region, the idea that the T to C mutation derived from an error during the sequencing process was discarded. The chromatogram also did not present a second peak in the transition position, a sign that indicates that the bases of a determined region are different in the two alleles (heterozygous), therefore the mutation did not correspond to a SNP of the wild type genome (SNPs are variations of one nucleotide between different individuals of the species that can be caused by random or point mutation events [80][81]). This transition mutation was therefore considered as a result

of the repair mechanism of the DSB generated by the Cas9 protein. In addition to this mutation, the TRA7-1-RVC sequence presented a deletion of 5 bases, that were also considered as an effect of the Cas9 induced base pair edition, since the chromatogram showed no background noise that could reflect a sequencing error of this region. Another colony of the TRA7 plant, TRA7-4-RVC, presented a different indel than the rest of analyzed sequences. A Cytosine deletion instead of the C insertion common to the rest of sequences was observed, and after a chromatogram check it was classified as a product of the Cas9 induced DSB due to the absence of background noise or overlaying peaks. To sum up, 2 of the 4 sequences of the TRA7 plant had either additional or different indels. This can be an indication of mosaicism in the SRR1 protein of the TRA7 plant. The presence of cells with different variants of the gene inside the same plant could be caused by a still active Cas9 nuclease, that can keep inducing DSBs that will introduce new indels every time that they are repaired by NHEJ [82]. The fact that the analyzed DNA proceeded from T_0 plants reaffirms the possibility of having individuals with an active Cas9 protein, since the selection of Cas9-free seeds is done with T_1 seeds [35].

Seeds from the 3 mutant plants were harvested (T_1 seeds) and the seeds derived from the TRA6 plant were used for the phenotypic characterization experiments. This decision was made based in the fact that this plant seemed to present a more stable version of the *SRR1* gene since the 2 aligned sequences presented the same type of indel, a Cytosine insertion, next to the PAM site of the region encoding the transmembrane domain of the SRR1 protein. Therefore, the T_1 seeds and organisms of this plant were labeled as TRA-KO, since they could possibly be Transmembrane knock-out lines of the SRR1 protein.

In addition, samples from KINgRNA transformed plants were also sequenced and after aligning them with the genomic DNA of the *SRR1* gene it was confirmed, as previously stated, that these plants did not have a mutation in the Kinase region of the SRR1 protein.

Finally, it should be mentioned that the process performed to obtain the TRA-KO plants did not consist of a set of individual experiments. That's the reason why it was decided to use these seeds for further experiments, and not focus in obtaining KIN-KO plants before characterizing the TRA-KO mutant lines. However, parallel experiments to generate this Kinase edited plants were done but unfortunately none of them produced KO or edited plants.

1.2.2 Phenotypic analysis, growth measurement

Once mutant plants that could present a knocked-out version of the *SRR1* gene were identified, it was decided to study how they behaved in comparison to the T-DNA KO1 line after exposing them to different sugars (sucrose and palatinose) and hormones. Two experiments were done for this purpose, first the growth of different lines was evaluated by measuring and comparing their root length (cm). Second, the specific phenotype and the hypocotyl length (cm) of those same lines were studied after growing the seeds in dark with ACC present in the MS media to determine possible interactions between Ethylene and the *SRR1* gene or protein. For both experiments, One-way ANOVA and Bonferroni's tests were performed.

1.2.2.1 Extracellular sugar influence over root length

The extracellular sugar signaling pathways in plants are still yet to be fully understood and discovered [83,84]. However, previous studies have shown that sucrose may be involved in these events [7,85]. That, together with the fact that the T-DNA KO1 and OX6 lines had previously shown to be sensitive to treatments with sucrose (Javad Najafi's PhD thesis, data not published) were the reason why sucrose was used to characterize the CRISPR mutant line TRA-KO. In addition, another disaccharide was used in this experiment. Palatinose (Isomaltulose) is a non-metabolizable analog of sucrose, that can mimic the signaling effect of sucrose independent of its metabolism [15,86]. Thus, it was used to study the signaling role of SRR1 in response to external sugars.

As it can be seen in Figure 5, where the bars of the graphs represent the mean root length of each plant line, sucrose induced the largest root growth of all the 3 treatments. In addition, this treatment presented significantly lower lengths for the 2 knock-out lines, KO1 and TRA-KO, when comparing them with the WT plants (Figure 5A). This indicated that those 2 mutant lines behaved in a similar way, thus indicating that the TRA-KO line could be a knock-out mutant. However, it should be mentioned that these plants had significantly longer roots than the T-DNA KO1 line (P value = 0.0193 from Bonferroni's test). This could be an indication that the newly developed mutant was more sensitive to sucrose than the original one or that the *SRR1* gene had not completely lost its function and therefore was knocked-down. This opposes what was observed with both the HRM and Sanger sequencing analysis, but it could be possible that the amount of data obtained with those experiments was not enough to fully conclude that the TRA-KO line was indeed a knock-out mutant since the studied seedlings belonged to the first generation

of transformed plants. It's very common to find a majority of heterozygous *SRR1* variation in this generation, thus not being completely reliable for the detection of knock-outs.

On the other hand, the palatinose treatment (Figure 5B) obtained shorter length results than the sucrose one, but a bigger root growth than the observed in the absence of sugar. Therefore, it could be that, since palatinose is supposed to induce similar signaling events to sucrose but this are independent of the latter disaccharide metabolism, sucrose can induce root growth, via SRR1 interaction, without being metabolized. This could be a sign of the role of SRR1 in sensing extracellular sugars.

Moreover, the KO1 and TRA-KO1 lines had significantly smaller roots than the WT plants, therefore confirming the apparent extracellular signaling effect of palatinose.

The non-sugar treatment (Figure 5C) had the shortest results of the 3 treatments. However, no significant differences in root length were observed for the KO1 and TRA-KO1 lines in comparison to the WT plants. This could be an indication that the *SRR1* gene is only involved in root growth pathways when sucrose is present. Therefore, sucrose may have an activating effect of *SRR1*, either interacting with the protein or regulating its expression.

Additionally, only the OX6 plants were significantly shorter than the WT plants. This overexpression line showed the shortest results in all 3 treatments, opposing what was expected from previous characterization experiments of these plants. Thus, an additional experiment to test the possibility that the OX6 plants had lost their characteristic phenotype was performed.

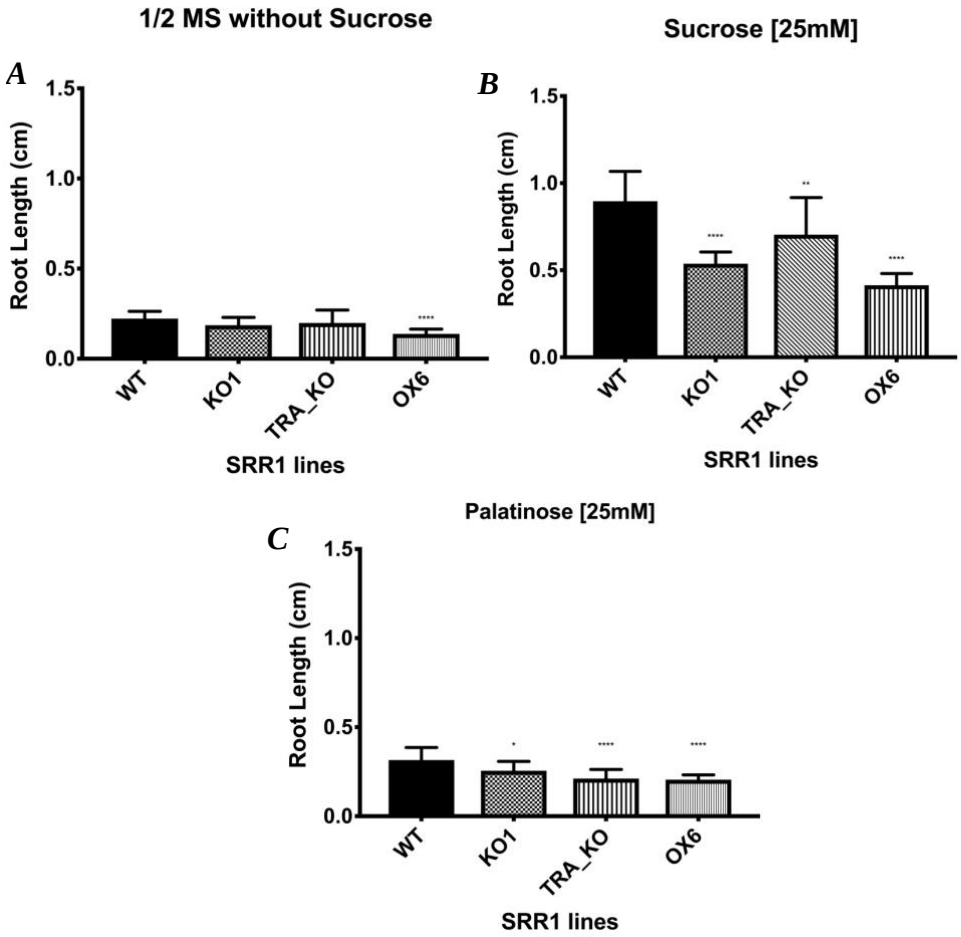

Figure 5 Root growth measurement (cm) of different SRR1 lines grown in presence or absence of sugar. *A) Plants grown in ½ MS media without sugar. B)Plants grown in the presence of 25 mM sucrose C) Plants grown in the presence of 25 mM palatinose respectively. In all cases the differences in root growth were calculated towards the WT plants and were labeled with a * when statistically significant. The scale of the Y axis of A and C were plotted based on the scale from B, since this graph presented the biggest root length values.*

In previous experiments, when the line was developed, the OX6 plants presented no difference in growth and development when grown in media with high sucrose concentration (100 mM) in comparison to growing them in ½ MS with normal in vitro sucrose concentration (25mM). Therefore, the OX6 line was classified as hyposensitive to high sucrose. A similar experiment was performed to check if indeed these OX6 plants retained their hyposensitive phenotype. Together with seeds from this line, WT, KO1 and TRA-KO seeds were grown in high sucrose conditions since in the previously mentioned assay the KO1 plants were characterized as hypersensitive to high sucrose concentrations. The additional experiment, as can be observed in Figure 6, showed that the OX6 plants had lost their previously observed hyposensitivity towards high sucrose concentrations, thus confirming the hypothesis that this line had lost its characteristic phenotype towards sucrose exposure. Moreover, the KO1 and TRA-KO plants behaved similarly since both

lines presented shorter root length when compared to WT plants. This result was considered as an additional suggestion that the TRA-KO line was a knock-out mutant of the transmembrane domain of the SRR1 protein.

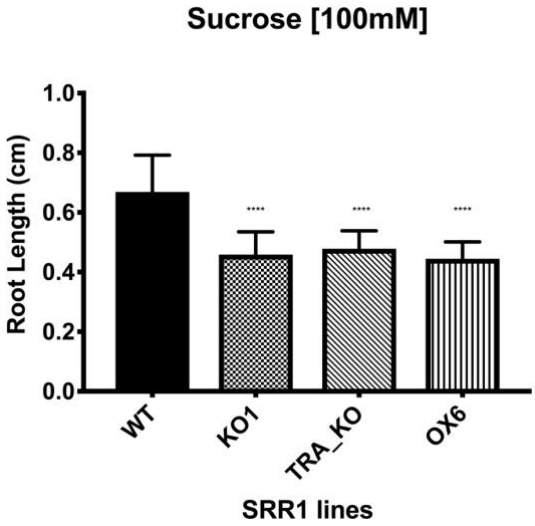

Figure 6 Root growth measurement (cm) of different SRR1 lines grown in high sucrose concentration (100mM). *The 3 mutant lines had significantly shorter roots when compared to the length of the WT plants even though the OX6 line (overexpression) was supposed to have larger roots than the other plants since it was shown to be hyposensitive to high sucrose in previous studies.*

1.2.2.2 Exogenous hormonal effect

The phytohormone ethylene is known to interact with sucrose in different metabolic pathways and levels [51,87]. Additionally, ethylene insensitive lines can also present altered sucrose response [88]. To observe the possible effect over the *SRR1* gene KO, OX and WT plants were grown in square petri dishes containing ½ MS media supplemented with the ethylene precursor ACC. The plates were placed in vertical position and covered with aluminum foil to avoid light exposition [52].

As it can be seen in Figure 7, where the bars of the graphs represent the mean hypocotyl length of each line, the ACC treatment (Figure 7B and D) induced a decreased growth of the hypocotyls of all plants, with exception of the *ein2* line. This line was used as a negative control, since it presents an insensitive phenotype towards ethylene (ACC) exposition [52]. The 2 knocked lines behaved similarly when ACC was present, thus indicating again that the TRA-KO line could be a knock-out mutant. Additionally, even though the TRA-KO line presented longer hypocotyls in absence (A) and presence of sucrose (B), this difference was not statistically significant (P value = 0.3156 and P value = 0.1727 respectively, from Bonferroni's test)

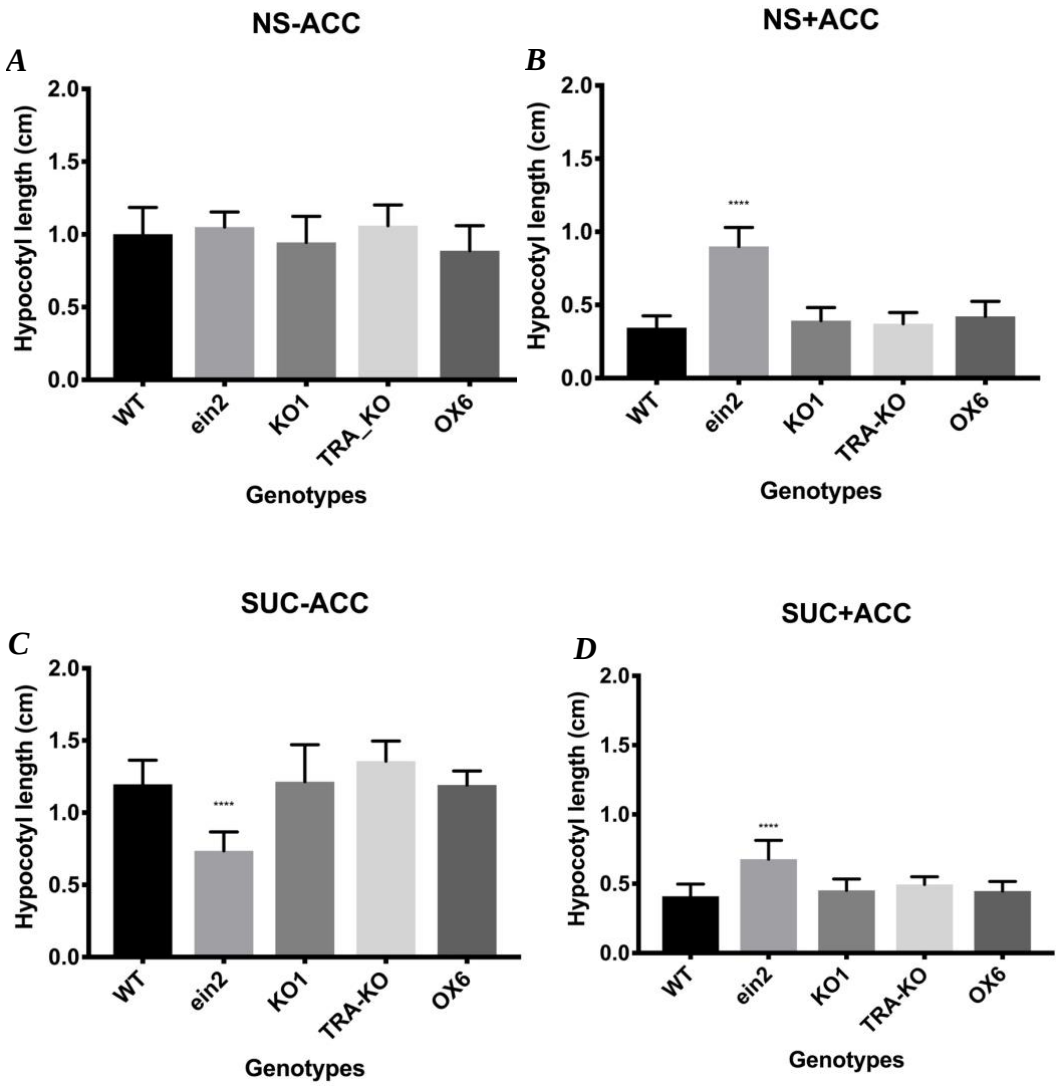

Figure 7 Hypocotyl growth measurement (cm) of different SRR1 lines grown in presence (C) and absence (A) of sugar or ACC (B and D). *A) The hypocotyl length calculated in absence sucrose. B) The phenotype observed when ACC was present in plates without sucrose. C) The root length obtained when sucrose (25mM) was added to the media. D) The phenotype observed when ACC was present in plates together with sucrose (25mM). The significant differences between mutant and WT plants were represented by a * symbol. Moreover, the Y axis scale of the graphs was based on the scale of C since this treatment had the biggest length values. The ein2 line is a mutant line insensitive to ACC and therefore used as a negative control of its effect.*

Moreover, the ethylene "triple response" was observed in the lines when treated with ACC as seen in Figure 8, and no noticeable differences between the reduced hypocotyl length and its thickening was observed between the presence or not of sucrose together with the hormone (Figure 8A and B respectively). But, the addition of sugar to the media seemed to decrease the effect of ethylene over the roots since the plants grown in presence of sugar (Figure 8A) had longer roots than the same lines grown without it (Figure 8B). Figure 9 shows the phenotype of WT and *ein2* plants when grown in media with ACC and with or without sucrose (Figure 9A and B respectively). Since the same effect of sucrose towards root growth was observed in WT plants (Figure 9A), this could be a

signal that SRR1 and ethylene interact, specifically it would reflect that perhaps SRR1 has an inhibitory or at least downregulating effect over ethylene.

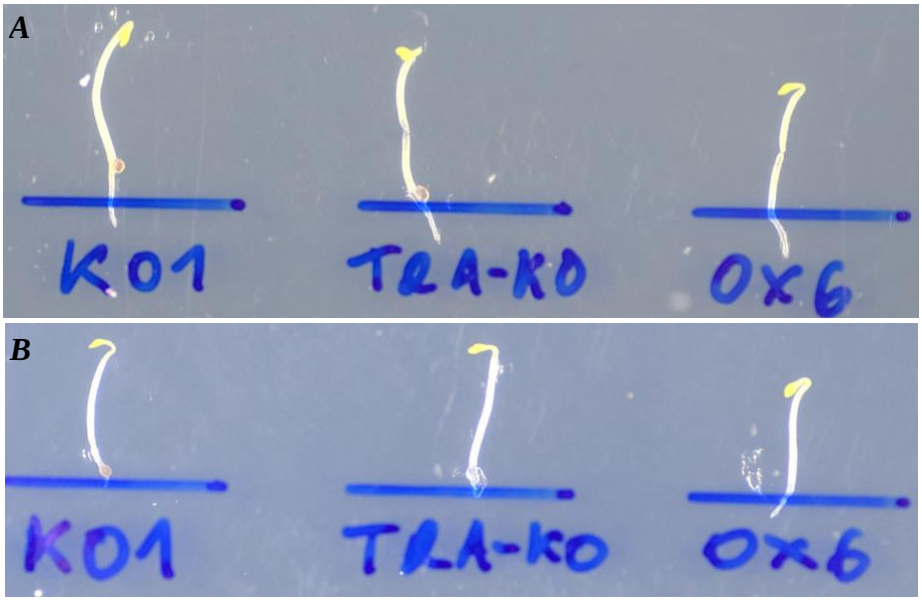

Figure 8 Effect of ACC over different SRR1 lines grown in presence (A) and absence (B) of sucrose. *All the SRR1 mutant lines presented the "triple response" to ACC treatment. They had inhibited growth of both roots and hypocotyl, thicker or swollen hypocotyls and a notable apical hook. But, the presence of sucrose (A) seemed to induce root growth or at least downregulate its inhibition by ACC since the seedlings from A presented longer roots than the plants grown without sucrose (B).*

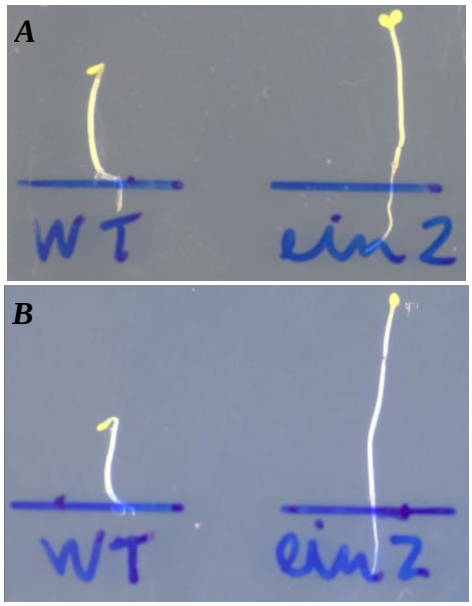

Figure 9 Effect of ACC over WT and ein2 lines grown in presence (A) and absence (B) of sucrose. *The "triple effect" of ethylene can be seen in WT plants both with and without sucrose. But, the presence of sucrose in the media (A) seemed to reduce the effect over roots when comparing it to the phenotype of plants grown without it (B) since longer roots were observed.*

1.2.3 Gene expression analysis

Once a characteristic phenotype for the TRA-KO and KO1 lines was determined after growing them in media with exogenous sugars (sucrose and palatinose), it was decided

to study what genetic changes these plants presented in comparison to WT plants. Thus, conducting a reverse genetics experiment, a powerful method to discover or study the function of genes [32,89], could help elucidate the role of *SRR1* in sugar signaling pathways related to sucrose metabolism and growth.

Since the OX6 line was not presenting the expected phenotype, as previously characterized by Javad Najafi in his PhD thesis (data not published), it was decided not to include that line in this experiment.

When performing a qPCR experiment aimed to obtain data that reflects gene expression levels, is necessary to use a reference gene whose expression is stable among different plant conditions [90,91]. For this experiment, as previously mentioned in the material and methods section, the TIP41-like gene was selected due to the high stability of its expression [92]. Additionally, the "starvation" conditions were used to determine if the possible observed interactions between *SRR1* and the studied genes was a result of extracellular sugar signaling since palatinose can mimic the responses of sucrose but can't be metabolized.

As it can be observed in Figure 10A, the genes involved in root growth mechanisms presented lower expression in comparison to the levels obtained for the sucrose response related genes shown in Figure 10B. These results were not surprising due the treatments and settings of the experiment. Since conditions similar to those of starvation or nutrient deficiency were induced, it was expected to observe low levels of expression of those genes related to developmental and growth pathways such as *PAP1* and *TPS9*.

On the other hand, since 3 sugar related treatments were used for the experiment, it was awaited that the genes connected to sucrose response, *DIN1/6* and *bZIP11* had higher expression levels.

In Figure 10A it can be observed that *PAP1* (black bars) was downregulated in the no sugar (NS) and palatinose (PAL) treatments of the two KO lines when compared to its expression in presence of sucrose (CTR). Moreover, this downregulation was not observed in the WT plants, therefore suggesting that *PAP1* may interact with *SRR1*. Furthermore, the low expression level generated by the palatinose treatment in the 3 lines suggests that sucrose probably interacts with *SRR1* and *PAP1* through its metabolic pathway and not by an extracellular sugar sensing response, since palatinose should mimic this sugar sensing mechanism. This correlates with studies where this anthocyanin-related gene was shown to be vital for the sucrose-induced synthesis of the pigment [15]. Additionally, the upregulation of *PAP1* in presence of sucrose observed in the 2 KO lines

in comparison to the expression of the WT plants could be a sign that *SRR1* has an inhibitory or downregulating effect over *PAP1*.

On the other hand, *TPS9* (Figure 10A, grey bars) had similar expression levels than the control group (WT-CTR) in the 2 studied mutant lines, for the no sugar and palatinose treatments. Additionally, the sucrose treatment of these lines (KO1-CTR and TRAKO-CTR) presented a downregulation of *TPS9* not observed in the control group. This gene is active when external sucrose is supplemented [93], therefore its downregulation in the 2 knocked-out lines suggests that an interaction between *SRR1* and *TPS9* could be possible. In addition, this interaction could mean that *SRR1* has an upregulating effect of *TPS9*. Moreover, it could corroborate the suggested role of *SRR1* in root growth induction since *TPS9* is active in this tissue [94]. This latter statement could confirm what Wu et al. (2017) had previously observed towards the role of *SRR1* in growth [95].

In relation to sugar response related genes (Figure 10B), *DIN6* was upregulated in the 3 different treatments of the 3 lines (WT, KO1 and TRAKO) (Figure 10B, grey bars). Nonetheless, the sucrose treatment generated lower expression levels of *DIN6*, which was expected since sucrose is supposed to repress this gene [96]. Therefore, the interaction of *DIN6* with *SRR1* would have been reflected in a downregulation of the *DIN6* expression in the KO lines of this treatment. The lack of down-regulation suggested the absence of a relationship between *SRR1* and *DIN6* or at least that *SRR1* does not participate in the regulation of *DIN6.*

The other *DIN* gene studied, *DIN1*(Figure 10B, black bars), was upregulated in the NS and PAL treatments of the 3 lines; but it was slightly downregulated in presence of sucrose (CTR) in the 2 knocked-out lines. It is known that there's a positive response of *DIN1* towards sucrose [96]. Therefore, its downregulation in the KO lines suggests that this response could be related to interactions with *SRR1*. Thus, maybe *SRR1* has a positive or upregulating effect over *DIN1*.

Finally, *bZIP11*, a gene that encodes a transcription factor upregulated by sucrose [97], was downregulated in the no sugar and palatinose treatments (Figure 10B, white bars). However, it was slightly downregulated in the sucrose (CTR) treatment of the 2 KO lines. This suggested that the expected sucrose upregulation could be positively mediated or regulated by *SRR1*. Additionally, the downregulation observed in the palatinose treatment of all lines could point out that if an interaction between *SRR1* and *bZIPP11* takes place in the presence of sucrose, this interaction is not related to the extracellular sensing of the disaccharide. These results seem to be contradictory to the observations of the "Root

growth measurement" experiment, since *SRR1* is supposed to induce root growth in presence of sucrose, as also demonstrated by Wu et al. (2017), and positive regulation of *bZIP11* would indicate the opposite since this gene is related to root growth inhibition [97].

Moreover, it should be noted that both KO1 and TRA-KO presented similar gene expression levels, thus providing more evidence to the theory that suggested that the TRA-KO line was a knock-out line.

To summarize, *SRR1* seems to downregulate *PAP1* and upregulate *TPS9*, *DIN1* and *bZIP11*.

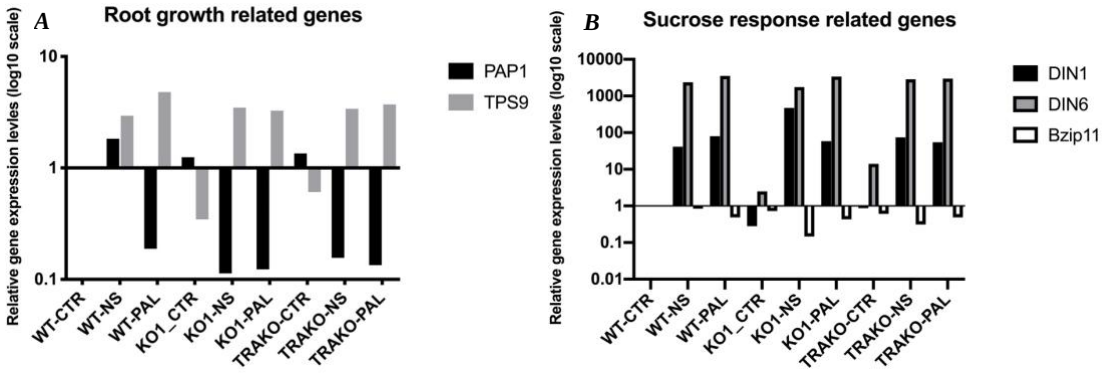

Figure 10 Expression of 5 different genes after 6 hours of simulating starvation conditions. *A) Gene expression of genes related to root growth. B) Gene expression of genes related to sucrose sensing. The scale of the Y axis was set to log10 to generate graphs that show up- (positive values) and down-regulation (negative or lower than 1 values) of the target genes, in comparison to the control group WT-CTR. NS: no sugar; PAL: palatinose.*

To furtherly study how the *SRR1* gene interacts with sucrose, two different sets of experiments were performed. Histochemical assays were done to investigate the possible relation of the *SRR1* gene with exogenous supplied sugars and phytohormones, and 2 different methods for protein-protein interaction observation were tested to help determine the function of the SRR1 protein in the sugar signaling pathway.

1.3 Histochemical assays

1.3.1 Sugar response of *SRR1*

The expression pattern of *SRR1:GUS* transgenic plants shows that sucrose (Figure 11B) has indeed an activating effect of *SRR1* and therefore confirms the results observed when growing KO lines with and without sucrose. This correlates with the fact that in absence of sucrose (Figure 11A) the gene expression is dramatically reduced and localized only in small patches of the vascular tissue of the leaves/cotyledons. Moreover, the sucrose-

induced expression was observed in leaves, root and the transition zone between the root and shoot. The expression in the transition zone and in the cotyledons could implicate that perhaps *SRR1* is also involved in sucrose transport from photosynthetic to non-photosynthetic tissues, since expression was also observed in the root and because sucrose is the form in which carbohydrates are transported [4].

The expression pattern of the palatinose treatment, where the vascular tissue all over the plant had signal, even though lower than the one produced by sucrose, supports the idea that *SRR1* is involved in extracellular sugar sensing events, thus confirming the suspicions arisen after observing the effect of palatinose over root growth (length). This theory was backed up by the fact that the sucrose treatment had an increased signal than palatinose in the same tissues. It's worth recalling that palatinose, by being a non-metabolizable analog of sucrose, can only mimic the expression of the latter in pathways that are independent of sucrose metabolism [15,86].

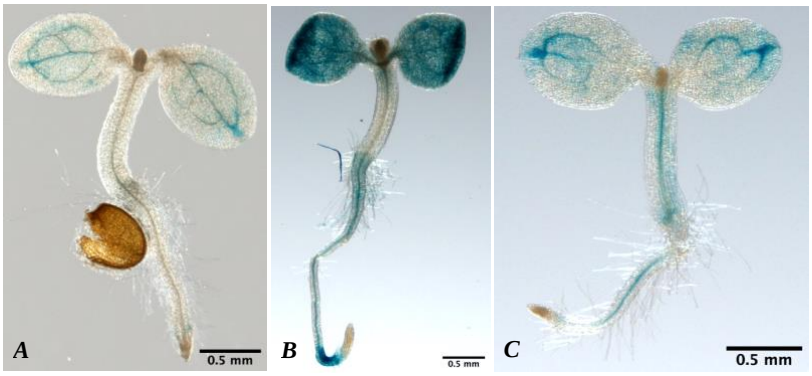

Figure 11 Localized expression of SRR1 after exogenous sugar exposure). *The SRR1:GUS transgenic plants showed different expression patterns when sugar at 25 mM concentration was added to the media (B sucrose and C palatinose). A) The absence of sugar induced weak signaling just in the vascular tissue of cotyledons. B) The sugar treatment presented gene expression localized in the cotyledons, root and root-shoot transition zone. C) Palatinose induced weaker signaling than sucrose (B) in cotyledons and root, but it additionally presented signaling in the shoot region.*

1.3.2 *SRR1* behavior towards phytohormone and sucrose interactions

Since a possible effect between the ethylene precursor ACC and *SRR1* was observed, after growing seeds in media supplemented with both sucrose and ACC, it was decided to use the *SRR1:GUS* transgenic plants for a second histochemical experiment. Besides using ACC and sucrose treatments that could help corroborate the previously observed interaction, other phytohormones were tested to study their possible relation with SRR1. All the tested hormones induced the expression of *SRR1:GUS* in absence of sugar (Figure 12, top), and with exception of Salicylic acid (SA), this expression was modified by the presence of sucrose together with the hormones (Figure 12, bottom). This could be an

indication of interactions between the other hormones (ABA, IAA, MeJA and Zeatin) and indication of interactions between the other hormones (ABA, IAA, MeJA and Zeatin) and *SRR1* in relation to sucrose related pathways. This relationship is plausible since most of those hormones are involved in either extracellular signaling or stress response pathways [1,98], and *SRR1* has been shown to be related to extracellular signaling towards nematode infection [95,99] thus connecting both pathways.

Regarding ethylene, the similar *SRR1:GUS* expression pattern between plants treated with ACC+sucrose and only sucrose (Control in Figure 12) correlates with the results observed when plants were grown under the same conditions ("Exogenous hormonal effect" section). In that experiment, the addition of sucrose to the medium seemed to reduce the inhibitory effect of ACC over the root growth of the seedlings. The expression pattern could confirm the hypothesis that there's an interaction between ethylene and *SRR1*. As previously indicated, perhaps *SRR1* has an inhibitory role over ACC or ethylene. In addition, when sucrose was not present ACC produced signal just in the root and not in the leaves/cotyledons. This could be a confirmation of the results obtained by Mendy et al. (2017) that indicated that *SRR1* is expressed towards nematode infection [99], since ethylene is also related to biotic stress [100]. Moreover, ethylene is known to inhibit root growth by stimulating auxin synthesis [101,102] and by reducing the sucrose-induced synthesis of anthocyanin [103]. This effect over the synthesis of anthocyanin could be the interacting point of *SRR1* and ethylene towards root growth regulation. Perhaps, the theoretical relationship of *PAP1* and *SRR1* observed in the "Gene expression" experiment is the connecting point between ethylene and *SRR1* since as previously mentioned, *PAP1* is induced by sucrose to participate in the synthesis of anthocyanin [15].

Similarly, ABA also induced an expression pattern in the presence of sucrose that was comparable to the signal shown by just treating the seedlings with sucrose. However, the ABA+sucrose signal was lower in the root zone than the obtained with sucrose or ACC+sucrose. Furthermore, *SRR1:GUS* was more expressed in the shoot region in the ABA+sucrose treatment than when only sucrose was present. These are signs that sucrose could inhibit or at least interfere with the effect of ABA over *SRR1*. The observed expression of the transgene in shoots could be related to the fact that ABA is synthesized in the leaves and transported through the xylem to the roots, where it stimulates growth [104]. This signal in the absence of sucrose could indicate that *SRR1* can be related to root growth not only when sucrose is present, as seen in the "Root growth measurement"

experiment. While, since when sucrose is also present in the medium the expression of the gene is observed in the shoot and vascular tissue of the cotyledons, ABA could the hypothetic role of *SRR1* in sugar transport from photosynthetic to non-photosynthetic tissues. Additionally, these results correlate to the positively regulated expression of *SRR1* by ABA observed by Wu et al. (2017) [95].

The MeJA and IAA treatments generated similar expression patterns when sucrose was added. In both cases, this addition produced a signal, weaker in the case of IAA, in the shoot and root of the seedlings together with a slight expression in the vascular tissue of leaves/cotyledons. This pattern differed from the one observed in seedlings treated just with sucrose, where the shoot region did not present GUS signal.

The auxin IAA is known to have an induction effect over root growth [105], therefore the presence of a signal in absence of sucrose could indicate an interaction between *SRR1* and IAA in terms of root growth stimulation. Thus, the effect of *SRR1* could be induced without sucrose and by IAA.

On the other hand, MeJA has an inhibitory effect on root growth [120]. Therefore, the presence of a signal in the absence of sucrose could also be an indication of an interaction between *SRR1* AND MeJA, and also the involvement of *SRR1* in root growth. However, in this case, the interaction with MeJA would probably inhibit its role.

Another evidence of the interaction of *SRR1* with IAA and MeJA could be their relation towards biotic stress since IAA and MeJA have been shown to interact with nematode infection responses[100,106–108] as well as SRR1 [99].

The Zeatin (Zt) treatment induced different expression patterns when sucrose was also present or not. This treatment generated a pattern different than the one observed in seedlings treated just with sucrose since in the Zt+sucrose treatment the shoot and not the root had *SRR1:GUS* expression. Zeatin is a Cytokinin (Ck) that can induce shoot growth [109], therefore this obtained pattern when sucrose was present could indicate that when *SRR1* is activated by sucrose, it also positively regulates Zeatin. This could relate to the findings of Barbier et al. (2015), that suggest that sucrose signaling pathway induces the shoot growth effect of Cks such as Zeatin [110]. Moreover, this expression pattern could also be a sign that confirms the hypothetic role of *SRR1* in sucrose transport from photosynthetic organs to non-photosynthetic ones.

Regarding the Salicylic acid (SA) transgene signal, this hormone seems to induce the expression of *SRR1* independent of sucrose in roots since no difference was observed between the presence or not of the sugar. The root expression could also be related to the

nematode response of *SRR1* observed by Mendy et al. (2017) since SA is also involved in biotic stress responses against pathogens [111,112].

In summary, *SRR1* seems to interact with all the hormones studied in different forms; mostly in relation to root growth or nematode infection response.

However, further studies using *SRR1* KO lines should be done in the future to confirm the suggested interactions between the different hormones used in this experiment and the SRR1 gene.

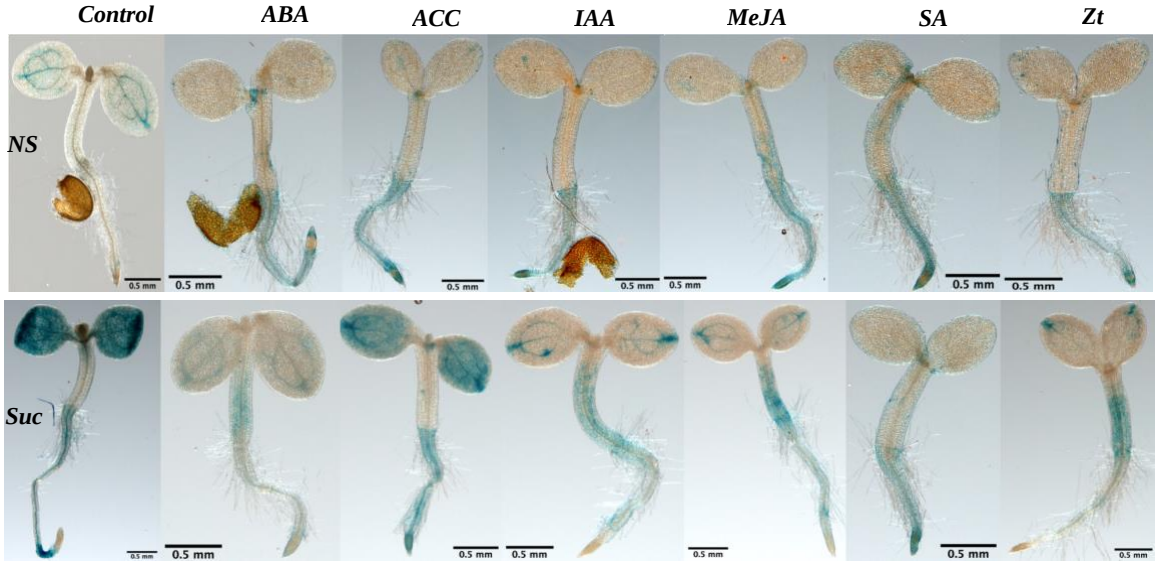

Figure 12 Localized expression of SRR1 after sugar or non-sugar was combined with hormonal treatment. *Top) The expression pattern of SRR1:GUS in absence of sugar (NS) and treated with different hormones Bottom) The expression of the transgene in presence of sucrose (Suc) and sucrose together with different hormones. ABA: Abscisic acid; ACC: 1-Aminocyclopropane-1-carboxylic acid; IAA: Indole-3-acetic acid; MeJA: Methyl Jasmonate; SA: Salicylic acid; Zt: Zeatin*

1.4 Protein-protein interaction (PPI) experiments

As described in the "Material and methods" section, 2 Bimolecular Fluorescence Complementation (BiFC) experiments were done. First, protoplasts from *A. thaliana* leaves were isolated and transfected, and then *N. benthamiana* leaves were infiltrated with three different A. tumefaciens GV3101 strains for transient expression analysis. In both cases the same set of vectors were used, pUC-SPYNE-*bZIP63* and pUC-SPYNE-*bZIP63*, to determine which method was more suitable to study PPIs of the SRR1 protein.

1.4.1 Protoplasts isolation and transfection

To isolate protoplasts, a protocol where different factors were modified for optimal results was used. One difference between the original protocol and the one described in the

"Material and methods" section was the centrifugation speed [60]. In this protocol, a higher speed, 120xg instead of 100, was used due to technical limitations of the centrifuges available in the laboratory. This higher speed could harm the protoplasts structure or induce them to aggregate, therefore it was decided to not use breaks to stop the centrifugation step. It's known that by doing this, better cellular pellets can be obtained when working with samples whose pellets are not completely solid, since this absence of sudden braking forces does not disturb the gradient formed between the supernatant and the pellet thus avoiding remixing [113]. Another modification of this protocol was done to the final dilution step. In the original protocol, the cell density is adjusted to $5x10^5$ cells/mL with MMg solution after counting the cells with the help of a hemocytometer. In the modified version, the protoplasts were dissolved in 5 mL of MMg solution and later the cell density was calculated using a Flow cytometer programmed to detect the autofluorescence of the protoplast's chlorophyll [114,115]. This modification was based in the obtention of higher protoplasts yields than the original protocol since cell densities of $1.5x10^6$ cells/mL were calculated. This increased yield was due to the use of 25 mL of enzyme solution instead of the 10 mL from the original protocol. The use of the Flow cytometer for calculating the cell density is not only a faster method than using a hemocytometer, but it also provides information about the state of the cells. The Flow cytometer calculates the cell density by measuring the fluorescence emitted only by alive cells since it's programmed to discriminate those cells whose plastids are not intact and thus have different fluorescence profiles [115]. The higher cell densities achieved with the modified protocol showed that it could be used in further experiments for protoplasts isolation.

Regarding the transfection of the isolated protoplasts, the methods described by Wu et al. (2009) and Mitula et al. (2015) [60,61] were used to create a new protocol (see "Material and methods: Protoplasts isolation"). This protocol was designed to determine which was the optimal cell density that should be used and which buffer, W1 or W5, provided better expression results. With this aim, the transfection was done used different cell densities, from $5x10^4$ to $2x10^5$ cells/mL, and using both buffers for each cell density. Also, the final incubation step was done in 6-well plates instead of using 2 mL tubes, thus preventing disturbance of the protoplasts in comparison to when incubated in tubes placed in a horizontal position. The tubes can be easily accidentally moved (roll), which can harm the stability of the protoplasts. Moreover, the incubation was done both in dark and light conditions, to check which situation provided better transfection results.

After visualizing samples of non-treated and treated protoplasts under light or dark conditions with either W1 or W5 buffer, no fluorescence results were obtained. This indicated that the transfection was not successful since the used vectors contained a fluorescent tag-protein fusion, YFP-bZIP63, that was supposed to emit fluorescence in the nucleus of the cells [70]. In addition, as it can be seen in Figure 11B, the protoplasts seem to aggregate and lose their structure after the transfection procedure was done. But this was not observed in non-transfected protoplasts (Figure 11A). This could be caused by the centrifugation step prior to the resuspension in W1 or W5 buffer because neither a swinging bucket nor low acceleration values were used; thus, not following the recommendations of the original protocols [73,74]. This was due to technical limitations of the centrifuges used for the pellet obtention.

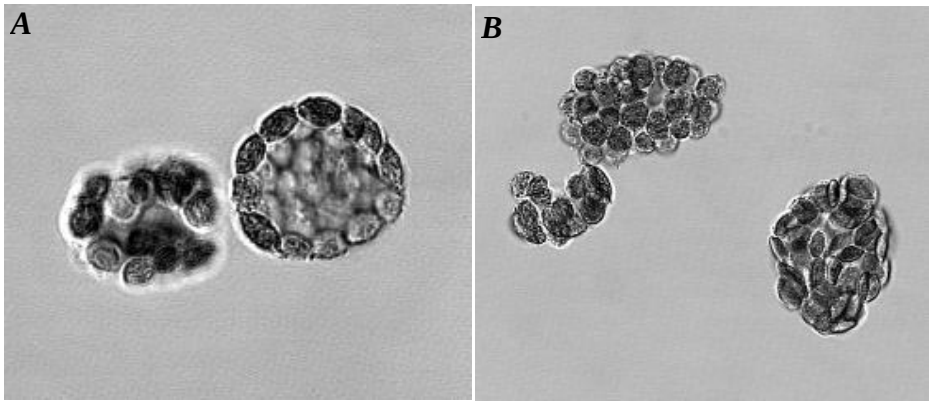

Figure 13 Protoplasts isolated from A. thaliana leaves*. A) Protoplast after isolation following the modified protocol presenting their characteristic round shape. B) Protoplasts after transfection aggregated and lost their round shape, forming a mass of cells that difficulted the observation of possible transient expression induced fluorescence.*

The absence of positive transient expression results in protoplasts led to the decision of stopping these experiments and instead of attempting a different approach for the PPI investigation.

1.4.2 *Nicotiana benthamiana* infiltration

Since the protoplast transfection experiment was not successful, it was resolved to perform a BiFC assay using Agrobacterium to infiltrate *N. benthamiana* leaves. Three agro lines were used. Two lines contained either the pUC-SPYNE-*bZIP63* or pUC-SPYCE-*bZIP63* vector and a third one presented a plasmid encoding the p19 protein, that has been shown to help in the transient expression of genes [76]. The observation of infiltrated leaves after 4 and 5 days of being treated did not show any cells with nuclear-localized fluorescence. A possible explanation for this absence of transient expression

could be the fact that the efficiency of the transformation is inversely proportional to the number of used vectors [70,76]. It was also suspected that the agro cells used were not GV3101 but C58 ones; or that maybe they had lost their helping vector. The first hypothesis was corroborated by growing non-transformed cells in LA plates containing just Gentamicin in order to select colonies with the helping vector, or Ampicillin to prove if they were C58 cells instead of GV3101. The cells did grow in the latter treatment and not in the presence of Gen, thus confirming that they belonged to the C58 strain. This fact made the colonies unsuitable for the experiments since there's no possible way of obtaining transformants that can be selectively grown to express the helping plasmid and the YFP containing vector. This problem could have been earlier detected by checking the presence of the pUC-SPYNE/SPYCE vectors running a gel electrophoresis with PCR products from a Colony PCR of cells electroporated to express these plasmids. But this was not possible since no primers were available to obtain the above-mentioned PCR products.

The experiments were stopped due to lack of suitable vectors that could be expressed in C58 cells and because no other agro strain was available to effectively express the pUC-SPYNE and SPYCE plasmids.

Concluding remarks

The approach used to generate mutant plants can be considered partially effective since 2 of the 3 desired vectors were successfully generated following a Digestion+Ligation based method. However, only one of those vectors, pKI1.1R-TRAgRNA, was able to produce mutant plants after *Agrobacterium* floral dip transformation.

The *SRR1* gene is positively regulated by sucrose, and this disaccharide could induce this effect without being metabolized. Both facts seemed to indicate the role of SRR1in extracellular sugar sensing. This was confirmed by the effects of palatinose in promoting both root growth and gene expression of the *SRR1:GUS* in the vascular tissue of transgenic plants.

There is an interaction between ethylene and the *SRR1* gene. It seems that this gene may have a negative regulation of ethylene since the root growth under hormone treatment was still active when sucrose was supplemented. Additionally, ACC does not inhibit the expression of SRR1 in presence of sucrose as it was shown by the signaling pattern of *SRR1:GUS* transgenic plants.

The *SRR1* gene seems to be involved in the regulation of genes related to both growth and sugar response such as *PAP1*, *TPS9*, *DIN1* and *bZIP11*.

An efficient protocol for protoplasts isolation that generates higher yields than the obtained by previous methods was developed. Unfortunately, this was not the case of the protoplast's transfection assay since this did not generate the expected nuclear-localized fluorescence and in addition, the procedure seemed to promote the aggregation and loss of structure of the protoplasts.

To summarize, the study showed signs that the SRR1 gene interacts with both sucrose and ethylene. It is activated or upregulated by the first one and inhibits or downregulates the latter. Moreover, this gene can recognize external sucrose signaling and could form part of extracellular sugar sensing pathways.

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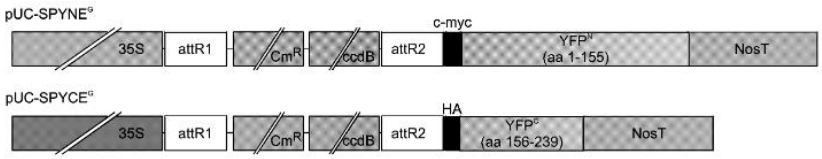
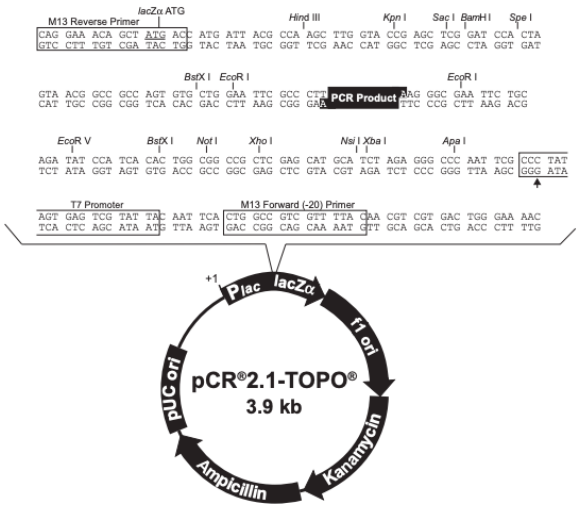
Appendixes

Appendix 1: Plasmid/vector maps

Map of the pKI1.1R plasmid (Addgene) The figure was generated with the SnapGene software (GSL Biotech)



Representation of the pUC-SPYNE and pUC-SPYCE vectors. The figure was modified from Walters et al., 2004



Map of the pCR®2.1-TOPO® vector from Invitrogen.

Appendix 2: List of oligos and primers

Guide RNAs

Primer Name	Sequence
Island gRNA antisense	AAACCCGTTCAACTTTGTATACG
Island gRNA sense	ATTGCGTATACAAAGTTGAACGG
Kinase gRNA antisense	AAACCACGAGGTTCGGATGTGCC
Kinase gRNA sense	ATTGGGCACATCCGAACCTCGTG
Transm gRNA antisense	AAACTAAGTACAATACCTGATAC
Transm gRNA sense	ATTGGTATCAGGTATTGTACTTA

PCR primers

Primer Name	Sequence
Island PCR FW	GGGGAGTAATCCTTCTCCAACA
Island PCR RV	ATGCTTGCAGGAATCTCACCT
Kinase PCR FW	AGTTCAGGTGGATCATCGCC
Kinase PCR RV	TGCCCATTCGACCAAACAC
pKIseq	GAAGTCCAGAACCGAGGAATG
Transm PCR FW	ATCAGGGAACAACACGAGGA
Transm PCR RV	CCCCTACCTACCACTCTCTCC

HRM primers

Primer Name	Sequence
Island HRM FW	GGTTCTGGTGAATGCTTGGCG

Island HRM RV	ACCGTGGAACCAGCAGAACA
Kinase HRM FW	AGGGAAGGCACAGAAGCAGA
Kinase HRM RV	CCCGCCTCCCATGTATTCGT
Transm HRM FW	TGGATTTCCTTGGCTCTTGCA
Transm HRM RV	GCCATGGCGATGATCCACCT

qPCR primers

Primer Name	**Sequence**
qbZIP11 FW	CGATTCAAACGTCGTCAGG
qbZIP11 RV	TCCGTTTACGTTTCCTCTGC
qDIN1 FW	GAAACTCAAACTTCCGATGGAG
qDIN1 RV	GGAATTTTAACTGCCTCTGCTG
qDIN6 FW	TGAACAAATTTTCTATATGGGTTTTTC
qDIN6 RV	CCCATTTCTCGATCCTTCCT
qPAP1 FW	AAATGGCACCAAGTTCCTGT
qPAP1 RV	TCAGAGCTAAGTTTTCCTCTCTTGAT
qTip41-like FW	GTGAAAACTGTTGGAGAGAAGCAA
qTip41-like RV	TCAACTGGATACCCTTTCGCA
qTPS9 FW	TGAACACGGATACTTCATAAGGTG
qTPS9 RV	GGTTCTACCATCGTCTTCCATT

Appendix 3: Thermal and Light cycler programs and Master mix composition

Digestion and Ligation Set-Up

Master mix composition

Component	**Volume (µL)**
Destination vector (pKI1.1R plasmid) (125 ng/µL)	1
Insert (gRNA mix) (0.1 µM)	2
10x AarI buffer	2
50x AarI oligo	0.4
AarI (restriction enzyme)	0.5
T4 ligase	1
ATP (5mM)	4
H2O	9.1
Total volume per reaction	20

Incubation program

Step	Description	Cycles
Linearization of the plasmid	37ºC for 10 minutes	25
Ligation	16ºC for 10 minutes	
Digestion to ensure that only ligated plasmid remains	37ºC for 10 minutes	1
Inactivation of the restriction enzyme	80ºC for 5 minutes	1
Cool down	4ºC	1

Colony PCR

Master mix composition

Component	Volume (µL)
10x DreamTaq™ Green buffer (Thermo Fisher)	2
Sense gRNA (FW primer) (5 µM)	1
pKIseq (RV primer) (10 µM)	1
dNTP mix (10µM) (Thermo Fisher)	2
DreamTaq polymerase (Thermo Fisher)	0.5
DNA template	5
H2O	8.5
Total volume per reaction	20

PCR profile/program

Step	Description	Cycles
Initial denaturation	95ºC for 5 minutes	34
Denaturation	95ºC for 30 seconds	
Annealing	55ºC for 30 seconds	
Extension	72ºC for 25 seconds	
Final extension	72ºC for 3 minutes	1
Final hold	4ºC ∞	1

PCR for plant DNA amplification

Master mix composition

Component	**Volume (µL)**
Ex-Taq buffer (Takara)	2
PCR FW primer (µM)	1
PCR RV primer (µM)	1
dNTPs (10µM)	2
DNA	1
Ex-Taq polymerase (Takara)	0.5
H2O	12.5
Total volume per reaction	20

PCR profile/program

Step	**Description**	**Cycles**
Initial denaturation	95ºC for 2 minutes	30
Denaturation	95ºC for 30 seconds	
Annealing	55ºC for 30 seconds	
Extension	68ºC for 1 minute per kb	
Final extension	68ºC for 5 minutes	1
Final hold	4ºC ∞	1

HRM analysis

Master mix composition

Component	**Volume (µl)**
H2O	0.6
HRM master mix	10
MgCl 25 mM	2.4
Primer (FW+RV)	2
Total volume	15

Light cycler profile/program

Step	**Description**	**Cycles**
Preincubation	95ºC for 600 seconds	1
3 Step amplification	95ºC for 10 seconds	45
	Touch-down 63ºC, 1 Cycle to 55ºC (-1ºC)	
	72ºC for 20 seconds	

High Resolution Melting	95ºC for 60 seconds	1
	40ºC for 60 seconds	1
	65ºC for 1 second	1
	95ºC for 1 second	1
Cooling	37ºC for 30 seconds	1

Synthesis of cDNA

Master mix composition

Component	**Volume (µl)**
Quantiscript RT	1
RT buffer	4
RT-primer mix	1
Total volume	6

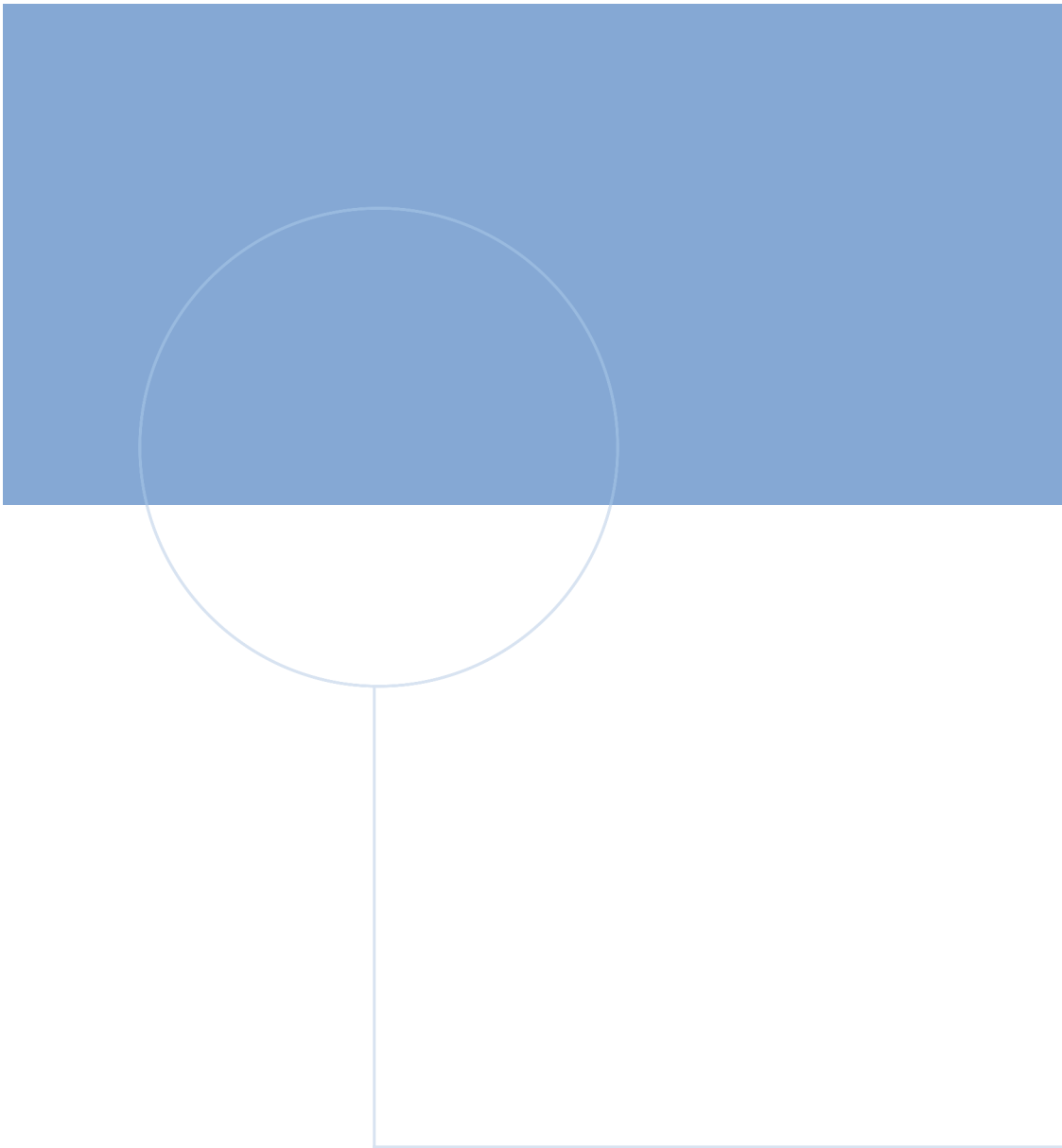
Quantitative PCR (qPCR)

Master mix composition

Component	**Volume (µL)**
H20	3
Primers (FW+RV) (5µM)	2
SYBR GREEN MIX 2x	10
cDNA	5
Total volume	15

Light cycler profile/program

Step	**Description**	**Cycles**
Preincubation	95ºC for 10 minutes	1
3 Step amplification	95ºC for 10 seconds	45
	55ºC for 10 seconds	
	72ºC for 10 seconds	
Cooling	37ºC for 30 seconds	1



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