

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**MIR396 KNOCKOUT VIA CRISPR TO ENHANCE EXPRESSION LEVELS
OF GROWTH REGULATING FACTORS (GRFS) IN TOMATO**



ALİ BURAK YILDIZ

A THESIS SUBMITTED FOR THE DEGREE OF MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

GEBZE
2022

T.C
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

CRISPR İLE MIR396 SUSTURULARAK DOMATES
BİTKİSİNDE BÜYÜMEYİ DÜZENLEYİCİ FAKTÖRLERİN
(GRFS) İFADELERİNİN ARTIRILMASI

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YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. YELDA ÖZDEN ÇİFTÇİ

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2022

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ÖZET

MikroRNA'lar genellikle 21-24 nükleotid uzunluğundan oluşan kodlama yapmayan küçük RNA'lardır. Oldukça hedefe özgü olmakla birlikte bitkilerde gen regülasyonu üzerinde kritik rollere sahiptirler. MiR396, bitkiler arasında oldukça korunmuş olan miRNA'lardan biridir ve çeşitli bitki türlerinde 60 üyeye sahiptir. Şu an için domatesti miR396'nın sadece 2 üyesi tanımlanmıştır, miR396a/b ve bitkiler üzerinde benzer isimlere ve benzer etkilere sahip olsalarda her biri farklı kromozomlarda ifade edilmektedir. Bu tezde, miR396 biogenezi durdurarak GRF'lerin ifadelerini artırmak hedeflenmiş ve miR396b biogenezi inhibisyonu için çift guide RNA'lı CRISPR/Cas9 protokolü tasarlanıp bu protokolün domates için optimize edilmesi planlanmıştır. *Arabidopsis*'te ve domatesde miR396b GRF'leri hedeflediğinden, bu miRNA bitki büyümesi ve gelişmesi, hücre çoğalmasının düzenlenmesi, meristem ve yaprakların boyutu ve stoma sayısı üzerinde doğrudan etkiye sahiptir. Bundan dolayı, CRISPR aracılığı ile miR396b'nin tam olarak susturulması domates bitkisinde olumlu sonuçlar doğurup geliştirilmiş bitki ıslahı sağlaması ile birlikte, fizyolojik gelişmelere yol açtığından sonuçların analizi ve sonuçlara ilişkin kararların alınmasını daha kolay hale getirmiştir. CRISPR ile geliştirilen mahsuller GDO'dan (Genetiği Değiştirilmiş Organizmalar) ziyade GEO (Genetiği Düzenlenmiş Organizmalar) olarak kabul edildiğinden, bitki ıslahı için CRISPR tabanlı protokollerin geliştirilmesi literatürde zaten büyük önem taşımaktadır. Bu tezde, dizileme ve fenotipleme yoluyla 11 transgenik bitkiden 2 mutant bitki tespit ettik. Mutant bitkiler sürgün büyümesinde önemli gelişme göstermiştir. Mutant bitkiler ortalama 207 cm sürgün uzunluğuna sahipken, yabani tip bitkiler ortalama 167.5 cm sürgün uzunluğuna sahiptir. Her ne kadar bitki ıslahı öncelikli amaç olsa da bu CRISPR-miRNA imha protokolü, gelecekte sadece bitki iyileştirme çalışmalarında kullanılmakla kalmayacak, aynı zamanda domates üzerinde fonksiyonel araştırmalar için de faydalı bir araç olacaktır.

Anahtar Kelimeler: miR396b, CRISPR/Cas9, *Solanum lycopersicum*, GDO, moleküler bitki ıslahı.

SUMMARY

MicroRNAs are small non-coding RNAs and generally 21-24 nucleotides in length. Besides being highly target-specific, miRNAs also have critical roles in gene regulation in plants. MiR396 is one of the highly conserved miRNAs among plants and has more than 60 members in various plant species. Currently, 2 members of miR396 family are classified in tomato plant, miR396a/b. They have similar sequences and targets in tomato plants. In this thesis, it was aimed to increase the expression of GRFs by inhibiting the miR396 biogenesis and for that CRISPR/Cas9 protocol with double guide RNAs was designed for tomato plants. Because miR396b form miRNA/target module with GRFs, miR396b has regularity role on various plant developmental mechanisms, such as plant growth and cell proliferation. Therefore, silencing miR396b via CRISPR lead to positive physiological results in tomato plants, and those physiological improvements allowed us to analyse the results easier. The development of CRISPR-based protocols for plant breeding is already of great importance in the literature, as crops developed with CRISPR are considered GEOs (Genetically Edited Organisms) rather than GMOs. In this thesis, we detected 2 mutant plants from 11 transgenic plants through sequencing and phenotyping. Mutant plants (MP) shown significant improvement on shoot growth. MPs averagely had 207 cm shoot length, on the other hand WT had 167.5 cm. Although plant breeding is the primary goal, this CRISPR-miRNA protocol will not only be used in plant improvement studies in future but will also be a useful tool for functional research on tomatoes.

Key Words: miR396b, CRISPR/Cas9, *Solanum lycopersicum*, GEO, molecular plant breeding.

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SYMBOLS and ABBREVIATIONS

<u>Index of Symbols And Abbreviations</u>	<u>Definitions</u>
µg	: Microgram
µl	: Microliter
µM	: Micromolar
mg	: Milligram
g	: Gram
ml	: Millilitre
mM	: Micromolar
L	: Liter
Rpm	: Return per minute
C	: Degree Celcius
bp	: Base pair
CRISPR	: Clustered Regularly Interspaced Short Palindromic Repeats
DSB	: Double streak break
HDR	: Homology directed repair
Mb	: Mega base
miRNA	: Micro RNA
NHEJ	: Non homologous end joining
PAM	: Protospacer adjacent motif
PCR	: Polymerase Chain Reaction
sgRNA	: Single guide RNA
TALEN	: Transcription activator-like effector nuclease
ZFN	: Zing Finger Nuclease

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1. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the most important tradable agricultural product due to its production-consumption value. Besides direct consumption as a nutrient, it can also be utilized as ketchup, tomato paste and canned after undergoing various processes. Since Turkey is sharing approximately %7 of the total tomato production in the World, the tomato has a critical role in the Turkish economy and agricultural industry [TEPGE, January 2022]. Due to this role and importance, tomato has drawn the focus of molecular breeders' attention.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated protein (CRISPR/Cas) system is the most popular genome editing technique among the others including Zinc finger nucleases (ZFNs) and Transcription activator-like effector nucleases (TALENs). Various advantages of CRISPR over other genome editing techniques have been pointed out and listed in the literature but the simplicity of design has been singled out as the main reason making CRISPR the most widely utilized genome editing tool [Shamshirgaran et al., 2022]. In addition to that, agricultural products that developed as a result of CRISPR/Cas are as considered Genetically Edited Organisms (GEOs), rather than Genetically Modified Organisms (GMOs) [El-mounadi et al., 2020]. Due to the mentioned reasons, CRISPR has been used by molecular breeders to manipulate metabolic pathways, enhance, or gain stress resistance and increase nutrient content and yield in tomatoes [Liu et al., 2020; Li et al., 2018; Martínez et al., 2020; Yu et al., 2017].

Micro RNAs(miRNAs) are the type of small RNAs that have a role in various plant biological processes. Due to their regulatory effect on various genes, they have recently become the focal point of researchers. Many miRNA studies have been performed with either classification [Tao et al., 2022; Zhang et al., 2021; Navarro et al., 2021] or plant development [Liebsch et al., 2020; da Silva et al., 2021; Lin et al., 2022] purposes. In combination with CRISPR, miRNA studies became simpler, faster, and more efficient

[Hong et al., 2021; Zhou et al., 2017; Zhao et al., 2016; Gupta et al., 2021; Deng et al., 2022].

Agrobacterium is a pathogenic bacterium that is capable of introducing genetic material into the plant cell and causing tumour formation in plants. With the removal of tumour causing gene, *Agrobacterium*-mediated transformation became harmless [Mehrotra Goyal., 2016]. Together with CRISPR, stable and efficient transformation and genome editing protocols have been specifically developed for tomatoes [Van Eck et al., 2018; Reem and Van Eck., 2019]. In addition to this, with CRISPR, targeting and editing of multiple genes simultaneously became possible [Ma et al., 2016; Lee et al., 2018].

1.1. Aim, Contribution and Content

In the literature, overexpression studies have been performed for functional classification or identification of genes and many promising results were demonstrated through these studies. However, due to the methodology of overexpression studies, developed products are transgenic and not suitable for field application. Therefore, this thesis was firstly aimed to build up a CRISPR/Cas protocol that could be utilized to enhance the expression of desired genes and avoid transgenicity. Since plant miRNAs are the highly target-specific and negative regulators of various genes, miR396b was targeted by two guide RNAs and complete inhibition of miR396b biogenesis was planned.

In tomatoes, miR396b targets Growth Regulating Factors (GRFs) together with miR396a. Although, miR396a has greater effect on plant development in tomato and its expression rate is higher during the plant development, it produces two miRNAs from single miRNA duplex which means inhibiting biogenesis of miR396a will silence two miRNAs. Therefore as a target we chose miR396b to silence only one functional miRNA. We thus secondly aimed to obtain tomato plants with enhanced growth and fruit size via double guide RNA knock-out of miR396b. Double guide RNAs were used to cause the

deletion of multiple bases and avoid the formation of unknown miRNA from the same gene.

Since the aim of this thesis was to build up a protocol that overcomes problems caused by overexpression of genes, generated protocol will not only be utilized in further plant development studies but also may become the main tool for tomato functional studies in the future.

2. LITERATURE REVIEW

2.1. Tomato (*Solanum Lycopersicum* L.)

Tomato is a member of the nightshade family, and it is cultivated for its vitamin C and lycopene rich fruit. The fruit is often consumed raw in salads or as cooked vegetables, used as an ingredient in various ready-to-eat meals, and pickled. It has the highest production-consumption value compared to other vegetables.

The origin of the tomato dates to the Aztecs and the word "tomato" is derived from the Aztec word "tomatl". The location of the origin is believed to be South America, generally around Peru and Ecuador. The first meeting of Europeans with tomato goes back to the early 16th century and was transported to Europe by Spanish travellers. In the beginning, tomato plants were cultivated only for ornamental purposes. In addition to that, since its roots and leaves contain the neurotoxin solanine, the tomato was labelled as poisonous and avoided to consume until the early 18th century. Firstly, French people cultivated tomato plants for consumption purposes. Later, around the 1770s, its cultivation started in Turkey. Firstly, located in Mediterranean Region, probably Adana, then move to Istanbul and Marmara, and then dispersed to other regions [Oraman, 1968].

2.1.1. Global and Local Production

Due to the rapid increase in the human population, sustainability in agriculture has become difficult. To prevent global hunger and feed humanity, along with the products, the arable lands were also tried to be increased and developed (Figure 2.1, Our World in DATA, 2017). Yet, land per capita continues to decline dramatically (Figure 2.2, Our World in DATA, 2017). Therefore, an increase in the production of valuable and nutrient-rich vegetables like tomato has become crucial and a necessity.

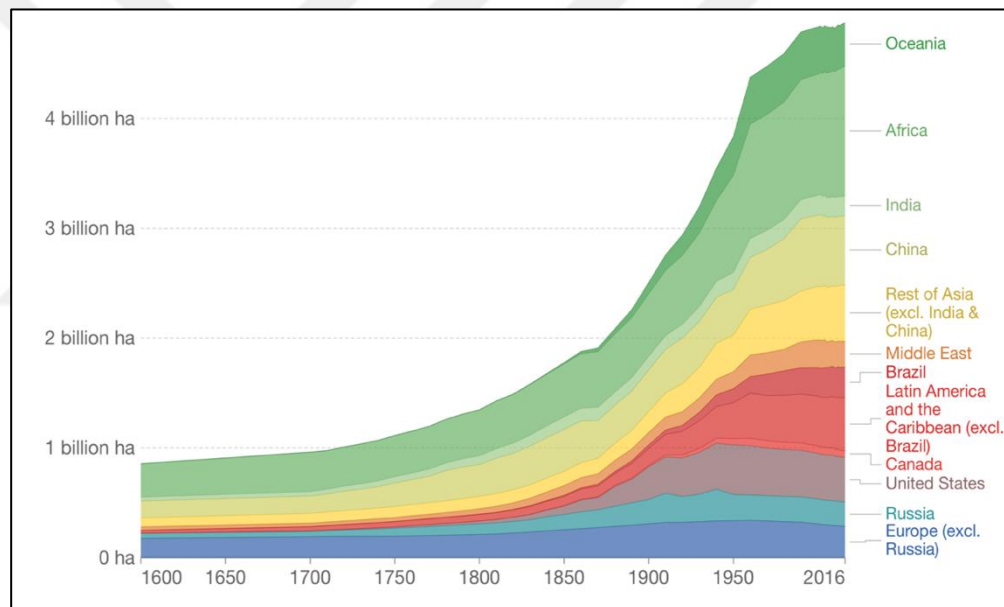


Figure 2.1: Arable lands over years, 1600-2016.

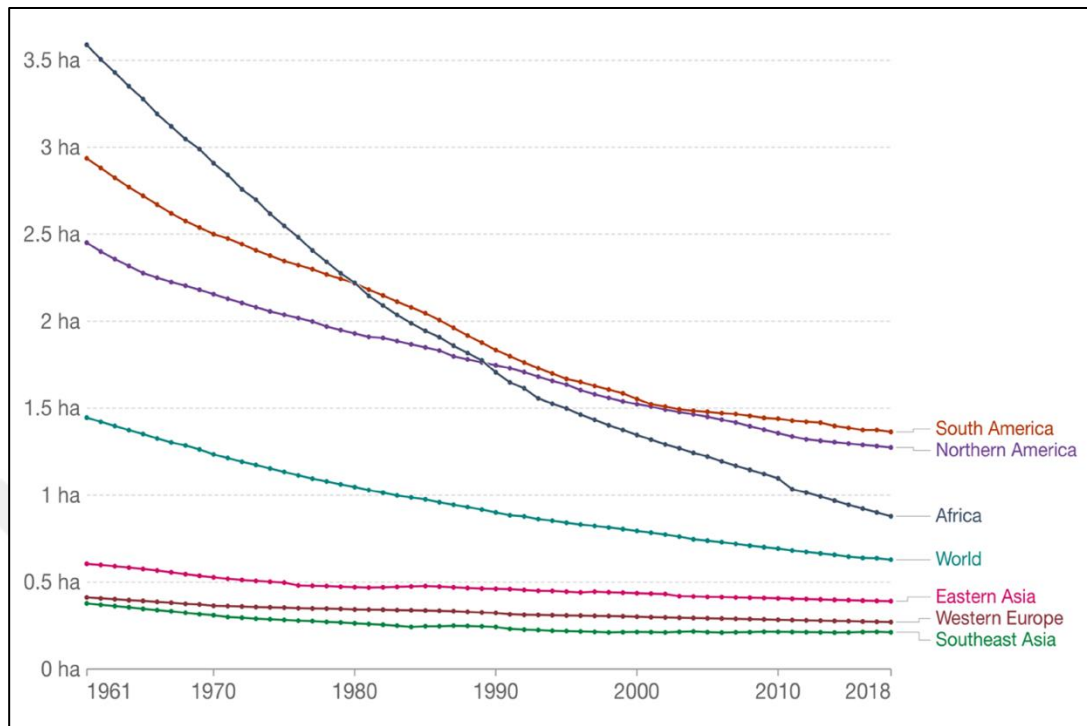


Figure 2.2: Agricultural Land per capita.

According to FAO data, 186.821 million tons of tomatoes were produced in 2020 and an increase of about 3 million tons was detected compared to the previous year. Along with an increase in production, 1.1% and 1.0% increase in arable land and yield were observed, respectively (Table 2.1).

Table 2.1: World Tomato Data (thousand ton).

	2016	2017	2018	2019	2020	Change (%) ¹
Area (t ha)	4.854	4.876	5.005	4.999	5.052	1,1
Yield (ton/ha)	36,54	36,51	36,01	36,61	36,98	1,0
Production	177.383	178.024	180.231	183.015	186.821	2,1
Imports	7.796	7.530	8.071	8.075	7.458	-7,6
Exports	8.323	8.009	8.354	7.855	7.774	-1,0

According to last year's statistical data with a production of 64.8 million tons of tomatoes, China ranks first in tomato production in the world. After China, which meets

approximately 35% of the world's tomato production alone, India ranks second with 20.6 million tons and Turkey ranks third with 13,2 million tons (Figure 2.3a). Interestingly, Nigeria ranks second in the usage of arable land for the tomato production, yet it ranks 11th in world tomato production with a share of only 2%. With 21,9% China ranks first in the usage of arable land for the tomato production and Turkey ranks fourth with 3,6% (Figure 2.3b).

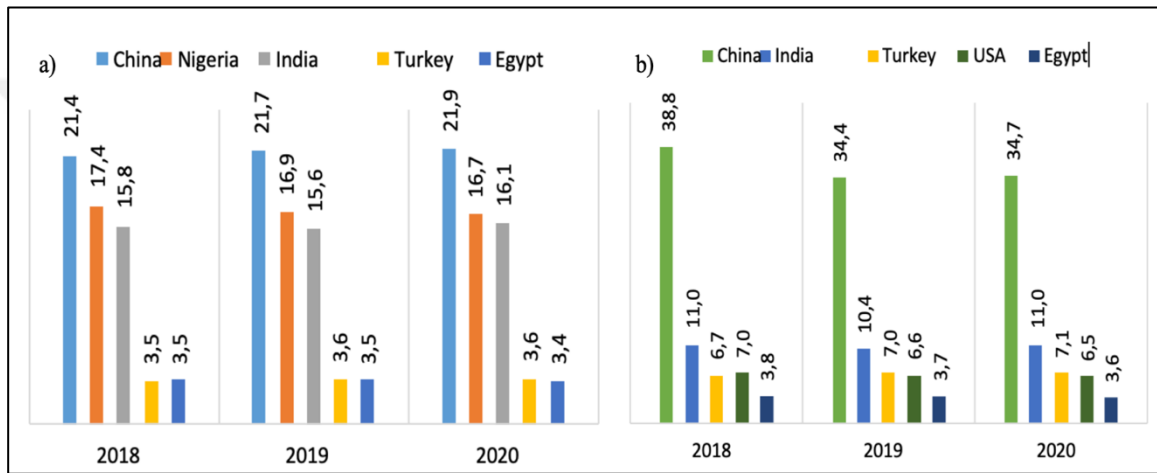


Figure 2.3. a) Important countries in tomato cultivation area (%). b) Important countries in tomato production (%) (TEPGE, January 2022).

Production in Turkey was 12.8 million tons in 2019 and increased by 2.8% to 13.2 million tons in 2020. In addition, 66% of this production was for raw consumption. On the other hand, according to TUIK data, tomato cultivation areas in Turkey decreased by 3.5% in 2020 compared to 2016 and increased by 1% compared to the previous year (Table 2.2, TEPGE, January 2022). When tomato production is evaluated on a provincial basis, Antalya ranks first with a production of 2.6 million tons (19.5%) in 2020 (Figure 2.4).

Table 2.2: Turkey Tomato Data (thousand ton).

	2015/16	2016/17	2017/18	2018/19	2019/20	Change (%) ¹
Production	12.615	12.600	12.750	12.150	12.842	5,7
Consumption	9.340	9.284	9.443	9.013	9.511	5,5
Imports	10,9	10,4	11,2	34,8	17,4	-50,0
Exports	1.195	1.246	1.205	1.155	1.220	5,6
Consumption per Capita (kg)	118,6	116,3	116,9	109,9	114,4	4,1

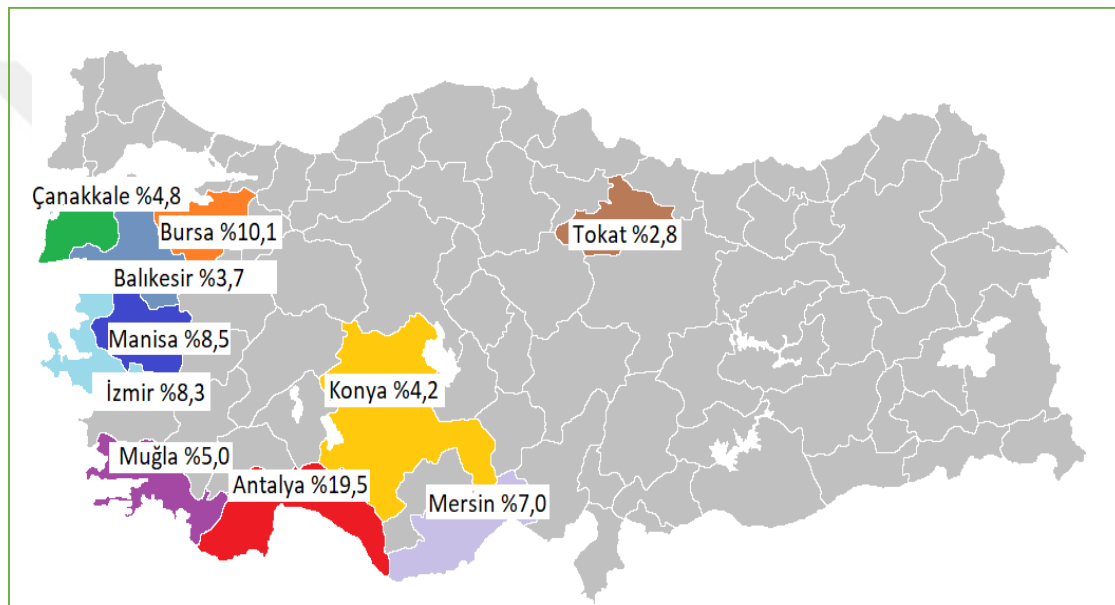


Figure 2.4. Turkey data by tomato cultivation area (TEPGE, January 2022).

2.2. Plant small RNAs

Until the discovery of plant small RNAs, it was general knowledge that DNA was encoded for the purpose of protein synthesis. Along with the discovery of 20-23 nucleotide RNA molecules that are not translated into protein but instead, have a regulatory role in gene expression, this general knowledge has also changed [D'Ario et al., 2017; Yu et al., 2020].

The first identified miRNA is *lin-4* and it is an endogenous miRNA in *Caenorhabditis elegans* (*C. elegans*) [Lee et al., 1993]. In the same year, the target of *lin-*

4 was also identified, and this identification also meant the discovery of the first miRNA/target module [Wightman et al., 1993]. By the virtue of these ground-breaking discoveries, a post-transcriptional level gene regulation mechanism was founded. On the other hand, the first plant small RNA was later found in 1999. Accumulation of this 25-nucleotide length small RNA was triggered by RNA viruses and classified as a post-transcriptional gene silencing (PTGS) factor [Hamilton et al., 1999]. At last, miRNAs and small interfering (siRNAs) are the two main types of plant small RNAs and are classified according to their biogenesis or associated proteins.

2.2.1. Biogenesis of miRNAs

Although miRNAs only compose a small part of plant small RNAs, they have a tremendous impact on diverse plant biological processes. Therefore, the deformation of miRNA biogenesis may have a direct effect on plant development or plant responses. Thus, miRNA biogenesis has become a major focus of miRNA characterization studies.

Each miRNA has a specific MIR gene which is at the very beginning of miRNA biogenesis, transcribed to a single-stranded primary transcript (pri-miRNA) by RNA POLYMERASE II (Pol II). At the same time, a polyadenylated (poly A) tail stabilizes this newly formed pri-miRNA [Yu et al., 2020; Gou et al., 2005]. Formed as a hairpin or stem-loop pri-miRNA has miRNA and miRNA* strands on each side [Gou et al., 2005]. This step is followed by the formation of precursor-miRNA (pre-miRNA) which is formed through the cleavage of the pri-miRNA by The RNase III enzyme DCL1. Then, DCL1 again takes charge and converts pre-miRNA into a miRNA/miRNA* duplex [Kurihara and Watanabe., 2004]. The diversity of the DCL family varies with different plant species. Different DCL family members produce miRNAs of different lengths. Most plant miRNAs are processed by DCL1 or DCL4 and are 21 nucleotides in length. The DCL2 and DCL3 proteins, on the other hand, tend to produce miRNAs that are 22 and 24 nucleotides in length, respectively. The molecular structures of DCL proteins are thought to be the main determinant of miRNA length [Huntley et al., 2016; Rajagopalan et al., 2006; Yu et al.,

2020]. The formation of the miRNA/miRNA* duplex occurs inside Dicing-bodies or D-bodies chambers in the nucleus. The miRNA / miRNA* duplex is then modified at the 3' end through methylation by Hua Enhancer 1 (HEN1). This methylation is important for the miRNA / miRNA* duplex stabilization and preventing degradation of the newly formed duplex by SMALL RNA DEGRADING NUCLEASE 1 (SDN1) [Ramachandran and Chen., 2008; Yu et al., 2005]. TRANSPORIN 1 (TRN1) helps ARGONAUTE (AGO1) for the incorporation of methylated miRNA and this process is followed by the removal of miRNA* [Cui et al., 2016]. Exportation of miRNA from the nucleus to the cytoplasm is driven by HASTY (HST) and interestingly a significant decrease in miRNA production was exhibited in *hst* mutant *Arabidopsis* lines [Park et al., 2005]. The biogenesis of miRNA is finalized by the assembly of the RNA-induced silencing complex (RISC) (Figure 2.5).

In addition to these listed key factors of miRNA biogenesis, there are various other factors to aid miRNA biogenesis. To assemble Pol II to MIR promoter, the interaction between coactivator Mediator and transcriptional activators is required [Kim et al., 2011]. CELL DIVISION CYCLE 5 (CDC5) is another protein that positively regulates the transcription process by occupying Pol II which binds with the MIR promoter [Zhang et al., 2013]. In addition to this, CDC5 also positively affect the activity of DCL1 by interacting with the PLEIOTROPIC REGULATORY LOCUS 1 (PRL1) [Zhang et al., 2014]. pri-miRNA recognition by DCL1 is driven by DAWDLE (DDL) which is a fork head-associated protein and also, stabilizes pri-miRNA [Morris et al., 2006]. HYPONASTIC LEAVES 1 (HYL1) and SERRATE (SE) are responsible for enhancing the efficiency of DCL1 in the miRNA processing [Dong et al., 2008]. On the other hand, enhancing the dicing activity of DCL1 is driven by TOUGH (TGH) [Ren et al., 2012].

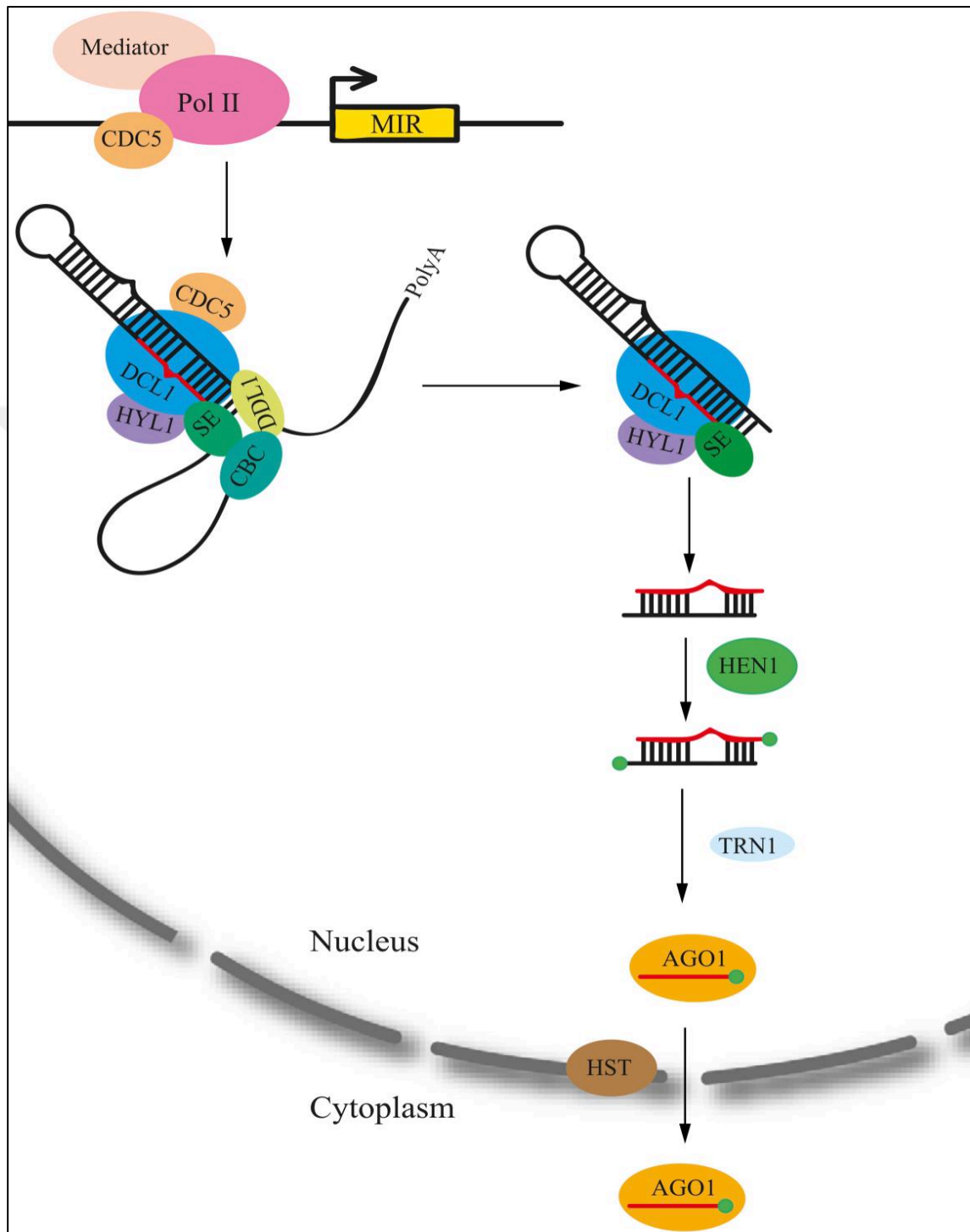


Figure 2.5: Biogenesis of a miRNA. CDC5: CELL DIVISION CYCLE 5, Pol II: RNA POLYMERASE II, DCL: DICER-LIKE, SE: SERRATE, HYL1: HYPOASTIC LEAVES 1, CBC: cap-binding complex, HEN1: Hua Enhancer 1, TRN1: TRANSPORIN 1, HST: HASTY, AGO1: ARGONAUTE 1.

2.2.2. Mode of Action

Plant miRNAs mostly regulate gene expressions through two PTGS ways: transcript cleavage and translation repression (Figure 2.6).

In the miRNA-guided transcript cleavage, the target is recognized by the sequence complementarity. After the recognition, cleavage occurs at the 10th and 11th nucleotides. The miRNA carried by the RISC complex binds to the target messenger RNA (mRNA) with a perfect or a near-perfect complementarity. Since cleavage leaves a 5' monophosphate behind, the classification of 5' monophosphated RNAs shown that transcript cleavage occurs in most of miRNA-guided PTGS regulation of genes [German et al., 2008].

Although translation repression by miRNA rarely occurs, a few miRNAs still regulate the gene expressions through translation repression. For example, miR172 regulates the expression of AP2 which is a flowering related gene in *Arabidopsis*. Excessive production and accumulation of this miRNA lead to a decrease in AP2 protein levels yet, the transcript level of AP2 was similar to the wild type [Chen et al., 2004]. This finding was the first evidence of miRNA-mediated translation repression in plants. Later on, other miRNAs with the same ability were identified. miR159 was one of them and this miRNA family targets MYB domain transcription factors in *Arabidopsis*. Excessive accumulation of this miRNA inhibited plant growth and promoted programmed cell death in *Arabidopsis* [Alonso-Peral et al., 2010]. Also, these discoveries indicate that miRNA-mediated translation repression regulates the target by preventing protein synthesis rather than degrading proteins [Li et al., 2013]. Since most miRNAs are polysome enriched, their gene expression regulation occurs through either miRNA-guided transcript cleavage or miRNA-mediated translation repression on the endoplasmic reticulum (ER) [Li et al., 2016].

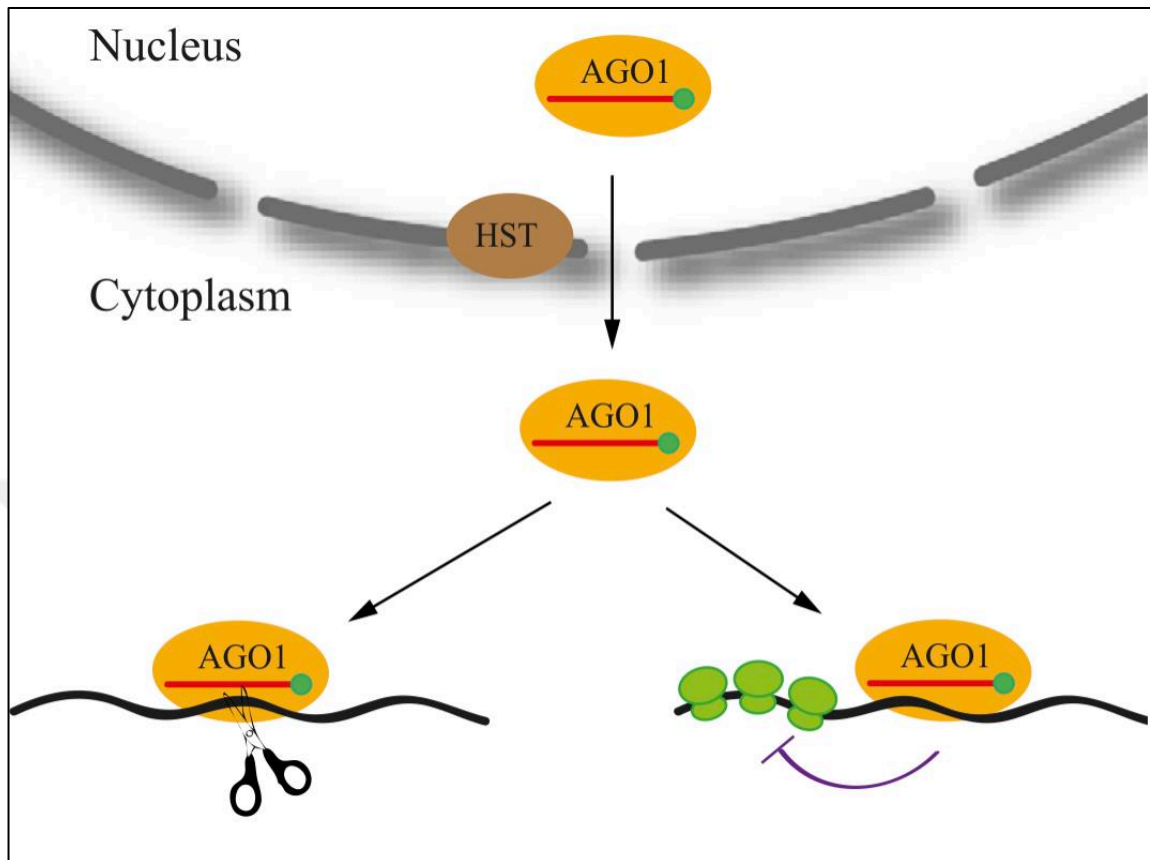


Figure 2.6: miRNA mode of actions. Two PTGS mechanisms; transcript Cleavage (left) and translation repression (right).

2.2.3. miRNAs in Plant Development

miRNAs generally regulate plant developmental processes through targeting transcription factors. These processes are listed as; plant growth, stress responses, plant immunity, hormone pathways and flowering (Figure 2.7) [D'Ario et al., 2017; Yu et al., 2020].

A substantial number of studies have been performed to identify miRNAs and their roles in plant development (Table 2.3). For example, miR394 is known to be one of the factors that have a role in meristem maintenance by targeting LEAF CURLING RESPONSIVENESS (LCR). Accumulation of miR394 occurs in the protoderm and this accumulation leads to restriction of its target LCR expression [Knauer et al., 2013].

miR160 has a role in the maintenance of auxin efflux by regulating the expression of auxin response factors (ARFs) [Pillot et al., 2010]. The establishment of leaf polarity is carried by miR390 which targets and restricts the expression of the ARF2 gene [Xu et al., 2007].

Flowering is another important stage of plant development. Several miRNA families have been identified along with their regulatory activity in Arabidopsis flowering process, such as miR156, miR159, and miR172 [Yamaguchi and Abe., 2012]. In the natural flowering process, a reduction in the accumulation of miR156 occurs which leads to an increase in the expression of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes. On the other hand, overexpression of the same miRNA family resulted in a delay in the flowering process [Gandikota et al., 2007]. In addition to that, maize miR156 has an impact on the formation of the grain by targeting the TSH4 [Chuck et al., 2010]. miR159 controls floral timing and transition by the regulation of Gibberellin regulated MYBs (GAMYBs). These regulations lead to changes in the expression of MYB33, MYB65 and MYB1101 genes which affect the activity of LEAFY [Achard et al., 2004]. Although miR172 targets the *AP2* gene and has a role in the flowering process, its function varies in other plants, such as seed development and floral organ development [Aukerman et al., 2003; Lauter et al., 2005; Yoshikawa et al., 2013].

miRNAs also have roles in the root formation and seed development. For example, miR164 regulates the lateral root formation in various plants by targeting NAC1 DOMAIN CONTAINING PROTEIN 1 (NAC1) which is a plant-specific transcription factor [Gou et al., 2005; Li et al., 2012]. In addition to that, miR166 family members target and regulate the expression of PHABULOSA (PHB) to shape the formation of the root [Miyashima et al., 2011]. Under stress conditions, the expression of miR393 is enhanced which leads to inhibition of the auxin receptors TIR1 and AFB2, resulting in reduced lateral root growth [Chen et al., 2012]. On the other hand, the same miRNA targets *GmTIR1* and *GmAFB3* and these miRNA/target modules determine the nodule development in soybean [Cai et al., 2017]. Overexpression of miR390 revealed an interesting result in *M. truncatula* which was an enhanced lateral growth along with

inhibited nodule formation [Hobecker et al., 2017]. In tomato, miR167 targets and regulates ARF6 and ARF8 and these miRNA/Target modules have a direct impact on the dispersal of seeds and floral organ development [Liu et al., 2014]. miR156 regulates grain size and branching by targeting SPL genes in rice [Tang et al., 2017].

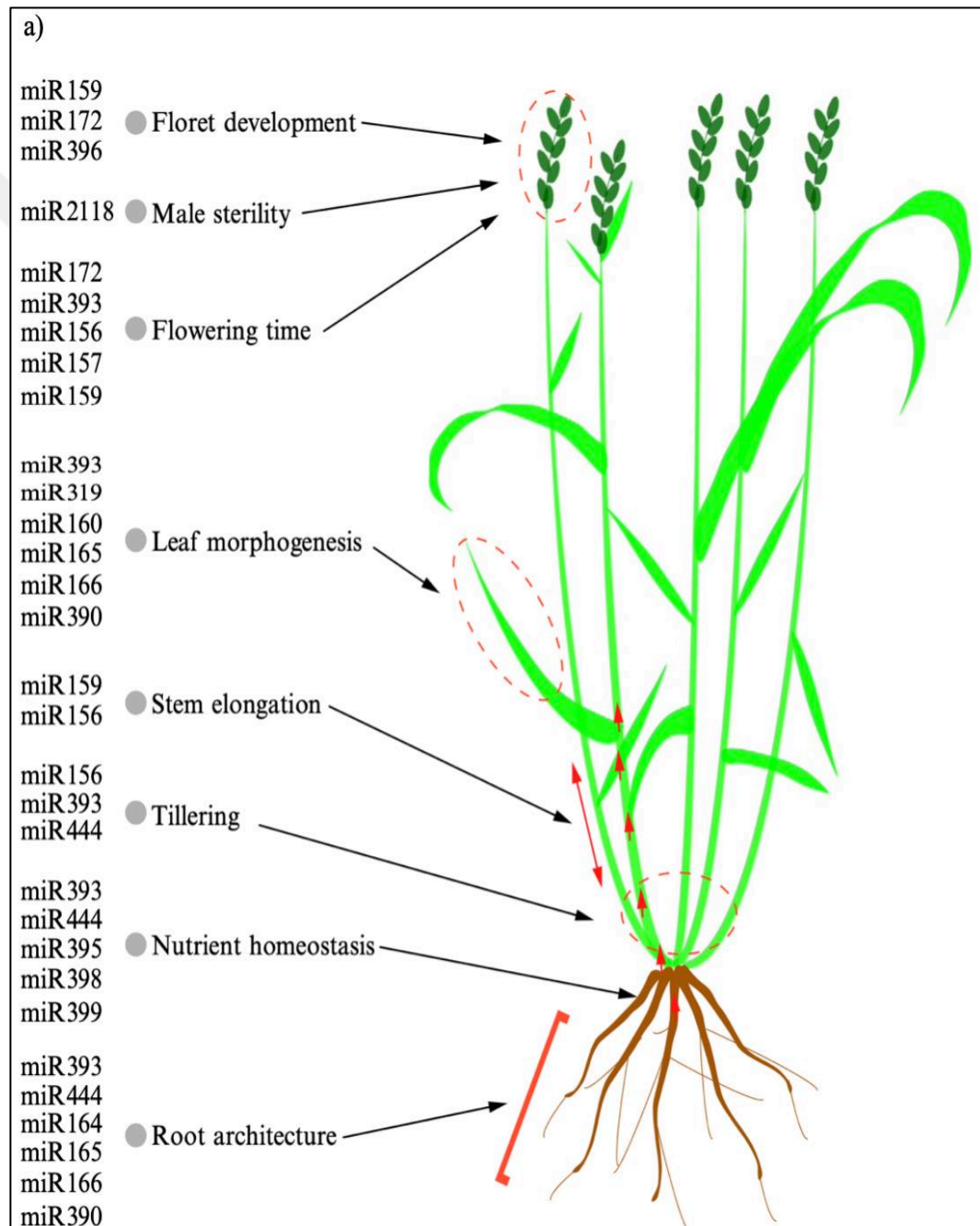


Figure 2.7: a) miRNAs roles in plant during the reproductive stage.

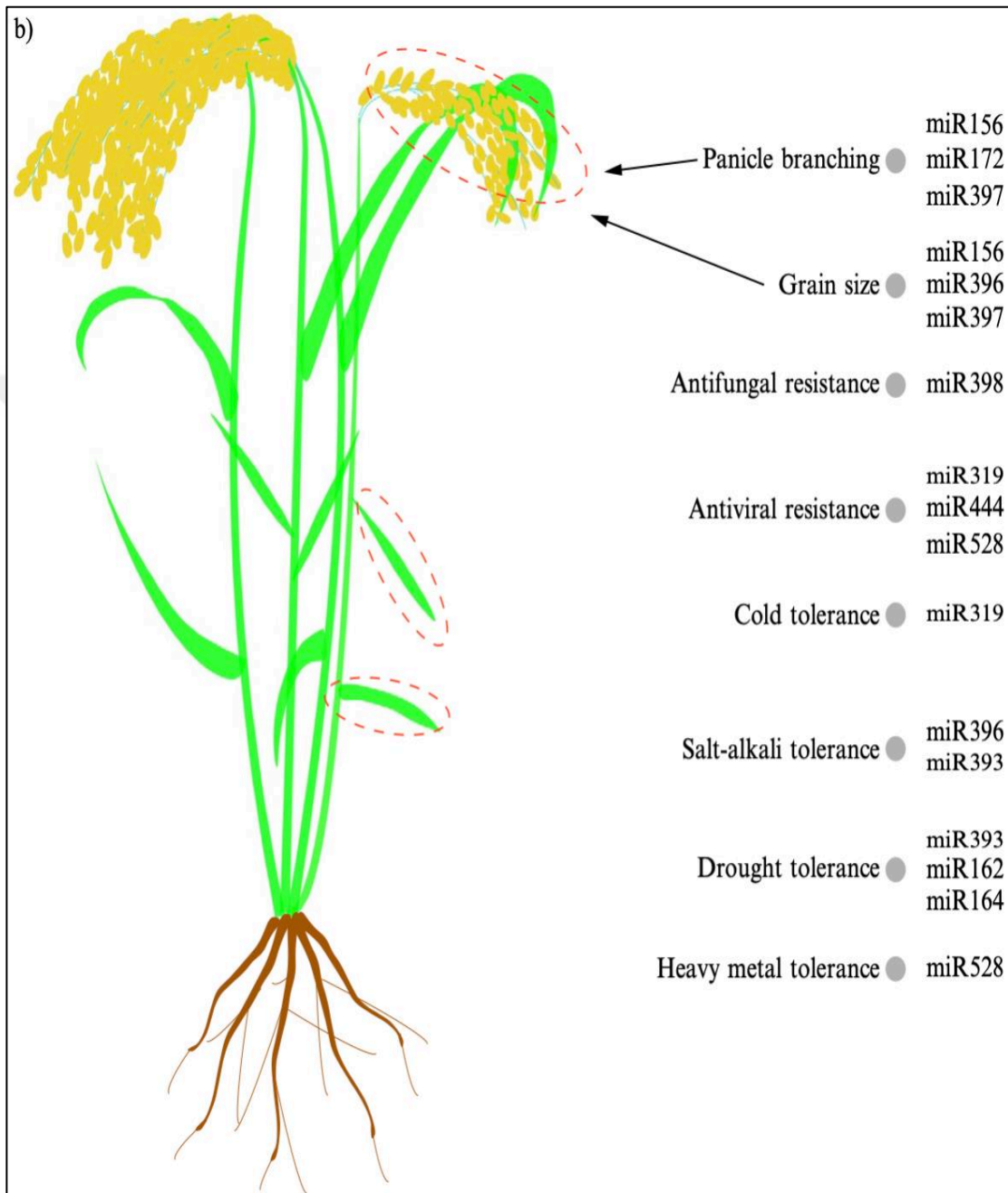


Figure 2.7: b) miRNAs roles in plants during the ripening stage.

Table 2.3: miRNAs and their roles in plants.

miRNA	miRNA targets	Functions of miRNA targets
miRNA156/157	SPL family	Flowering, tillering, ear development, aging, GA response, grain size, panicle branching
miRNA159	MYB33/65/101	Flowering, anther, silique, and seed development
miRNA160	ARF10/16/17	Auxin response, root cap formation, seed development
miRNA164	CUC/NAC	Meristem boundary identity, auxiliary meristem formation, leaf serration, lateral root formation
miRNA165/166	HD-ZIP III family	Meristem maintenance, leaf patterning, root architecture, seed germination
miRNA167	ARF6/8	Auxin response, seed dispersal and floral organ development, male organ development
miRNA169	HAP2	Nodule cell differentiation
miRNA172	AP2 family	Flowering, seed development, floral organ development
miRNA319	TCP family	Cell growth and proliferation, leaf and petal shape, GA response, nitrogen fixation
miRNA390/TAS3	ARF2/3/4	Leaf patterning, lateral root development
miRNA393	TIR1/AFB	Lateral root development, nodule development, seed development
miRNA394	LCR	Meristem maintenance
miRNA396	GRF	Cell proliferation in leaf, grain size
miRNA397	LAC	Grain size

2.2.4. miR396

So far 174 members of miR396 have been identified according to miRbase, and only two members of miR396 are identified in tomato and Arabidopsis. In various plants, this miR396 members target and regulate GROWTH REGULATING FACTOR (GRF). This miR396/GRF module represses the activity of GRF genes and influences plant growth (Figure 2.8) [Liebsch et al., 2020; Kim et al., 2019; Rodriguez et al., 2016].

GRFs are composed of WRC and QLQ domains and WRC is a domain that is required for DNA-binding and consists of Trp-Ar-Cys motif. This QLQ-WRC specific combination can be only found in plant GRFs [Kim et al., 2019; Rodriguez et al., 2016]. miR396 precisely recognises the WRC domain of plant GRFs. Other regulatory roles of miR396 are known in plants but only miR396/GRF module is conserved in various seed plants [Debernardi et al., 2012; Bologna et al., 2014].

In tomato, two members of miR396 are identified, miR396a and miR396b. The difference between these two miRNAs appears at the end of the miRNAs as a single base. On the other hand, their expression rates are dramatically different and miR396a has a higher expression rate than miR396b [Cao et al., 2016]. Destruction of the miR396/GRF module led to enhanced cotyledon growth, larger flower, altered sepal formation and also influenced the fruit and shoot size [Cao et al., 2016]. In addition to these, in miR396 downregulated tomato plants, less water loss, and activated jasmonate and GABA pathways were observed. Thus, miR396 downregulated tomato plants had higher drought stress resistance due to the enhanced water use efficiency [Fracasso et al., 2021].

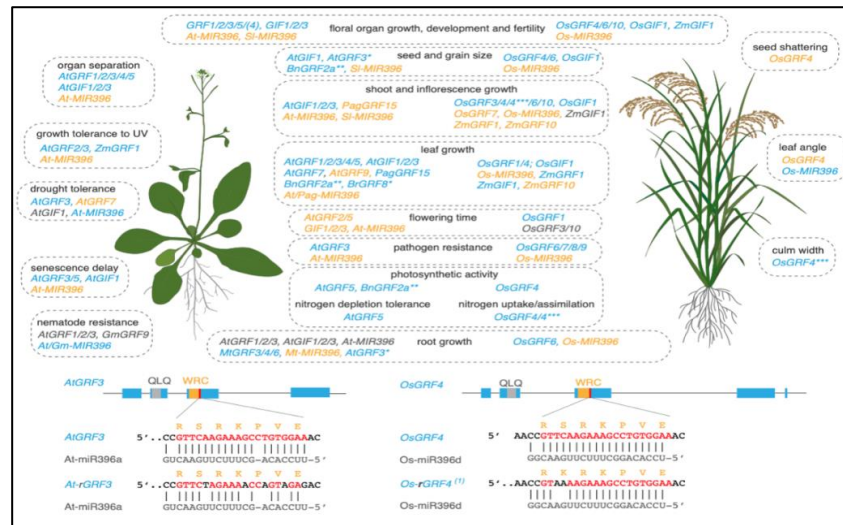


Figure 2.8: Roles of miR396 in various plant biological process and their bindings with the target loci in Arabidopsis and maize.

2.3. Genome Editing Technology

The rapid increase in the population, climate changes, increasing hunger levels, and the reduction of arable lands are the biggest problems that make it difficult to live on earth. Since these changes are happening very quickly, plants can not have adequate time to adapt to changing environment. Therefore, genome editing techniques have become important tools to overcome this problem. Thanks to their precise targeting systems, it is now possible to introduce mutations to plants in a couple of weeks which would happen evolutionarily in hundreds of years.

Zinc finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and CRISPR are the major next generation genome editing techniques. These techniques are used for introducing mutations on a specific target through Double-strand breaks (DSBs) on the target DNA sequences. Those DSBs are mostly repaired by either homologous recombination (HR) or non-homologous end-joining (NHEJ) [Shah et al., 2018]. Since NHEJ is an error-prone repair mechanism that can completely shut down a gene, it causes deletions or additions in the amino acid sequence and amino acid substitutions. HR, on the other hand, is preferred for replacing the defective gene with the intact/unmutated allele or adding a new gene to the genome (Figure 2.9).

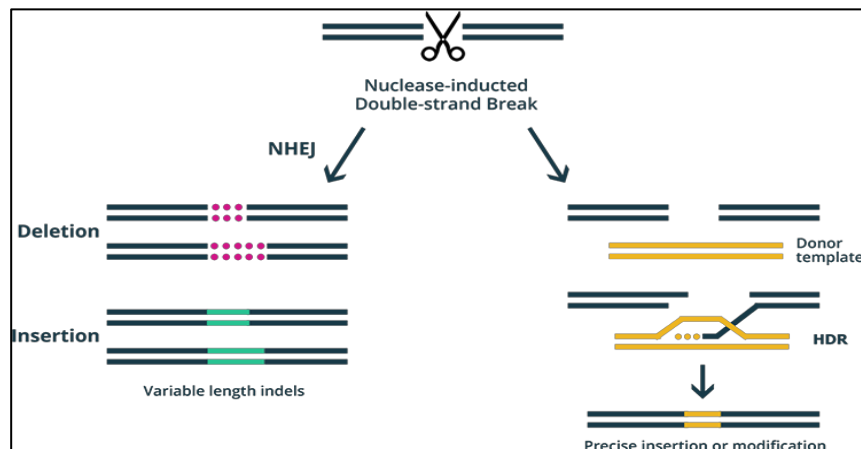


Figure 2.9: DNA repair system, non-homologous end-joining (NHEJ) on left and homologous recombination (HR) on right.

2.3.1. Zinc finger nucleases (ZFNs)

Zinc finger nuclease is the initial genome editing technique that was invented in 1985 [Miller et al., 1985]. ZFN was developed by a combination of an engineered zinc finger (ZF) domain with a nuclease(N) domain. *FokI* is used as a nuclease which causes a single-strand break (SSB) on the targeted sequences in the ZFN system. In addition to that, *FokI* endonuclease has been isolated from the bacterium called *Flavobacterium okeanoicoites* [Wah et al., 1998]. On the other hand, Zinc finger protein domains are in a sequence, and each recognizes three specific nucleotides so that a specific region of 18-24 bases can be targeted. These domains consist of engineered Cys₂-His₂ residues, and they are stabilized by Zn⁺² ions [Albà et al., 2017]. Due to the SSB activity of *FokI*, ZFNs are used as pairs to obtain double-strand breaks on the target location (Figure 2.10) [Rahim et al., 2021].

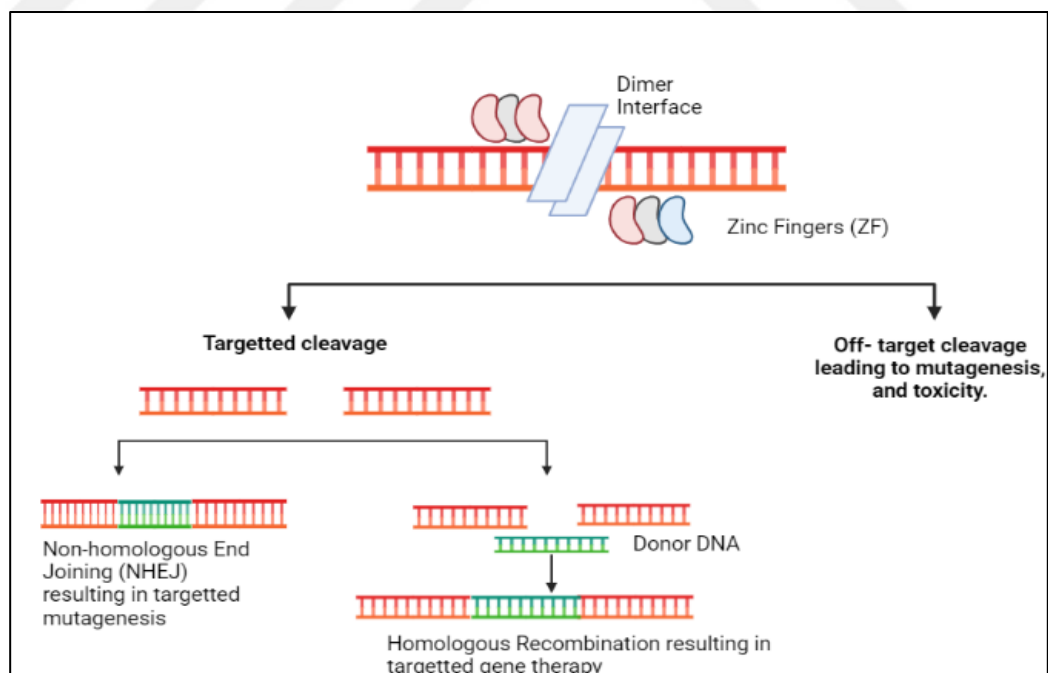


Figure 2.10: Zinc Finger Nucleases mode of action.

Due to the challenging construction process, ZFN is not a preferred technique for genome editing. On the other hand, mainly two methodologies have been followed to construct zinc finger systems; (i) Construction of new ZFN combinations through analysing the ZFs libraries, which is called context-sensitive selection strategy [Durai et al., 2005]. (ii) Construction of ZFN pairs by selecting ZFs from an identified pool according to target sequences. This method is called modular assembly [Cathomen and Joung, 2008]. A downside to working with ZFN is the need to regenerate ZFN for each region. This is both time-consuming and costly. 64 different zinc finger protein motifs are required for all triplet sequences in the target sequence. Since the amount of differently combined ZF protein motifs is limited, this technique is not applicable to every desired target sequence [Chen and Gao., 2013]. In addition to this, the high rate of mutation in off-target sequences, the need for high amounts of endonuclease enzymes, high cost of experimental setup and long experimentation periods are among the disadvantages of this technique.

2.3.2. Transcription activator-like effector nucleases (TALENs)

TALEN originated from *Xanthomonas* which is a pathogenic bacterium for various plants. When *Xanthomonas* causes infection in a plant, it transports transcription activator-like (TAL) proteins into the host cell via type III secretion system. After this translocation, TALs act as transcription factors of the host organism and they cause developmental changes which lead to accelerated infection and severe damage to plants [Joung and Sander, 2013].

Transcriptional activation and central tandem repeats are the two main domains of TALs. In addition to these two domains, TALs also consist of nuclear localization signals [Boch et al, 2009]. Repeat domains are driving the DNA binding and they consist of 33-35 amino acids. Hypervariable residues are the main determinant of target, and they are the 12th and 13th amino acids of the repeat domain. Hypervariable residues are named repeat-variable di-residue (RVD) and each one of these RVDs corresponds to one of four

bases [Cermak et al., 2011]. Like in ZFN, in this technique *FokI* is merged with TALs to cleavage DNA and together with *FokI*, TALs are also used as pairs (Figure 2.11) [Joung and Sander, 2013]. Because of the low efficiency of TALEN, a tremendous amount of time and effort has been spent on increasing and improving its applicability. The greatest improvement occurred after the truncation of the C-terminal domain. Before this finding, *FokI* was located next to the C-terminal domain and with the truncation of these domains, the activity of *FokI* was greatly increased [Joung and Sander., 2013]. In addition to that, to decrease the time consumption of this technique, researchers have also focused on improving the construction of TALEN structures. With Golden Gate assembly it is possible to construct a vector that consists of up to 31 RVDs. By following two steps this can be achieved, (i) assembly of up to 10 RVDs into an array and (ii) insertion of 10 RVDs consisting of arrays to the final plasmid [Cermak et al., 2011].

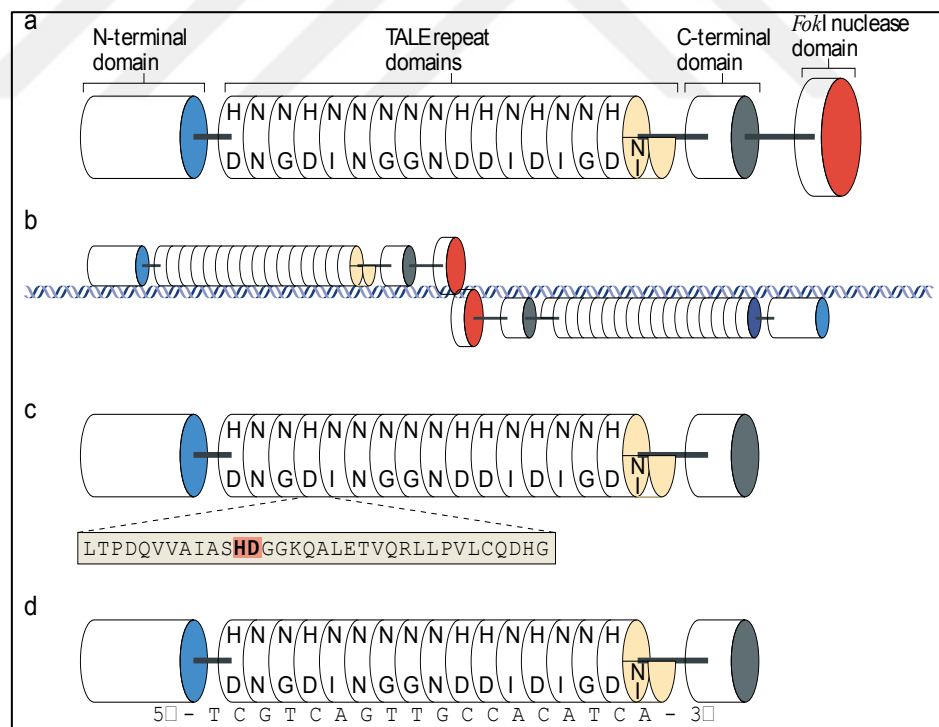


Figure 2.11: Schematic views of TALENs. a) TALEN structure, b) TALENs mode of action, c) Position of RVDs, d) TALEN amino acid sequence with corresponding nucleotides increased.

TALEN is preferred more than ZFN. First, TALEs are relatively less toxic than ZF proteins, and second, specific TALEs are easier to generate than specific ZF proteins. Because recognizing each DNA nucleotide simply relies on using a TALE repeat with the corresponding RVD [Malzahn et al., 2017]. In addition to that, the off-target binding rate is relatively low compared to ZFN.

2.3.3. Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR associated protein (CRISPR/Cas)

The CRISPR/CAS system is used by most bacteria and archaea. This system is an adaptive immune system that allows foreign genetic material to be cut by RNA-guided nucleases and allows organisms to recognize foreign genetic material. Thanks to this immune system, bacteria and archaea protect their genomes from integrations of foreign nucleic acids. CRISPR nomenclature was first defined by Jansen et al. (2002).

The CRISPR adaptive immune system occurs in three steps. The first step is the integration of foreign DNA fragments from the phage into the CRISPR array. The second step is the transcription of the pre-crRNA and the production of individual crRNAs. The final step is the degradation of foreign DNA by CAS proteins (Figure 2.12) [Semenova and Severinov, 2017; Jiang and Doudna, 2017].

During infection, short viral DNA fragments called protospacers can be selected by the Cas1-Cas2 protein complex and integrated into the CRISPR sequence. The CRISPR sequence is expanded, and spacer sequences are inserted between each repeat sequence. Later, CAS genes are activated and CRISPR sequence transcription creates pre-crRNAs. Pre-crRNA contains identical repeat sequences and spacer sequences which is processed to create single, short CRISPR RNAs. Each of the previously formed crRNAs associates with CAS proteins and forms an 'effector complex'. Each formed effector complex fights against invading pathogens. When the same phage infects the cell, the effector complex recognizes and degrades the phage DNA.

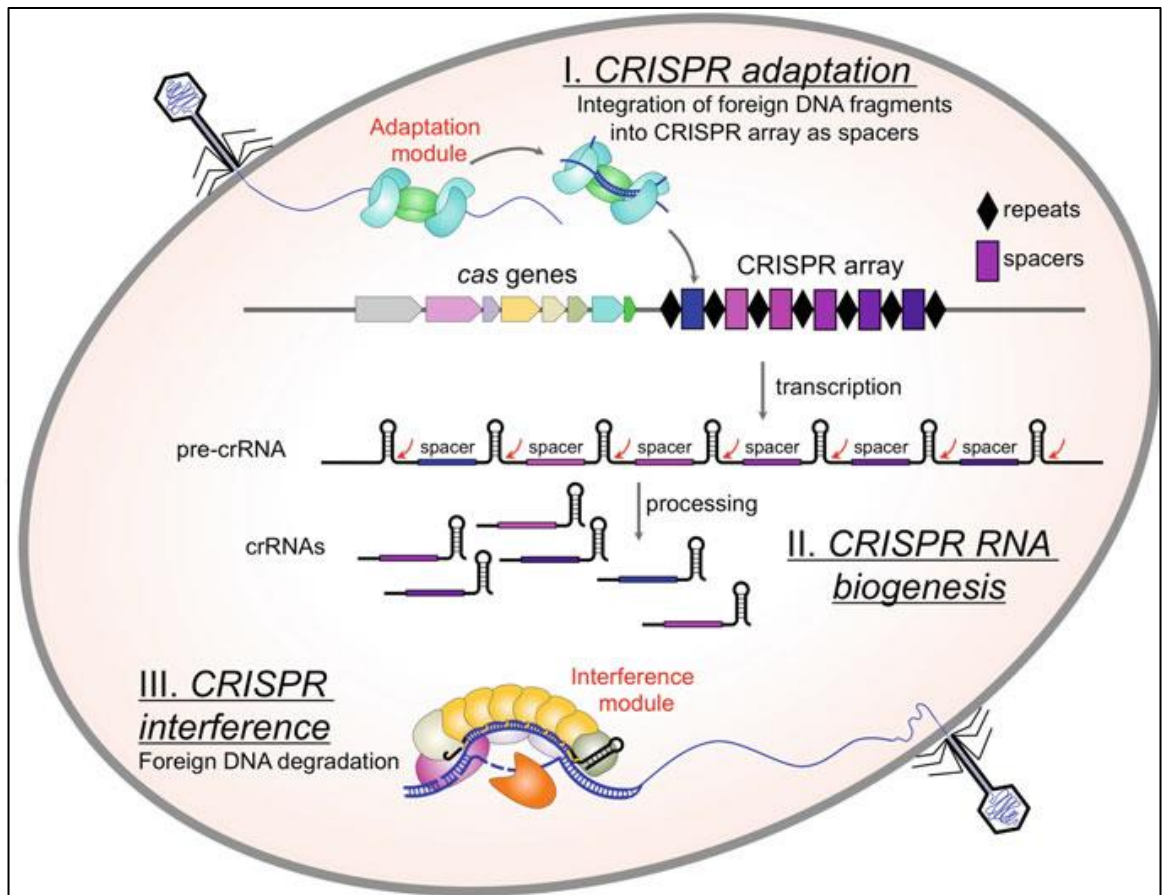


Figure 2.12: CRISPR adaptive immune system. Schematic representation of the three steps of the CRISPR/CAS system.

2.3.3.1. Utilization of CRISPR/Cas in genome editing

The use of CRISPR/Cas systems has greatly accelerated genome editing studies. Diversity of Cas proteins and the presence of multiple CRISPR loci have complicated the classification of these systems. Since not all the CRISPR related genes are conserved between all the CRISPR systems, it is impossible to make a simple and straightforward classification. For the classification of CRISPR systems, along with CRISPR key genes, conserved genes, loci, and similarity of sequences should be also considered [Makarova et al., 2020]. It is possible to divide all the Cas genes into four modules that are partially overlapping but different. CRISPR–Cas systems are classified primarily by differences in

Cas protein composition and sequence diversity between effector modules. Essentially, CRISPR effector modules are divided into two classes, Class I and Class II (Figure 2.13). Both classes have multiple subtypes. For example, Type I, Type III, and Type IV are the subtypes of Class I. These three subtypes have similar pre-crRNA processing and target recognition. On the other hand, they don't share any similarities in their sequences [Özcan et al, 2019]. The distantly related RNA recognition (RRM) motif and the repeat-associated mysterious proteins (RAMPs) which are Cas5 and Cas7, serve as the basis of the effector complexes in all three class 1 types. On the other hand, other RAMP, Cas6, does not have to be necessarily physically associated with the complex and it is responsible for RNase activity for crRNA processing. In addition to these RAMPs, although Cas8 and Cas10, the major subunits of type I and III effector complexes, respectively, retain similar locations in the complexes there is no sequence similarity between them. Another RAMP Cas3, a massive protein with merged helicase and HD nuclease domains that is responsible for target DNA cutting in type I systems, is a crucial component of the effector module (Figure 2.14) [Makarova et al., 2020].

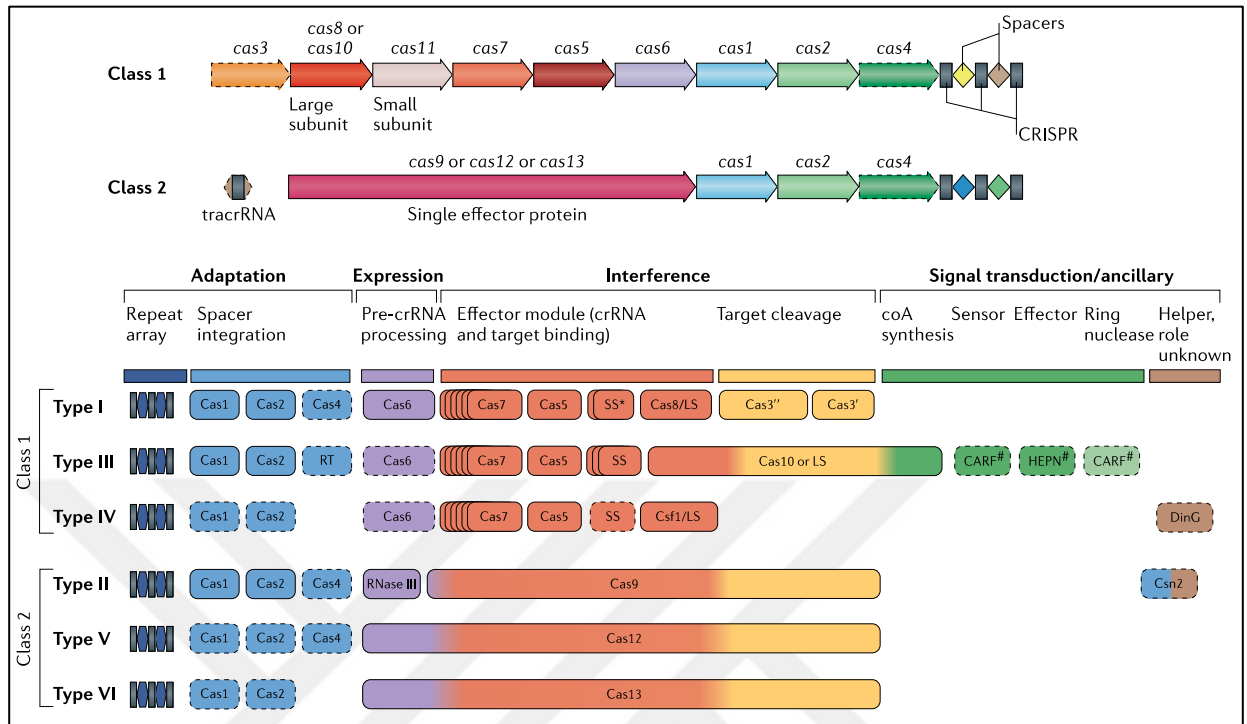


Figure 2.13: Classification of CRISPR systems. Class I and Class II along with their subtypes.

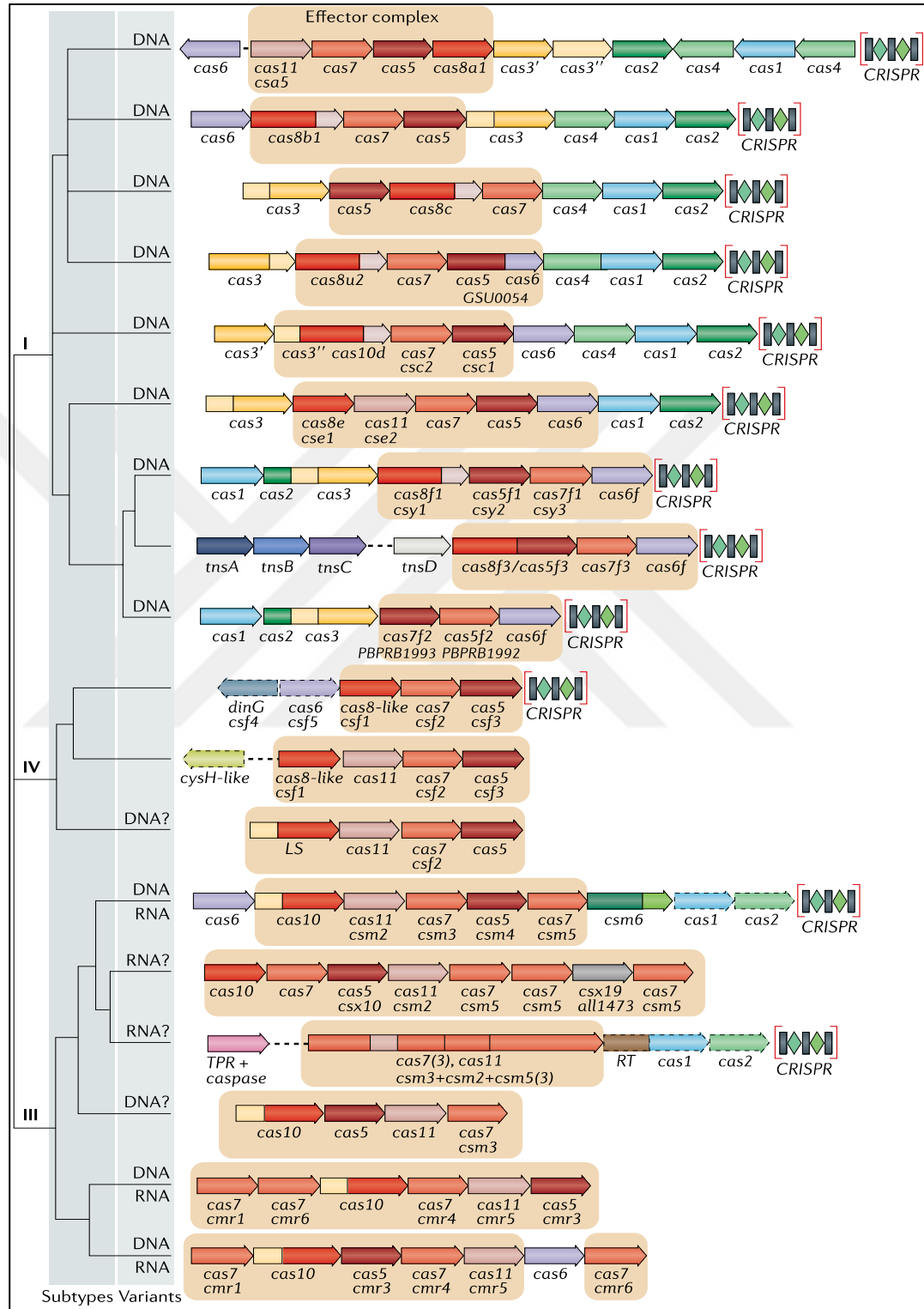


Figure 2.14: Detailed classification of CRISPR Class I systems. Structure and the combination of each subtype vary by organism.

On the other hand, the effector modules in class 2 are single big proteins with distinct domain structures that distinguish type II, V, and VI systems [Shmakov et al., 2017]. The processes of pre-crRNA processing vary markedly between the subtypes of class 2 CRISPR modules [Faure et al., 2019]. For example, the effector protein also includes the pre-crRNA processing RNase function in type VI and V systems, while in type II, this function is often assigned to RNase III [Chyou et al., 2019; Fonfara et al., 2016; East-Seletsky et al., 2016]. In addition to this, the trans-activating CRISPR (tracr) RNA, that generates persistent duplexes with the partly identical direct repeat of the pre-crRNA, is included in the effector module. The adaptability and effector modules, which consist of Cas1 through Cas13 proteins, determine the types and subtypes of CRISPR/Cas systems, and hence define the basis of class 2 CRISPR/Cas systems. This basis is surrounded by many other proteins that are only tenuously connected to CRISPR/Cas (Figure 2.15) [Makarova et al., 2020].

The type II CRISPR/Cas system is one of the Class II systems, that uses Cas9 endonuclease, and the system is designated as CRISPR/Cas9. Among the CRISPR systems, the most preferred is the CRISPR/Cas9 system [Rani et al., 2016]. In this CRISPR system, which we use as a genome editing method, there is only one guide RNA (sgRNA). Guide RNA is formed by binding the 5' end of the tracrRNA to the 3' end of the crRNA. For Cas9 to be directed to the target site, it is sufficient to design a sequence of 20 nucleotides in length. However, for Cas9 to be directed and degraded to the target sequence, the protospacer-associated motif (PAM), a short-conserved sequence (2-5 nucleotides), requires following 3' of the crRNA complementary sequence [Richter et al., 2012]. Since NGG sequences are common in any coding sequence, CRISPR/Cas9-based genome editing can be applied to almost all organisms.

CRISPR/Cas9 genome editing technology has many advantages over other genome editing technologies. It is quite effortless to create double-strand breaks. gRNA design can be performed easily and quickly, specific to the desired target. While the TALEN system cannot introduce mutations on methylated DNA, there is no such restriction in the CRISPR/Cas9 method. Compared to TALEN and ZFN, CRISPR/Cas9

technology is easy, inexpensive and has high efficiency. Considering the disadvantages, the off-target binding and off-target degradation rates are high, and plus the requirement of the PAM sequence to bind to the target may limit the targets [Malzahn et al., 2017].

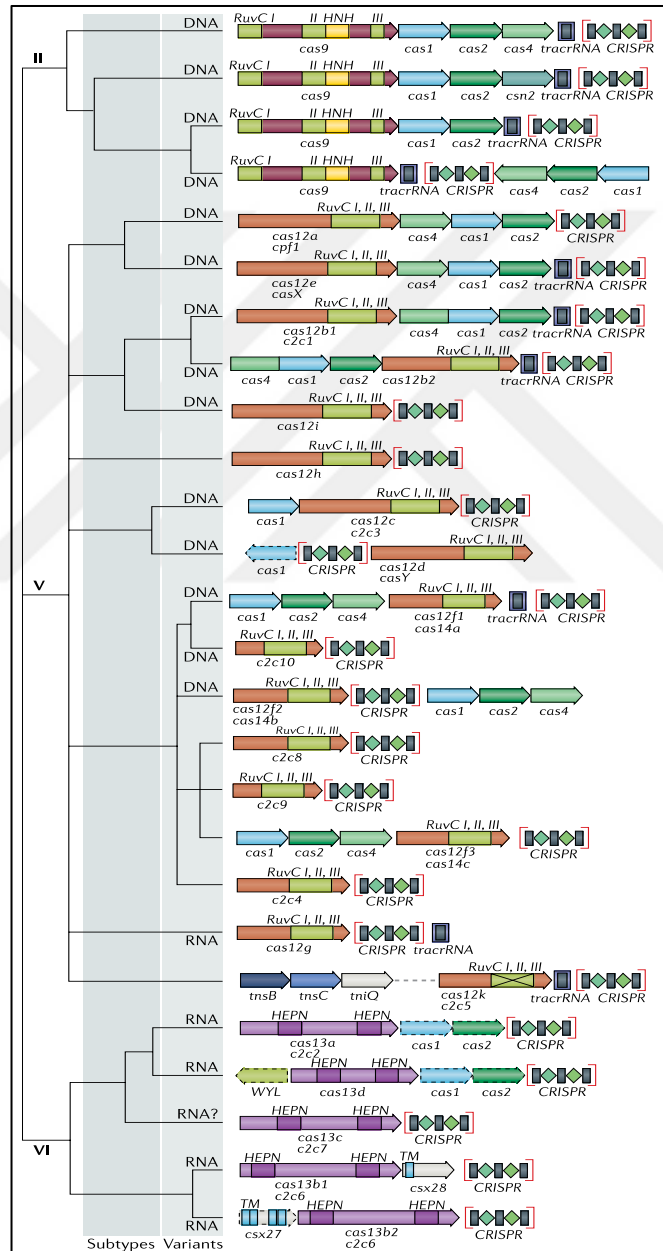


Figure 2.15: Detailed classification of CRISPR Class II systems. Structure and the combination of each subtype vary by organism.

Plant breeding techniques commonly used in modern agriculture can be listed as crossbreeding, mutation breeding, and transgenic breeding. In crossbreeding, two plant lines are selected and crossed. In mutation breeding, with chemical or radioactive agents, mutations are introduced randomly. In transgenic breeding, the gene of interest is for transfer into plant cells with the help of a plasmid. All three methods are time-consuming and difficult. The development of CRISPR/Cas9 technology has started a new era in plant breeding. This technology enables the improvement of metabolic pathways, provides biotic and abiotic tolerance in plants, increases nutrient content, produces high yield and quality, haploid seeds, and improves resistance to herbicides. On the other hand, utilization of this technique is quite restricted in Europe under the GMO regulations of the European Union (EU). Due to the usage of bacteria originated endonucleases, even non-transgenic products produced by CRISPR/Cas system are considered GMOs in the EU. This situation prevents implementation of the promising studies in the field. Interestingly, other regions of the world, have a more positive approach toward CRISPR/Cas systems. For example, Argentina with other South American countries do not consider non-transgenic GE products GMO. In addition to that Russia considers GE as an equivalent of conventional breeding. Japan Governmental Advisory Committee, on the other hand, indicated that there is no significant difference between GE technology and conventional breeding methods (Table 2.4) [Cebraioğlu et al., 2019]. The positive approaches of other countries and the pressure of European researchers strengthen the idea that EU CRISPR/Cas and genome editing regulations will be restructured and mitigated in the near future.

Table 2.4: Regulations of Genome Editing (GE) technologies by countries.

Countries	Regulation Decisions
Australia	Most genome editing techniques will be explicitly regulated. But the technique known as SDN-1 [generation of small deletions or insertions (indels) at a precisely defined location] will be excluded* / WTO Statement
Argentina, Brazil, Canada, Chile, Colombia, USA	Non-transgenic GE crops are not GMO / WTO Statement
Dominican Republic, Guatemala, Honduras, Jordan, Paraguay, Uruguay, Vietnam, Economic Community of West African States (ECOWAS)	WTO Statement
Japan	There is little difference between traditional breeding methods and gene editing in terms of safety (Japan Governmental Advisory Committee)
Russia	GE technologies as equivalent to conventional breeding methods.
The European Court of Justice (ECJ)	Using recombinant nucleases cause GMO
Belgium, Sweden, UK	Calling to update EU GMO laws / Gave permission for field trials before ECJ ruling
Cyprus, Estonia, Finland, France, Germany, Greece, Netherlands, Italy, Portugal, Slovenia, Spain	Calling to update EU GMO laws

3. MATERIALS and METHODS

3.1. Plant Material

In this thesis, cv. Alyanak F1 hybrid, which is a commercial tomato variety of E-Z Tohumculuk company, tomato (*Solanum Lycopersicum* L.) seeds were used. The seeds were taken into tissue culture and germinated in the plant growth room of Gebze Technical University, Faculty of Science, Department of Molecular Biology and Genetics, Plant Biotechnology Laboratory. The germination conditions are 22-23 °C, the light intensity of about 6000 lux, 16 hours light cycle and 8 hours dark cycle period.

3.2. Chemicals, Devices and Kits

The devices used in the thesis study are shown in Table 3.1, the chemicals used in Table 3.2 and the kits used in Table 3.3.

Table 3.1: Devices used in thesis.

Devices	Model	Company
Laminar Cabin		Heraeus
Spectrophotometer	UV-1800	Shimadzu
Nanodrop	EC 1000 XL	Thermo Scientific
Azure Imager	C150	Thermo Scientific
PCR	T100	Biorad
Electrophoresis	EC 1000 XL	Thermo Scientific
Incubator	EC160	Nüve
Centrifugal	MPW-150R	MPW MED.INSTRUMENTS

Table 3.2: Chemicals used in the thesis.

Chemical	Company	Catalog No
KH_2PO_4	Chem Cruz	7778770
K_2HPO_4	Alfa Aesar	AA11593A7
Glycerol	Merck	1040932500
D-Glucose	Duchefa Biochemie	5996101
LB Broth Miller	BD Difco	244620
Casein	Sigma Aldrich	91079402
Agar	Duchefa Biochemie	012290.02
$(\text{NH}_4)_2\text{SO}_4$	Sigma Aldrich	80K5421
MES Buffer	Caisson	03172005
MS	Duchefa Biochemie	P17840.01
Sucrose	Multicell	800-081-LG
KNO_3	Duchefa Biochemie	011852.14
Myoinositol	Duchefa Biochemie	015677.02
Chloroform	Süpelco	K52822245040
Octanol	Applichem	163386
Zeatin	Cassion	03192033
Folic Acid	Duchefa Biochemie	F0608
D-biotin	Merck	851209
TDZ	Sigma Aldrich	048K3794
2-IP	Duchefa Biochemie	0055582.01
Acetosyringone	Sigma Aldrich	2478-38-8
Timentin	Duchefa Biochemie	010953.04
Kanamycin	Sigma Aldrich	SLCC4961
Gentamicin	Sigma Aldrich	1406-41-0
AgNO_3	Sigma Aldrich	S6506

Table 3.2: Continued.

Nicotinic Acid	Sigma Aldrich	59H0621
Pyridoxine HCl	Sigma Aldrich	80K1103
Thiamine HCl	Sigma Aldrich	20K094025
CTAB	Applichem	0U010499
Tris HCl	Applichem	R10532
β -merkaptoetanol	Applichem	9X003976
NaCl	Sigma Aldrich	ZBE630V31434
Na EDTA	Sigma Aldrich	E6635
Glycine	Merck	M1.04201
Agarose	Bio Basic Canada	L6132170KC
DNA Marker	Thermo Scientific	SM0311
DNA Loading Dye	Intron Biotechnology	21161
RedSafe	Intron Biotech	21141

Table 3.3: Kits used in thesis.

Kit	Company	Catalog No
DNA Clean & Concentrator™	Zymo Research	D4013
Zyppy Plasmid Miniprep Kit	Zymo Research	D4019
High Fidelity PCR Kit	NEB	E0553S

3.3. Bioinformatical Process

To edit the MIR gene of miR396b via CRISPR/Cas system, sgRNA design, vector design, Golden Gate assembly, *E. coli* transformation, *Agrobacterium* transformation, *Agrobacterium* mediated tomato transformation, and in vitro germination steps were followed.

3.3.1. Verification of MIR gene

To verify MIR gene of miR396b (GeneID:104795610, miRBase ID: MI0029124) two designed primers are shown in Table 3.4. Primers were designed according to sequence that obtained from NCBI (Figure 3.1) by NCBI Primer-Blast. Primers with optimum melting temperature and GC% were chosen. Primers then were dissolved in nuclease free water to obtain 100 μ M (0.1 nmol/ μ l) primer stocks. Next, 10 μ M working primers were prepared by 1:10 dilution of 100 μ M primer stocks. 'High Fidelity PCR Kit (NEB E0553S)' was used for amplification of the MIR gene. Since the product of this PCR was going to be used for sequencing, the total volume of PCR solution was adjusted to 50 μ l. PCR components, their concentrations and volume have taken are shown in Table 3.5. First, a master mix containing nuclease-free water, 5X Phusion® HF Buffer, dNTP, Phusion® High Fidelity Polymerase, and forward and reverse primers was prepared and vortexed. 2 μ l of DNA samples were placed in each PCR tube and then 48 μ l of the master mix was added to the PCR tube. The conditions of the PCR reaction were determined, T_m was adjusted to 48°C, and the reaction was carried out according to the PCR conditions given in Table 3.6.

```

2629531 GGGGACAGGGCGAAACCACCTTGGTATAAGGGAGTTCATTCGAACCTCTTTTGAATACTATTATATATTAACAATAGTTTATGTATAT
2629441 ATAGTAGATATCGGATTCTCTTAGCTATTTCATGTGTCTAACTATATAAATTTGAATCTCCTTATTGAAAATTTCTGACTCCACCACCTGA
2629351 TCGGGGATATGTTTAGGTACCCACATAGCACTCACAAAATAAGAAAGTAGGCATTATATGTGGGGAAAATGAACCTACTTCCCTTAG
2629261 TCCCCCTATAAAGCTGGTTAAATTTACACAAATAAAATTTATATATTGCTAGTGTGGTCCATTCAACACATTAATTGTTTTCATCTCT
2629171 ATATCTATATGACCTATGTCTTTTTTTAACTCTTCTCTCTCACAAATCAAATATTTCTCAAATTTCTCTCCTTTTTTTTAAAAAA
2629081 AAATTGTTCAATTTTTTCTTATATTAATCTTTAAGAATTGACAAGTTTTACGCTAGCTGTGATCGATGACGATGACCAAGCTCCTAGTC
2628991 ATGCTTTTCCACAGCTTTCTTGAACCTCTTCTTGTCTAAATTTTGATCTCTAAATTTGATAATTTTGAGATGAGATTGAAGCTATGAAAGT
2628901 CCAAGAAAGCTGTGGGAAAAGATGGCAAGCTTTGGACTTATTTTTTTTTTACATTTTCTTCTATGGAGTACACTTGAAGATAATCA
2628811 AGCCAAATTTTCATGGTAAATCTTTTAAATTTTATAATATATAGTTTTTATTGTTTATTAAATTTACTTTTAAATTGTCATGAGTT
2628721 AGATAATTCAATTACTTAGCATACTTATTATTGTGAATTCTCTTAATTAATTACCTATTGTACACCTTTATATAGTGAATTATGGTTTTT
2628631 AAGACTTGCTGAAATTAGTTTTGAAAAATCAGCTAGAGCATATATCTTTTCGCTCGAAAATTACATTTAATAATATATGTCGGTTTAAAA
2628541 TTATATATAGAGAGTAAATGTTAGTTTTATGGGTAAAATTTCTACTTCTGTCAC TAGGAATTAATTGTAATAAATATATTTTACTGA

```

Figure 3.1: MIR gene of tomato miR396b. Green sequence is the MIR gene of miR396b.

Table 3.4: Designed primers for the amplification MIR gene.

Primers	Primer Sequences (5'-3')	Primer Lengths
miR396b F1	TTTACGCTAGCTGTTGATCG	20 bp
miR396b R1	AGTGTACTCCATAGGAAGGAA	21 bp

Table 3.5: Designed primers for the amplification MIR gene.

PZR Components	Added volumes	Final concentrations
Phusion® High Fidelity Polymerase (2U/μl)	0,5 μl	0,02 U/μl
5X Phusion® HF Tampon	10 μl	1X
10 μM Forward Primer	2,5 μl	0,5 μM
10 μM Reverse Primer	2,5 μl	0,5 μM
10 mM dNTPs	4 μl	200 μM
Template DNA (100 ng)	2 μl	4 ng/μl
Water	28,5 μl	
Total	50 μl	

Table 3.6: PCR conditions for MIR gene amplification.

Temperature	Time	Cycle
98 °C	30 seconds	1
98 °C	10 seconds	32
48 °C	60 seconds	
72 °C	30 seconds	
72 °C	5 minutes	1

3.3.2. Utilization of CRISPR/Cas9 systems

3.3.2.1. Designing

After the verification of MIR gene in cv. Alyanak tomato plants, designing process was started. The sgRNA design for the MIR gene was performed using the CRISPOR-TEFOR program. Many criteria were considered for sgRNA design and basically, three steps were followed for submission of the target area, i) entering target sequence, ii) selection of genome and iii) selection of suitable Cas protein (figure 3.2). In figure 3.3, the program predecessors are shown by giving the interface of the CRISPOR-TEFOR program.

The screenshot displays the CRISPOR-TEFOR web interface with three main steps:

- Step 1:** Planning a lentiviral gene knockout screen? Use [CRISPOR Batch](#). It includes a text input for 'Sequence name (optional):', a text input for 'Enter a single genomic sequence, < 2300 bp, typically an exon', and a 'Clear Box - Reset to default' link. Below the input is a sample DNA sequence: `CTTGATGATCTTCAAAGTGTACTCCATAGGAAGGAAATGTAAAAAATAAGTCCAAAGCTTCCCATCTTTTCCACAGCTTTCTTGAGCTTCTAGCTTCAAACTCATCTCAAAATTCATTTAGAGATCAAAATTGACAGAGAGAGTTCAAGAAAGCTGTGGAAAGCATAGGAGCTTGGTATGCTCATCATCAACAGCTAGCTAAAGCTGTCAATCTTAAGATTAAAT`. A note at the bottom states: 'Text case is preserved, e.g. you can mark ATGs with lowercase. Instead of a sequence, you can paste a chromosome range, e.g. chr1:11,130,540-11,130,751'.
- Step 2:** Select a genome. It features a dropdown menu currently set to 'Solanum lycopersicum - Tomato - Solgenomics.net SL3.0 (TAG3.0)'. A note below says: 'We have 794 genomes, but not yours? Search [NCBI assembly](#) and send a GCF_ID to [CRISPOR support](#).'.
- Step 3:** Select a Protospacer Adjacent Motif (PAM). It includes a dropdown menu set to '20bp-NGG - Sp Cas9, SpCas9-Hifi, eSpCas9 1.1'. A note says: 'See [notes on enzymes](#) in the manual.' Below the dropdown is an orange 'SUBMIT' button.

Figure 3.2: CRISPOR-TEFOR submission steps of target sequences.

Position/ Strand	Guide Sequence + PAM + Restriction Enzymes ☐ Only G- ☐ Only GG- ☐ Only A-	MIT Specificity Score	CFD Spec. score	Predicted Efficiency Doench '16 Mor.-Matebs	Outcome Out-of-Frame Lindel	Off-targets for 0-1-2-3-4 mismatches + next to PAM
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Figure 3.3: CRISPOR-TEFOR program predecessors.

The criteria that should be considered for sgRNA design are as follows:

- The specificity scores (MIT and CFD) should be high because the higher the specificity score, the fewer off-target regions in the genome.
- The GC content should be between 40% and 70%.
- Specific sequences that do not contain 4 or more T nucleotide sequences should be selected (figure 3.4).
- The location of the target directory should be between the beginning and middle of the entire sequence.
- Regions near the 5' end of the sequence should have an appropriate site for restriction enzymes.
- Self-complementary should be prevented.

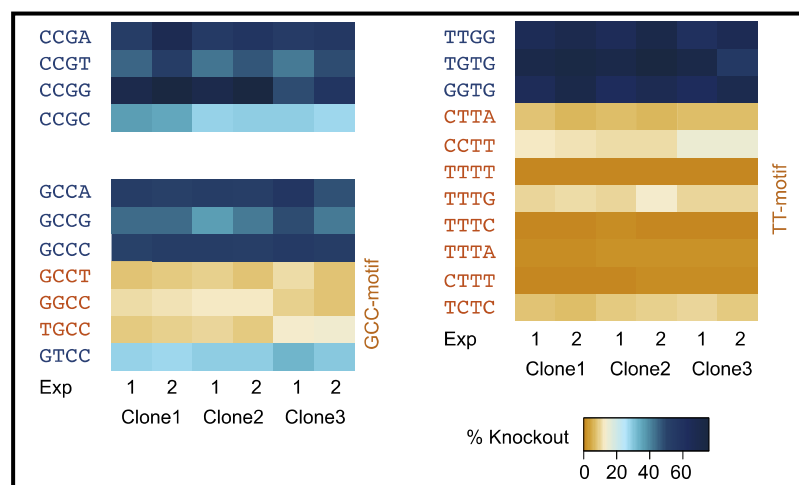


Figure 3.4: sgRNA motives and their effects on knockout rates. Higher TT motives lower the efficiency of CRISPR knockout systems.

Guide sequences were provided using CRISPOR-Tefor Software according to the sequence of the MIR gene, from highest to lowest according to the specificity score. In the selection of the guide RNA, the first column indicates the position of the guide RNA. The second column gives the guide RNA sequence with a PAM string. In addition, it offers users the connection between cloning and PCR primers. Also, targets with low GC content (<20%) and guidelines with high GC content (>80%) were marked. Various studies have reported that both conditions lead to low efficiency of guide RNA targeting. The third column gives the specificity score. The higher the score, the lower the off-target binding rate. Since no off-target effects are desired, the highest-scoring guide sequences should be chosen. This scoring ranges from 0 to 100 and the best score is 100. The fourth column shows the productivity scores. The efficiency score is an estimate of how well this target can be cut by the RNA guide sequence. It ranges from 0-100, with 100 being the best. The sixth column is an estimate of the probability that a grid will result in out-of-frame deletions. This is a scoring system that applies when gene knockouts are made with a single guide. The last column gives the off-target mismatch scores. This is a summary of the whole genome search for sequences similar to the guide target sequence. The guide sequences that give the lowest number of mismatches should be chosen. Figure 3.5 shows predicted guide RNAs for targeting MIR gene which are ranked according to specificity scores in the CRISPOR-Tefor sgRNA Design Software.

The predicted guide sequences for PAMs supplied by the CRISPOR-Tefor sgRNA Design Software were selected considering all the above-mentioned guidelines. In this study 193/Fw and 172/Fw were selected.

Position/ Strand	Guide Sequence + PAM + Restriction Enzymes Only G- Only GG- Only A-	MIT Specificity Score	CFD Spec. score	Predicted Efficiency Doench '11 Mor-Mateu	Outcome Out-of-Frame Lindel	Off-targets for 0-1-2-3-4 mismatches + next to PAM
32 / fw	TCAAAGTGTACTCCATAGGA AGG Enzymes: BstI Cloning / PCR primers	98	97	49	33	63 78 0-0-0-2-21 0-0-0-0-0 23 off-targets
187 / fw	AGCTGTGGAAAGCATGACT AGG Enzymes: MaeI Cloning / PCR primers	98	99	63	39	37 74 0-0-0-1-12 0-0-0-0-0 13 off-targets
78 / rev	ATGAAAGTCCAGAAAGCTG TGG Enzymes: AluBI, XcmI Cloning / PCR primers	95	94	62	30	64 76 0-0-0-1-35 0-0-0-0-0 36 off-targets
58 / rev	TGGGAAAGATGGCAAGCTT TGG ⚠ inefficient Enzymes: AluBI, HindIII, BstC8I Cloning / PCR primers	93	97	43	53	73 83 0-0-0-2-49 0-0-0-0-0 51 off-targets
77 / rev	TGAAAGTCCAGAAAGCTGT GGG Enzymes: AluBI, XcmI Cloning / PCR primers	91	94	61	42	62 79 0-0-0-9-41 0-0-0-0-2 50 off-targets
90 / fw	ATCTTTTCCACAGCTTCT TGG ⚠ Not with USU3 ⚠ inefficient Enzymes: XcmI Cloning / PCR primers	90	97	13	18	65 89 0-1-1-8-47 0-0-1-2-3 57 off-targets
193 / fw	GGAAGCATGCTAGGACT TGG Enzymes: PfuI Cloning / PCR primers	90	94	55	29	48 55 0-0-0-2-32 0-0-0-0-1 34 off-targets
28 / fw	ATCTTCAAAGTGTACTCCAT AGG Enzymes: BstI Cloning / PCR primers	83	90	57	55	54 69 0-0-1-7-107 0-0-0-0-0 115 off-targets
172 / fw	GAAGAAGTTCAGAAAGCTG TGG Enzymes: AluBI Cloning / PCR primers	75	86	66	41	67 76 0-0-3-11-92 0-0-2-1-0 106 off-targets

Figure 3.5: Predicted guide RNAs ranked by specificity scores in the CRISPOR-Tefor sgRNA Design Software.

3.3.2.2. Self-complementary analysis

After the selection of guide RNAs, to prevent self-complementary with scaffold sequence, RNA fold analysis was done. For this purpose, RNA Folding Form V2.3 software was used. sgRNA without PAM sequence was submitted along with the scaffold sequence to RNA Folding Form V2.3 submit window and temperature was adjusted to 22 °C [Ma et al., 2015] (Figure 3.6).

For each guide RNA, self-complementary analysis was done (Figure 3.7). It is important to avoid the usage of sgRNAs that has more 6 bonds in a row with scaffold sequence. It is possible to obtain multiple structures and it is important to check and compare all the shown structures. Beside amount of the bonds, motives of the structures are also important factors that should be considered.

RNA Folding Form V2.3

[DNA mfold server](#). [Quikfold](#). Fold many short RNA or DNA sequences at once.

Enter sequence name:

Enter the sequence to be folded in the box below. All non-alphabet characters will be removed.
FASTA format may be used.

```
GAAGAAGTTCAGAAAGCTGGTTTATAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT
```

Figure 3.6: RNA fold sequence submitting together with sgRNA and scaffold sequences.

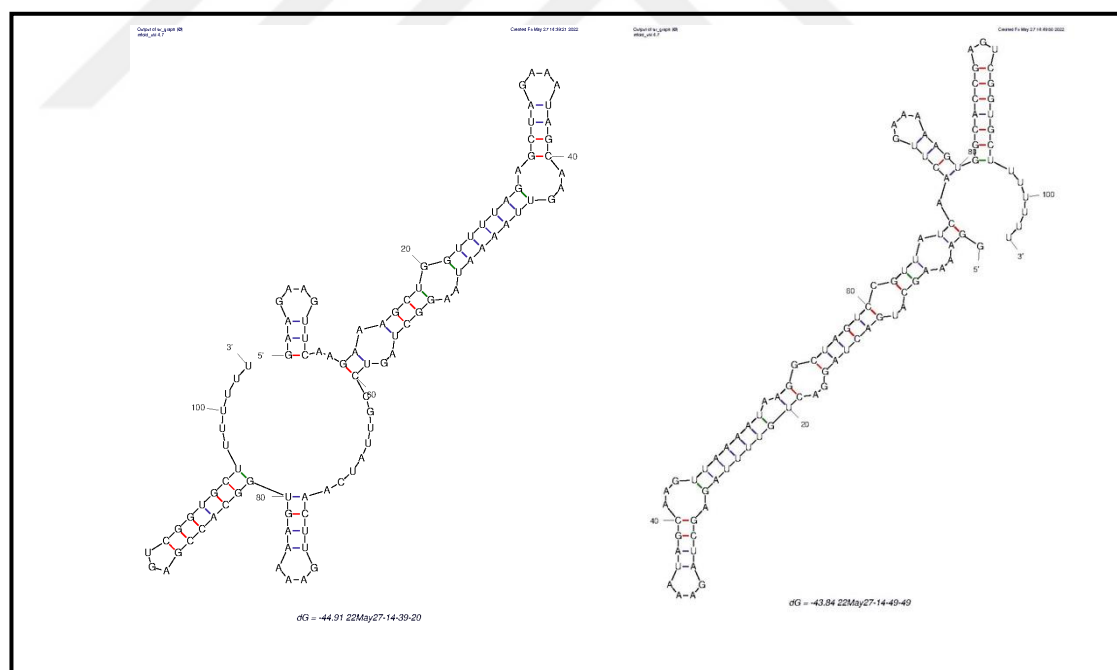


Figure 3.7: Secondary structures of guide RNAs, 172/fw on the left and 193/fw on the right.

3.3.3. Plasmid construction

The plasmid construction protocol consists of the transfer of sgRNA expression cassettes into the pYLCRISPR/Cas9 binary plasmid by the Golden Gate Assembly cloning.

3.3.3.1 Adaptor Primer Designing

The adaptor sgRNA primers given in Table 3.7 were designed by adding 5'-ATTG-3' (for the U6 promoter) to the 5' end of the designed and obtained sgRNA sequences. 100 μ M stock primers were prepared by adding the calculated amount of ddH₂O to the lyophilized primers. Working primers were obtained by diluting the stock primers 1/10 with H₂O (nuclease-free). Both stock and working primers were stored at -20 °C. For the adaptor ligation, the reaction conditions given in Table 3.9 were applied with the components given in Table 3.8 (Figure 3.8A).

Table 3.7: Adaptor Primers sequences and lengths.

Primer		Primer Sequences (5'-3')	Primer Lengths
U6 mir396b- 193	Forward Primer	ATTGGAAAAGCTGACTAGGACT	22 bp
	Reverse Primer	AAACCAGCTTTCTTGAATTCTT	23 bp
U6 mir396b- 172	Forward Primer	ATTGAATTCAAGAAAGCTG	19 bp
	Reverse Primer	AAACCAGCTTTCTTGAATTCTT	23 bp

Table 3.8: Ligation mix contents.

Components	Volume
10× CutSmart Tampon	1 µl
pYLsgRNA sgRNA cassette plasmid (20 ng/µL)	1 µl
Adaptor primer mix (1µM)	0,5 µl
10X T4 DNA ligase reaction tampon	0,5 µl
BsaI (10 U/µl)	0,15 µl
T4 DNA Ligase (20 U/µl)	0,2 µl
dH ₂ O	6,65 µl

Table 3.9: Ligation cycles and conditions.

Temperature	Durations	Cycles
37 °C	5 minutes	5
20 °C	5 minutes	

3.3.3.2 Construction

To construct CRISPR cassettes, two PCR reactions were performed for each sgRNA cassette [Ma et al., 2016]. U-F and RP (reverse primer) primers given in Table 3.10 were used in the first PCR reaction, while FP (forward primer) and gR-R primers were used in the second reaction (Figure 3.8B). The ligation product was diluted 1:10 and used as template DNA in this reaction. PCR mixing ratio for 12.5 µl reaction was prepared in microcentrifuge tubes (0.2 mL) as given in the table. The first PCR reaction was carried out with a standard thermal cycler (Bio-Rad T100) with the PCR components given in Table 3.11 under the conditions shown in Table 3.12.

Table 3.10: First PCR primer sequences.

Primers	Primer Sequences (5'→3')
U-F	CTCCGTTTTACCTGTGGAATCG
gR-R	CGGAGGAAAATTCCATCCAC-3

Table 3.11: First PCR components and their volumes.

PCR Components	Volume
5× Primestar GXL Tampon	2,5 µl
2,5 mM dNTPs mix	1 µl
Ligation product (1:10 diluted)	1 µl
FP primer (2.5 µM)	1 µl
RP primer (2.5 µM)	1 µl
Milli-Q water	5,75 µl
PrimeSTAR GXL DNA polymerase	0,25 µl
Total	12,5 µl

Table 3.12: Conditions of the First PCR.

Temperature	Duration	Cycles
95 °C	10 secs	28
60 °C	15 secs	
68 °C	20 secs	

In the second (Overlap) PCR reaction, to create the sgRNA cassettes, the amplified promoter region and the sgRNA sequence are combined. For this reaction, as shown in Table 3.13, the products obtained as a result of the first PCR reaction were

used as templates and the primers given in Table 3.14 were used. PCR was performed with the PCR reaction conditions given in Table 3.15 (figure 3.8C).

Table 3.13: Overlap PCR components and their volumes.

PCR components	Volume
2X Thermo scientific Phusion High Fidelity PCR Master Mix	25 µl
First PCR product (U6, Obtained by UF – RP primers)	1 µl
First PCR product (U6, Obtained by FP -gR-R primers)	1 µl
Forward Primers – Pps-2 (2 µM)	1 µl
Reverse Primers – Pgs-L (2 µM)	1 µl
Milli-Q Water	21 µl
Total	50 µl

Table 3.14: Primers for overlap PCR.

Primers	Primer Sequences (5'→3')
Pgs-2	AGCGTGGGTCTCGTCAGGGTCCACTCCAAGCTC
Pps-2	TTCAGAGGTCTCTGACACTGGAATCGGCAGCAAAGG
Pps-R	TTCAGAGGTCTCTACCGACTAGTATGGAATCGGCAGCAAAGG
Pps-L	AGCGTGGGTCTCGCTCGACGCGTATCCACTCCAAGCTC

Table 3.15: Overlap PCR conditions.

Temperature	Duration	Cycles
98 °C	30 secs	1
98 °C	10 secs	22
58 °C	15 secs	
72 °C	35 secs	

After the PCR reaction, 10 μ l of PCR product was run on 2% agarose gel electrophoresis and the result was checked. PCR products were stored at -20 $^{\circ}$ C.

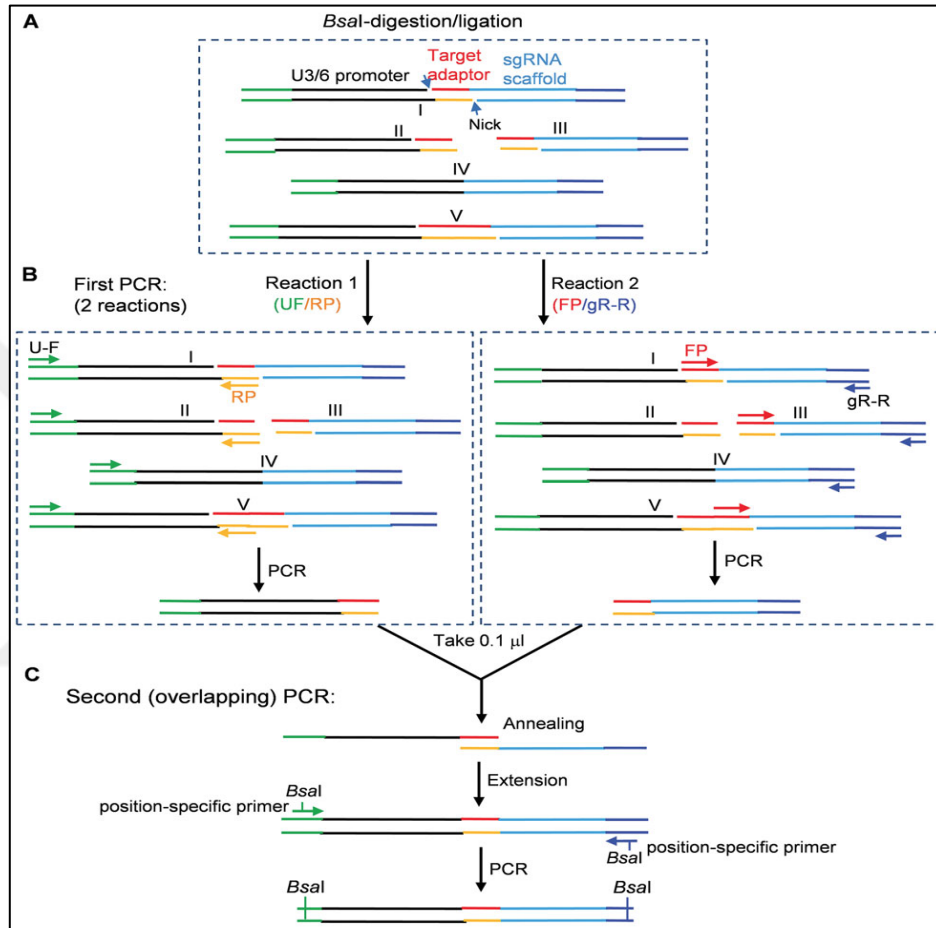


Figure 3.8: Summary of guide RNA cassettes construction. A) ligation of target adaptors, B) amplification of ligation products to prepare templates for overlap PCR, C) Second (Overlap) PCR to construct sgRNA cassettes.

DNA Clean & ConcentratorTM-5 Kit (Zymo Research, catalogue no: D4013) was used to purify the obtained PCR products. DNA binding buffer and PCR product were added in a 5:1 ratio (binding buffer: sample) in a 1.5 mL microcentrifuge tube. 90 μ l of DNA binding buffer was added to 18 μ l of PCR product. It was mixed by vortex for a short time. The resulting mixture of buffer and PCR product was transferred to the column on the collection tube and centrifuged at 11000 x g for 30 seconds. 200 μ l of DNA washing buffer was added to it. It was centrifuged again at 11000 x g for 30 seconds. The washing

process was repeated 2 times. The column was transferred to another tube and 10 µl of water was added to it. It was incubated for 1 minute at room temperature and then centrifuged at 11000 g for 30 seconds. The concentration of the purified PCR products was measured using the NanoDrop Lite Spectrophotometer.

3.3.3.3 Golden Gate Assembly

U6-sgRNA expression cassettes were prepared after the second PCR reaction and PCR products were purified by purification steps. Then, the U6-sgRNA cassettes were simultaneously transferred into the pYLCRISPR/Cas9P35S-N [Ma et al., 2015] vector by Golden Gate Cloning method. The PCR reaction mixture was vortexed and carried out with the reaction conditions given in Table 3.16 using the PCR components shown in Table 3.17. Then the products were stored at -20 °C. In addition, the full sequence map of the pYLCRISPR/Cas9P35S-N vector together with insert materials and position are shown in Figure 3.9.

Table 3.16: Reaction conditions.

Temperature	Duration	Cycle
37 °C	10 mins	3
10 °C	5 mins	
20 °C	5 mins	
98 °C	3 mins	10
10 °C	5 mins	
20 °C	5 mins	

Table 3.17: Reaction Components and their volumes.

Reaction components	Volume
10X CutSmart Tampon	1,5 µl
PYLCRISPR/Cas9 plasmid (100 ng)	1 µl
U6-sgRNA PCR product (10 ng)	1 µl

Table 3.17: Continued.

10 X T4 DNA Reaction Tampon	0,5 µl
<i>Bsa</i> I (20 U/µl)	0,5 µl
T4 DNA Lega (40 U/µl)	0,5 µl
Milli-Q Water	4,5 µl
Total	15 µl

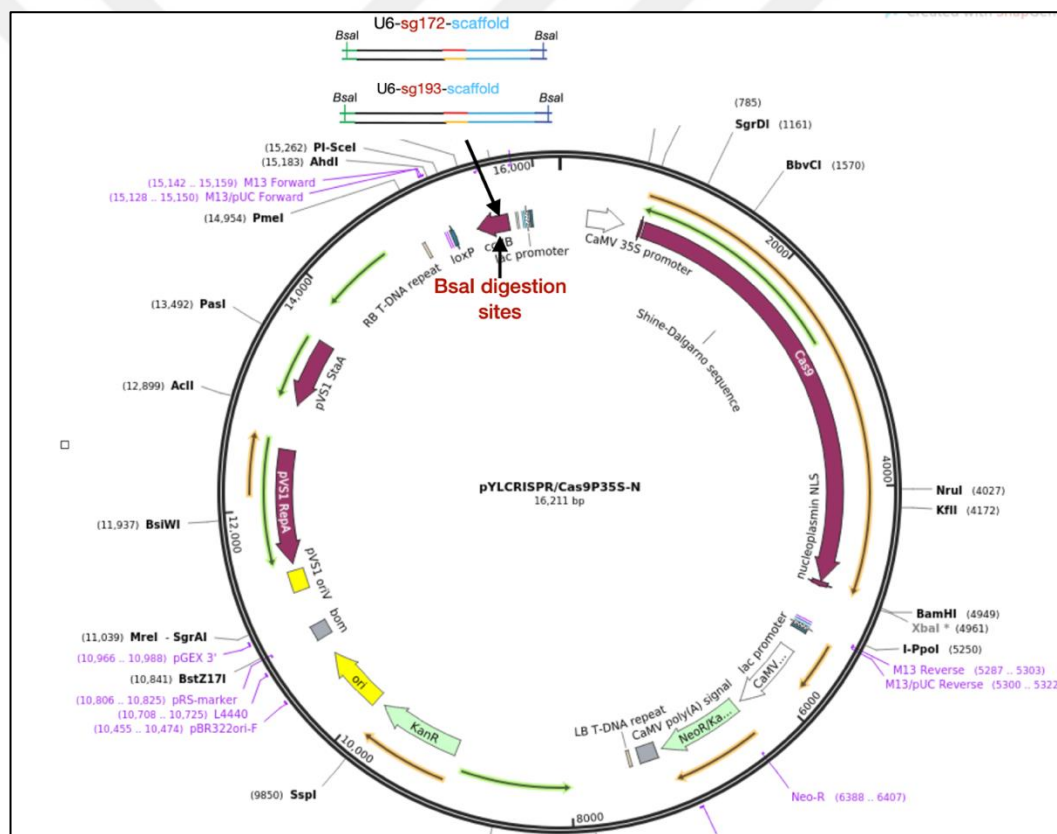


Figure 3.9: Full sequence map of the pYLCRISPR/Cas9P35S-N vector together with sgRNA cassettes and their insert position.

3.4. *Escherichia coli* Transformation

Competent cells stored at -80°C were taken and mixed gently after adding 4 µl of ligation product to 100 µl of competent cells in a 1.5 ml Eppendorf tube. It was incubated on ice for 5 minutes. Then, immediately after incubation in a water bath at 42 °C for 1 minute, the mixture was placed on ice and incubated again on ice for 5 minutes. 1 mL of liquid LB medium was added to the mixture. Mixture was incubated and shaken at 200 rpm for 1 hour at 37 °C. After incubation, the mixture was centrifuged at 800 x g for 5 minutes and the supernatant was discarded. *E. coli* cells were resuspended with LB medium remaining in the Eppendorf tube. *E. coli* cells were planted on LB agar plates containing 50 mg/L kanamycin. It was incubated at 37 °C for 24 hours. It was selected from the colonies formed after incubation and inoculated into liquid LB medium containing 50 mg/L kanamycin. Bacteria were grown by shaking at 37 °C for 16 hours at 200 rpm. After the bacteria proliferated, 500 µl of bacterial culture and 500 µl of 50% glycerol were taken into a 1.5 ml Eppendorf tube to obtain stocks from the bacterial culture. It was mixed by turning it upside down and immediately after that, the bacterial stock was moved to -80 °C.

3.4.1. Luria-Bertani (LB) Medium Preparation

LB medium was used to grow *E. coli* and *Agrobacterium*. The contents of liquid and semi-solid LB medium are given in Table 3.18.

Table 3.18: LB Components.

Components	Concentrations
Tryptone	10 g/L
NaCl	10 g/L
Yeast Extract	5 g/L

25 g of LB (Miller, Difco) medium containing 10 g of tryptone, 10 g of sodium chloride (NaCl) and 5 g of yeast extract was weighed on a precision balance and approximately 1 litre of distilled water was added to it. LB chemicals were mixed in the beaker until dissolved in water. After the medium was completely dissolved, the pH was adjusted to 7.0 ± 0.2 using 1 M HCl. The volume was made up to 1L and the medium was bottled. It was sterilized by autoclaving at 121°C for 20 minutes.

To prepare semi-solid LB, 8 grams of agar in addition to the above chemicals was weighed and placed in the bottle. The prepared medium was transferred to the agar bottle. It was sterilized by autoclaving at 121°C for 20 minutes. After sterilization, the media were stored at room temperature.

3.4.2. Plasmid Isolation

Zyppy Plasmid Miniprep Kit (Zymo Research, catalogue no: D4019) was used to isolate plasmids from the *E. coli* bacterial culture.

Bacterial culture was taken into a 1.5 ml Eppendorf tube and centrifuged at $11,000 \times g$ for 30 seconds. The supernatant was discarded, and the centrifuge was repeated by adding the bacterial culture again. The supernatant was discarded and 600 μL of nuclease-free water was added on top of the bacterial pellet. 100 μL of 7x lysis buffer was added to it. Mixing was done by turning the tube upside down and followed by vortex. The mixture was incubated for 2 minutes at room temperature. Then 350 μL of neutralization buffer was added. The task of this buffer is to terminate cell lysis. The lysis process is finished when the colour of the mixture turns yellow. When the colour of the mixture turned yellow, the mixture was centrifuged at $11000 \times g$ for 4 minutes. The supernatant was removed without touching the pellet and it was transferred to the Zymo-Spin™ IIN column. The column was placed in an Eppendorf tube and centrifuged at $11000 \times g$ for 15 seconds. The tube under the column was discarded and the column was placed in a new Eppendorf tube. 200 μL of Endo-Wash buffer was added to the column. It was centrifuged for 30 seconds at $11000 \times g$. 400 μL of Zyppy Wash buffer was

added to it and centrifuged at 11000 x g for 1 minute. The column was taken into a new 1.5 ml Eppendorf tube, and 45 µL of water was added directly onto the column matrix. It was incubated at room temperature for 1 minute and then centrifuged at 11000 x g for 30 seconds. Plasmid amount was measured using NanoDrop Lite Spectrophotometer.

3.5. *Agrobacterium* Transformation

Agrobacterium tumefaciens strain EHA105 was used for *Agrobacterium* transformation.

50 µL of competent *A. tumefaciens* bacteria were thawed on ice. Approximately 500 ng of plasmid DNA was added to the bacteria and mixed by tapping the tube. This mixture containing plasmid DNA and *A. tumefaciens* bacteria was thrown into liquid nitrogen and incubated for 5 minutes. Then, it was immediately taken to 37 °C, incubated again for 5 minutes and then put on ice. 2 minutes after being put on ice, 1 mL of LB broth was added. It was incubated for 2 hours at 30 °C with shaking at 200 rpm. The mixture was centrifuged at 3000 rpm for 5 minutes. The supernatant was decanted, and the bacterial pellet was resuspended with LB medium remaining in the tube. The mixture was spread into semi-solid LB medium plates containing the antibiotics gentamicin (30 µg/mL) and kanamycin (100 µg/mL). Petri dishes were incubated at 28 °C for 2 days. After 2 days, they were selected from bacteria colonies on the petri dish and inoculated again into liquid LB medium containing antibiotics. It was grown at 30 °C for 1 day with shaking at 200 rpm. When the bacterial density (OD600) reached 1, the bacteria stock was made by adding 30% glycerol at the same rate, mixed by turning it upside down and moved to -80 fridge °C, immediately.

3.6. Plant Tissue Culture

3.6.1. Seed Sterilization

Plant tissue culture studies were based on the protocol developed by van Eck [van Eck, Keen and Tjahjadi, 2019]. Firstly, sterilization solution was prepared by adding one drop of Tween 20 to 20% bleach solution. After the solution was prepared, 0.8-1 g of seeds were collected and immersed in sterilization solution (25 ml). Then, solution was agitated for 20 minutes at 250 RPM. After agitation, seeds were washed three times with deionized water.

3.6.2. Preparation of Media

During the plant tissue culture studies, seed germination medium, liquid co-culture medium, preculture/co-culture medium (2Z preculture), primary regeneration medium (2Z), secondary regeneration medium (1Z), and rooting medium (RM) were subsequently used, and their components are shown in; Table 3.19, Table 3.20, Table 3.21, Table 3.22, Table 3.23, and Table 3.24, respectively.

Table 3.19: Seed germination medium components.

Components	Concentrations
Murashige and Skoog (MS) salts	2.15 g/L
Myoinositol	100 mg/L
Thiamine HCl (1 mg/mL)	2 mL/L
Pyridoxine HCl (0.5 mg/mL)	1 mL/L
Sucrose	10 g/L
Agar	8 g/L

Table 3.20: Liquid co-culture medium components.

Components	Concentrations
MS salts	4.3 g/L
Myoinositol	100 mg/L
Thiamine HCl (1 mg/mL)	0.4 mL/L
Sucrose	20 g/L
pH	5.8

Table 3.21: Preculture/co-culture medium (2Z preculture) components.

Components	Concentrations
MS salts	4.3 g/L
Myoinositol	100 mg/L
Nitsch Vitamin*	1 mL/L
Sucrose	20 g/L
Trans-zeatin (1 mg/mL)**	2 mL/L
pH	6.0
Agar	8 g/L

* Nitsch Vitamin solution is prepared by dissolving 0.5g nicotinic acid, 0.025g Pyridoxine HCl, 0.025g folic acid, 0.1g glycine, 0.002 g d-biotin in 50 mL of water.

** Added after autoclave

Table 3.22: Primary regeneration medium (2Z) components.

Components	Concentrations
MS salts	4.3 g/L
Myoinositol	100 mg/L
Nitsch Vitamin	1 mL/L
Sucrose	20 g/L
Trans-zeatin (1 mg/mL)**	2 mL/L
Timentin (350 mg/mL)**	1 mL/L
Kanamycin (50 mg/mL)**	2 mL/L
pH	6.0
Agar	8 g/L

** Added after autoclave

Table 3.23: Secondary regeneration medium (1Z) components.

Components	Concentrations
MS salts	4.3 g/L
Myoinositol	100 mg/L
Nitsch Vitamin	1 mL/L
Sucrose	20 g/L
Trans-zeatin (1 mg/mL)**	1 mL/L
Timentin (350 mg/mL)**	1 mL/L
Kanamycin (50 mg/mL)**	2 mL/L
pH	6.0
Agar	8 g/L

** Added after autoclave

Table 3.24: Rooting medium components.

Components	Concentrations
MS salts	4.3 g/L
IAA (100 mg/mL)	100 µL/L
Nitsch Vitamin	1 mL/L
Sucrose	20 g/L
Timentin (350 mg/mL)**	1 mL/L
Kanamycin (50 mg/mL)**	2 mL/L
pH	6.0
Agar	8 g/L

** Added after autoclave

3.6.3. Utilization of Plant Tissue Culture

3.6.3.1. Preparation of Explants

After the sterilization of the seeds, 50-60 mL of seed germination medium was dispensed to each magenta boxes. 30 sterilized seeds were planted to each box (figure 3.9). After the plantation, waited 7 days to obtain proper tomato cotyledons. 7 days old cotyledons were excised from petiole and tips were removed. Remaining part of cotyledons were cut into 0.5-1 cm sections. Explants were placed on 2Z preculture medium as adaxial side down. Each plate had 50 explants. Filter papers were moistened and put on the each 2Z preculture mediums. Plates were sealed with parafilm and cultured for a day at 23 °C in growth chamber.

3.6.3.2. Preparation of *Agrobacterium* for tomato transformation

Since growth of *Agrobacterium* takes up to two days on solid medium, previously transformed and stocked *Agrobacterium* was streaked onto selective LB bacterial medium. Streaked plates were incubated until the formation of proper colonies at 28 °C. For colonies were selected and moved to 50 mL LB liquid medium which contained selective antibiotics, 100 µL kanamycin and 50 µL gentamycin. Prepared liquid mediums were put in a shaking incubator and cultured overnight at 28 °C and 250 rpm. After the overnight incubation, OD₆₀₀ checked and adjusted to 0.6. In the case of exceeding the 0.65, dilution was done to lower OD₆₀₀. When the optimum OD₆₀₀ was reached, liquid medium was moved to centrifuge at 8228 x g for 10 minutes at 20 °C. After the centrifuge, supernatant volume was discarded and recorded. After the removal of supernatant, 10 mL of 2% MSO were added on the pellet. Pellet resuspended with 10 mL of 2% MSO and vortexed. Added previously recorded amount of 2% MSO to adjust volume.

3.6.3.3. *Agrobacterium* mediated transformation of tomato explants

25 mL of *Agrobacterium* solution was pipetted either into magenta boxes or 50 mL tubes. For each 25 mL *Agrobacterium* solution, 2 plates of explants (approximately 100 explants) were used for transformation. Explants were incubated in *Agrobacterium* solution for 5 minutes and shaken. For controls, 2% MSO solution without *Agrobacterium* was used. After the *Agrobacterium* inoculation, explants were transferred to sterile filter paper and left to dry. When explants were dried enough, they were transferred to 2Z preculture medium adaxial side down. Plates were incubated at 19 °C, under dark conditions of two days. Then, 2Z selective medium plates were prepared and 25 explants were transferred to each 2Z selective medium plates from 2Z preculture medium, adaxial side up. Plates were incubated for 2 weeks, 16 hours photoperiod and at 25 °C. 1Z selective mediums were prepared and 15 explants were

moved to 1Z plates from 2 weeks old 2Z selective medium and 1Z plates were refreshed every 2-4 weeks. This process continued until the formation of shoots began and started to touch the lid of plates, then explants are moved to magenta boxes which contained same medium. After the formation of the initial shoots in the magenta boxes, shoots were cut and placed in RM medium containing magenta boxes. After the formation of roots, explants with sturdy shoots were moved to greenhouse.

3.6.3.4. Acclimatization

Plants were moved to Gebze Technical University, Plant Biotechnology Laboratory greenhouse for acclimatization under 25 °C and 16 hours photoperiod conditions. Firstly, they were placed into small pots and to reduce damage from the changing environment they were covered with stretch film for couple days. Then, 2-3 weeks old seedlings were moved to bigger pots and pollination was made by hands.

3.7. Detection of Transgenic Lines

3.7.1. DNA Isolation

DNA was first isolated from plants to identify transgenic lines and then mutant plants were confirmed with sequencing. DNA isolation was performed using the CTAB (cetyl trimethyl-ammonium bromide) protocol of Dooley et al.[Dooley et al., 2004], which was optimized previously in our laboratory. Firstly, plant samples were placed in 2 mL Eppendorf tubes together with 3-4 iron balls and homogenized using a homogenizer. Then, 600 µL of CTAB (2% β-mercaptoethanol) extraction buffer which kept at 65°C was added on each sample. Then, samples were vortexed and incubated at 65°C for 15 minutes. After the 15 minutes of incubation, samples were kept at room temperature for 5 minutes and 600 µL of chloroform/octanol (24:1) was added. The samples were then inverted 20-25 times and centrifuged at 13,000 rpm for 15 minutes.

The supernatants (approximately 500 µL) were taken into a new tube, 250 µL of 5 M NaCl and 750 µL of 95% EtOH were added to them and incubated at +4 for a day. The next day, centrifugation was performed at 13,000 rpm for 5 minutes, the supernatants were removed, and the pellets were washed with 1 ml of 76% ethanol. Then samples were centrifuged again at 13,000 rpm for 5 minutes and waited for drying. After complete drying, the pellets were dissolved with 30 µl of ddH₂O. Then, the amounts and purity of the samples were measured with Nanodrop. DNA samples were stored at -20 °C until use.

3.7.2. T-DNA amplification via PCR

For the verification of transgenic lines, PCR that aims to amplify T-DNA gene was planned. To obtain more reliable results, 3 primers used in 2 PCR with different combinations. DNA samples that we previously isolated from tomato leaves used as template. One of the forward primers was designed according to sequence that located at the outside of T-DNA region. Other forward primer was designed according to sequence that located at inside of T-DNA region. One reverse primer was used for the both PCR reactions which was located at the inside of T-DNA region of *Agrobacterium* plasmid. Primers and PCR reaction conditions are shown in Table 3.25 and Table 3.26, respectively. PCR reactions that gave band only with T-DNA inside primers are labelled as “Transgenic”.

Table 3.25: TDNA verification primers and their sequences.

Primers	Sequences
RB TDNA-F	GAAGGCGGGAAACGACAATC
pYL PmeI F1	TGGACGAACGGATAAACCTT
PI-SceI R	CTATGTCTGGGTGCGGAGAAA

Table 3.26: TDNA verification PCR conditions.

Temperature	Duration	Cycles
98 °C	30 secs	1
98 °C	10 secs	30
52 °C	15 secs	
72 °C	35 secs	

3.7.3. Amplification of MIR gene in transgenic tomato plants

A new PCR with primers, components and conditions which are shown in Table 3.5, Table 3.6, and Table 3.7, respectively was designed to amplify MIR gene. Transgenic lines DNA samples were used as template. 50 µL DNA samples were moved to 1,5 ml Eppendorf tubes from PCR tubes. Samples then sent to BM Yazılım Danış. ve Lab. Sis. Ltd. Şti. with forward primer for sequential analysis.

In this thesis 160 tomato seeds were used, and tissue culture process was initiated with 250 tomato cotyledon explants.

4. RESULTS

4.1. Method Development

In this thesis, we designed two guide RNAs for the target region. One of the guide RNAs was targeting a sequence inside of the MIR gene but outside of the pre-miRNA duplex forming sequence. On the other hand, the other guide RNA was targeting the pre-miRNA duplex sequence (Figure 4.1).

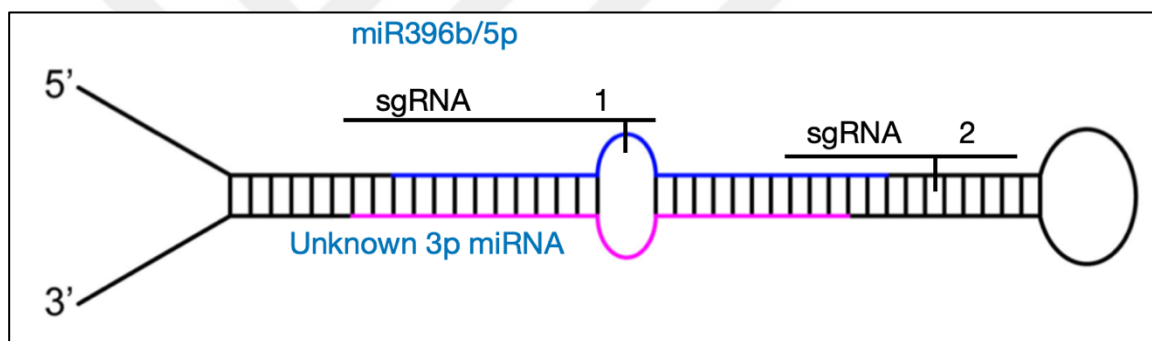


Figure 4.1: Targeting MIR gene of miR396b on both inside of the duplex forming sequence and outside of the duplex forming sequence in the MIR region.

4.2. miRNA analysis

Since we aimed to inhibit the biogenesis of miRNA, it was important to analyse possible side effects that could be caused by this inhibition. Firstly, we obtained the MIR sequence of miR396b from miRbase. Through this sequence, the pre-miR396b forming region and hairpin location were also underlined (Figure 4.2).



Figure 4.2: RNA sequence of miR396b MIR gene.

Although there is no miR396b-3p production from the MIR gene, we used a complementary sequence of miR396b-5p to define targets of possible miR396b-3p production. The complementary sequence was firstly used in psRNATarget to find possible targets (Figure 4.3). Then, possible target sequences were used in BLAST to find if any of those possible target sequences were matching with a coding region (Figure 4.4). Except of a few undefined protein genes, no significant match was found through BLAST runs.

miRNA Acc.	Target Acc.	Expect	UPE	Alignment	Target Description	Inhibition	Multiplicity
AAAGGGTGTGCGAAGAACCTGAA	Solyc02g069070.3.1	3.0	N/A	miRNA 23 AAGUCCAAGAAAGCUGUGGAAA 1 : : : : : : : : : : : : : : : : : : Target 47 UUAAGGUUUUUUGAUACUGUU 69	pacid=41468868 locus=Solyc02g069070.3 ID=Solyc02g069070.3.1.ITAG3.2 annot-version=ITAG3.2	Cleavage	1
AAAGGGTGTGCGAAGAACCTGAA	Solyc09g011750.3.1	3.0	N/A	miRNA 23 AAGUCCAAGAAAGCUGUGGAAA 1 : : : : : : : : : : : : : : : : : : Target 2876 CUAAUGUUUUUUGGACACCUUU 2898	pacid=41459358 locus=Solyc09g011750.3 ID=Solyc09g011750.3.1.ITAG3.2 annot-version=ITAG3.2	Cleavage	1
AAAGGGTGTGCGAAGAACCTGAA	Solyc02g093190.3.1	3.0	N/A	miRNA 23 AAGUCCAAGAAAGCUGUGGAAA 1 : : : : : : : : : : : : : : : : : : Target 1214 UCUUGAUUUUUUGACACCUUU 1236	pacid=41470335 locus=Solyc02g093190.3 ID=Solyc02g093190.3.1.ITAG3.2 annot-version=ITAG3.2	Cleavage	1
AAAGGGTGTGCGAAGAACCTGAA	Solyc08g077320.2.1	3.5	N/A	miRNA 23 AAGUCCA-AGAAAGCUGUGGAAA 1 : : : : : : : : : : : : : : : : : : Target 258 UUCAGGUUUUUUUAACACCCUUU 281	pacid=41486540 locus=Solyc08g077320.2 ID=Solyc08g077320.2.1.ITAG3.2 annot-version=ITAG3.2	Translation	1
AAAGGGTGTGCGAAGAACCTGAA	Solyc12g009470.2.1	3.5	N/A	miRNA 23 AAGUCCAAGAAAGCUGUGGAAA 1 : : : : : : : : : : : : : : : : : : Target 312 AUCAGUUUUUUUUAACCCUUU 334	pacid=41480648 locus=Solyc12g009470.2 ID=Solyc12g009470.2.1.ITAG3.2 annot-version=ITAG3.2	Translation	1
AAAGGGTGTGCGAAGAACCTGAA	Solyc01g090340.2.1	3.5	N/A	miRNA 23 AAGUCCAAGAAAGCUGUGGAAA 1 : : : : : : : : : : : : : : : : : : Target 135 UUCGUAUUUUUUGAAACUUU 157	pacid=41483352 locus=Solyc01g090340.2 ID=Solyc01g090340.2.1.ITAG3.2 annot-version=ITAG3.2	Cleavage	1
AAAGGGTGTGCGAAGAACCTGAA	Solyc01g108790.2.1	3.5	N/A	miRNA 23 AAGUCCAAGAAAGCUGUGGAAA 1 : : : : : : : : : : : : : : : : : : Target 224 AUUAGCUUUUUUGAUACUUU 246	pacid=41484419 locus=Solyc01g108790.2 ID=Solyc01g108790.2.1.ITAG3.2 annot-version=ITAG3.2	Cleavage	1

Figure 4.3: psRNATarget results. Beside possible targets of submitted miRNA, their mode of action is also shown either as Cleavage or Translation.

BLAST results have proven that inhibition of miR396b biogenesis will not affect the production of unknown miRNA that targets any coding region or exon. Thus, we used the MIR gene of miR936b as the target sequence in CRISPOR-Tefor web software to find optimum guide RNAs according to our approach.

Descriptions	Graphic Summary	Alignments	Taxonomy						
Sequences producing significant alignments									
Download									
Select columns									
Show									
100									
☒ select all 4 sequences selected									
GenBank									
Graphics									
Distance tree of results									
MSA Viewer									
	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	PREDICTED: Solanum lycopersicum uncharacterized protein C12B10.15c (LOC101254055), m...	Solanum lycopersicum	1448	1448	100%	0.0	100.00%	831	XM_004233179.4
☒	Solanum lycopersicum cultivar I-3 chromosome 2	Solanum lycopersicum	492	1468	100%	1e-136	99.63%	55344765	CP023758.1
☒	Solanum lycopersicum genome assembly, chromosome: 2	Solanum lycopersicum	492	1468	100%	1e-136	99.63%	54735237	OU640345.1
☒	Solanum lycopersicum chromosome.ch02, complete genome	Solanum lycopersicum	486	1452	100%	6e-135	99.26%	49921527	HG975514.1

Figure 4.4: BLAST results of predicted miRNA target sequences.

4.3. Designing of guide RNAs to target tomato miR396b

Two sgRNAs targeting MIR gene of tomato miR396b of the tomato (*Solanum lycopersicum*) were designed using the online program CRISPOR (<http://crispor.tefor.net/>). While selecting the guide RNAs, criteria such as having high GC content (40-70%), low off-target regions and TT-motives were considered during the selection. Two guide RNA sequences which we named miR172 and miR193, were selected (Figure 4.5).

Position/ Strand	Guide Sequence + PAM + Restriction Enzymes ☐ Only G- ☐ Only GG- ☐ Only A- ☒	MIT Specificity Score	CFD Spec. score	Predicted Efficiency Show all scores	Out-of-Frame Lindel	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score ☐ exons only ☐ SL3.0ch07 only
193 / fw	GGAAAAGCATGACTAGGACT TGG Enzymes: <i>PfI</i> Cloning / PCR primers	90	94	55	29	48 55 0-0-0-2-32 0-0-0-0-1 34 off-targets	3:intergenic:mRNA:Solyc12g019930.1.1-mRNA:Solyc12g019940.1.1 4:intergenic:mRNA:Solyc12g020000.2.1-mRNA:Solyc12g020020.1.1 4:intergenic:mRNA:Solyc12g020020.1.1-mRNA:Solyc12g020030.1.1 show all...
172 / fw	GAAGAAGTCAAGAAAGCTG TGG Enzymes: <i>AluI</i> Cloning / PCR primers	75	86	66	41	67 76 0-0-3-11-92 0-0-2-1-0 106 off-targets	2:intergenic:mRNA:Solyc12g009630.2.1-mRNA:Solyc12g009640.1.1 4:intergenic:mRNA:Solyc03g044200.3.1-mRNA:Solyc03g044210.3.1 4:intergenic:mRNA:Solyc05g041170.2.1-mRNA:Solyc05g041200.3.1 show all...

Figure 4.5: Selected guide RNAs, their sequences and scores.

During the selection, location of the target region was the priority. Since one of the guides RNAs should have targeted duplex forming region, 172/fw (Figure 4.7) was selected even though it has relatively low MIT specify score in CRISPOR software. Other guide RNA was selected by its distance to target of first guide RNA. Since we aimed to introduce 20 bp deletion on the target gene, we picked 193/fw for the second guide RNA as it has the highest score. After the selection of guide RNAs through CRISPOR-Tefor, to prevent self-complementary, together with guide RNA scaffold sequences were submitted to RNA Folding Form V2.3 software. Results showed that the self-complementary chance of guide RNA 172/fw was relatively low since it had few bonds with scaffold sequence on the RNA level. On the other hand, guide RNA193/fw posed some risk in the case of self-complementary since it had 6 bonds in a row with the scaffold.

4.4. Plasmid Construction

Adapter sgRNA primers were constructed by adding the 5'-ATTG-3' sequence (for the U6-1 promoter) to the 5' end of the designed guide sequences. The sequences of the adapter primers are given in Table 3.7. As a result of the reaction established for the preparation of the adapter primers, the high temperature used broke all the hydrogen bonds and new bonds were allowed to form between the sequences with the cooling process at room temperature. For adapter ligation, pYLsgRNA sgRNA cassette, adapter primers mixture and T4 DNA ligase were used. 5 min at 37 °C for 5 cycles followed by 5 min incubation at 20 °C were done for ligation reaction. In Figure 3.8, adapter ligation, first and second PCR steps and vector generation steps are shown. The expected size of the product of PCR reaction 1 (in which primers UF and RP are used) is 360 bp. In addition, the expected size of the PCR reaction 2 product (using FP and gR-R primers) is 135 bp. The expected band sizes were obtained, and the first PCR reaction was successfully completed.

In the second PCR reaction, the first PCR products were used as template DNA. Forward primer Pps-2 and reverse primer Pgs-L were used for the U6 sgRNA cassette. In this way, cassettes were formed by combining the promoter region and the sgRNA sequence. The expected band size is 490 bp. At the end of PCR, the reaction products were run on a 2% agarose gel at 110 volts for 40 minutes. The Agarose gel image is given in Figure 4.6.

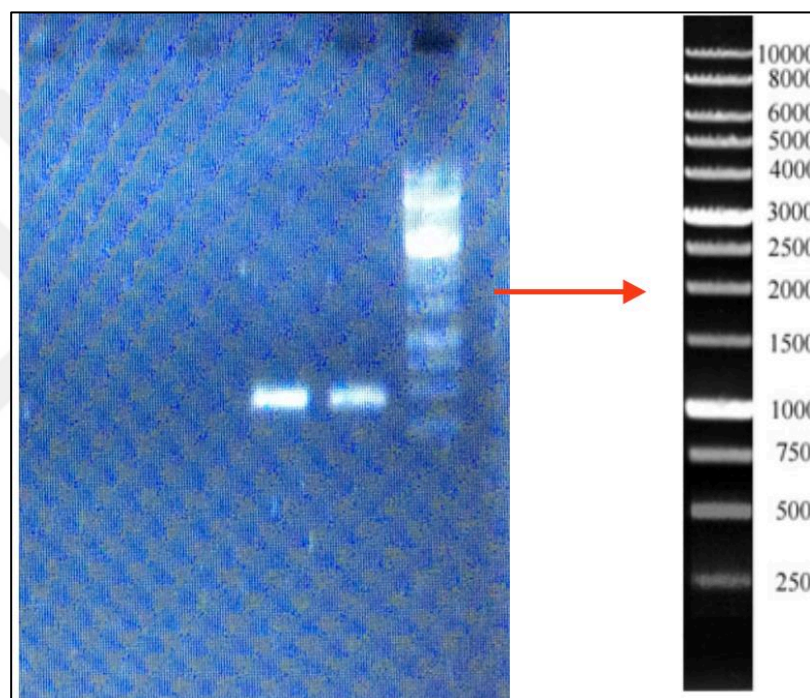


Figure 4.6: Agarose gel image of CRISPR cassettes. Marker: Intron 1 Kb ladder

After the confirmation of CRISPR cassettes via agarose gel electrophoresis, cassettes were inserted into the pYLCRISPR/Cas9 plasmid by Golden Gate assembly. Transformation of pYLCRISPR/Cas9 plasmid generated by Golden Gate Cloning into *E.coli* was carried out. Then, colony PCR was performed using UF/172/Rw and 172/Fw /gR-R primers from the colonies grown on the selective medium and were run and visualized on an agarose gel. (Figure 4.7). Then, plasmid isolation was made from the 1st and 2nd colonies. For control purposes, digestion was performed with the BamHI enzyme and visualized on the gel. A 20% glycerol stock was then made from the colonies and stored

at -80. Finally, the generated vector was transformed into *Agrobacterium tumefaciens* and grown in selective media (Figure 4.8).

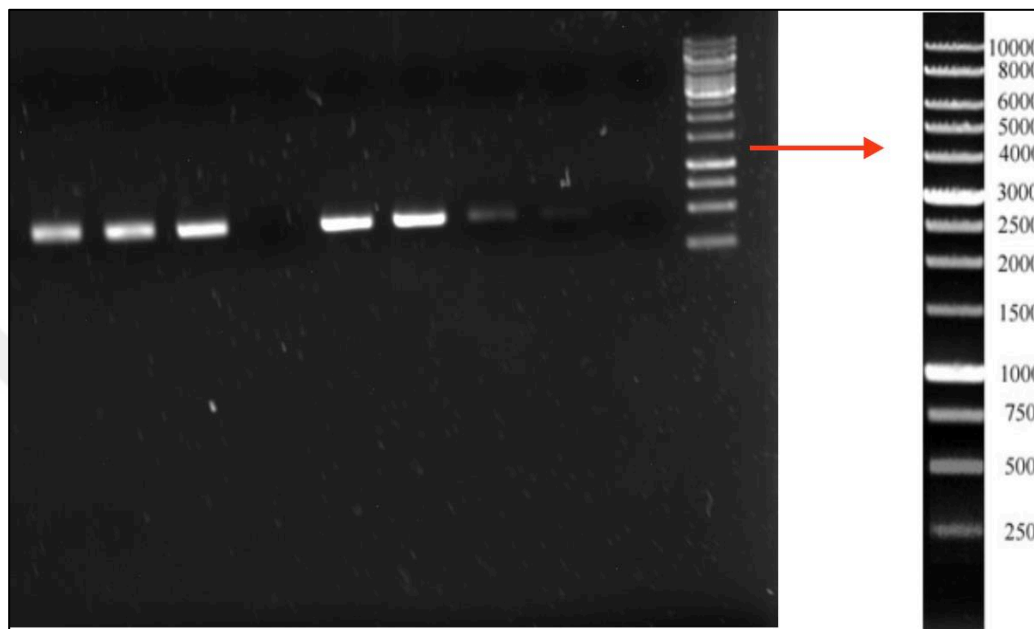


Figure 4.7: Gel image of *E. Coli* colony PCR. First 2 colonies were picked for *Agrobacterium* transformation. Marker: Intron 1 Kb Ladder.

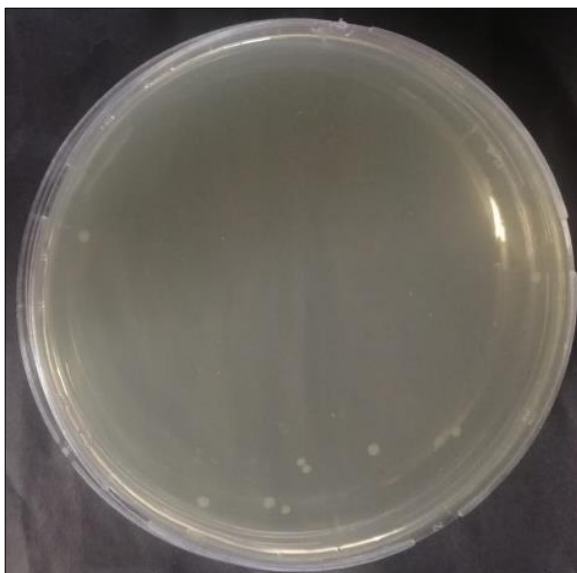


Figure 4.8: *A. tumefaciens* colonies on LB medium containing gentamicin and kanamycin.

4.5. Generation of Transgenic Tomato Lines from Cotyledons

800 mg of seeds were planted to germination medium (Figure 4.9A). With the appearance of cotyledons, explants were prepared by cutting cotyledons into 1 cm pieces. Each explant was planted in pre-culture medium (Figure 4.9B) and after the pre-culture process explants were transformed with *Agrobacterium* (Figure 4.9C). When shoot or leave formation started and touched the lid of plate, explants were moved to regeneration medium (Figure 4.9D). Explants were cultured in regeneration medium for 2-6 weeks (Figure 4.9E). After the regeneration medium, newly formed shoots were cut from nodes and moved to rooting medium (Figure 4.9F). With the formation of root and elongated shoots, plants were moved to green house (Figure 4.9G).

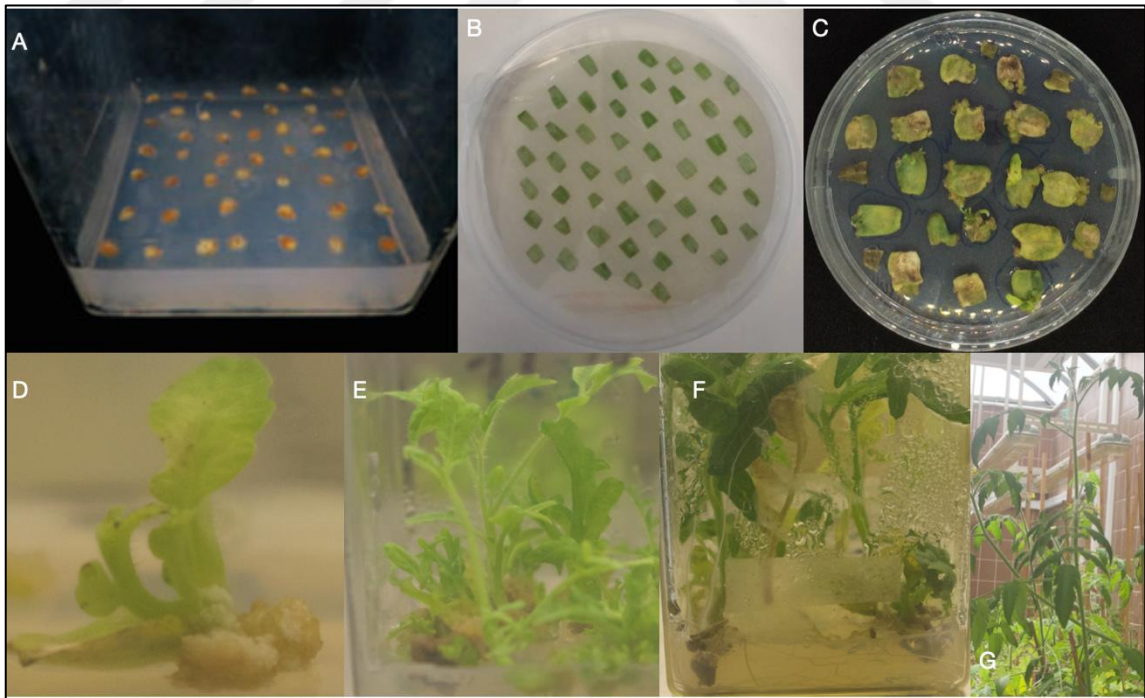


Figure 4.9: Tissue culture steps; A) Plantation of seeds, B) Pre-culture, C) Callus formation and regeneration, D) Formation of shoots and leaves, E) Elongation of shoot, F) Rooting medium, G) Growing plants in green house.

4.5.1. Preparation of Explants and Regeneration Ratio

Without controls, 250 explants were prepared for this study and tissue culture studies were initiated. Approximately 50 of them were lost as a result of problems with the growth chamber. Thereby, tissue culture studies were continued with 9 Petri dishes (Figure 4.10).

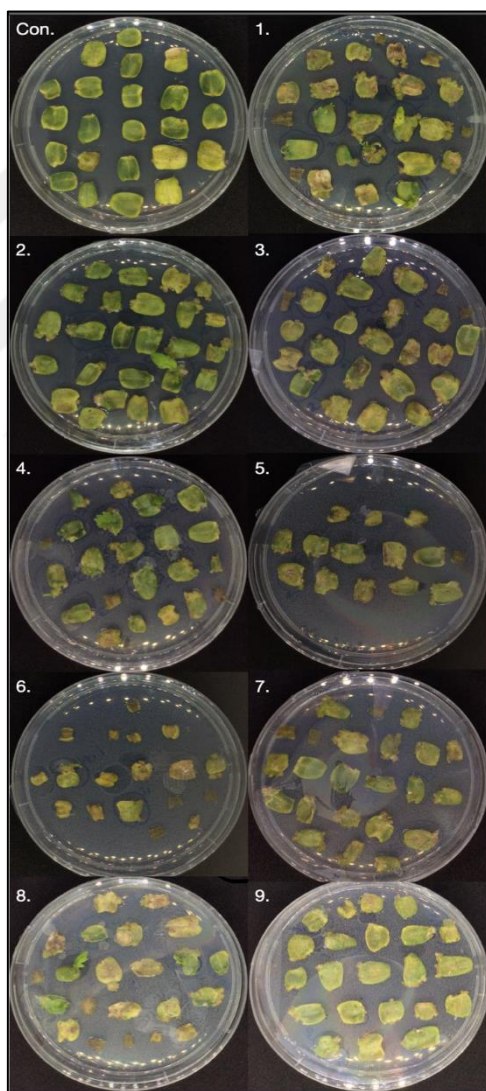


Figure 4.10: Explants used in the tissue culture study. Comparing with control (Con), callus formation can be clearly seen on the transformed explants (Petri number: 1-9).
Con: Negative control.

Regenerated explants were counted before starting the transfer of the first explants to the magenta boxes. In total, regenerations of various sizes were observed in 154 explants (Table 4.1). Ratio of the regeneration was 77%.

Table 4.1: Number of explants and regenerated explants on each petri.

Petri	Explant Amount	Number of Regenerated Explants	Frequency (%)
Petri 1.	25	20	80%
Petri 2.	25	20	80%
Petri 3.	25	18	72%
Petri 4.	25	21	84%
Petri 5.	14	10	71%
Petri 6.	18	14	77%
Petri 7.	23	16	69%
Petri 8.	22	19	86%
Petri 9.	23	16	70%
Total	200	154	77%

4.5.2. Transfer and Growth of Explants in Magenta Boxes

When calluses grew big enough and started to touch the lid of petri dishes, explants were moved to magenta boxes. In the beginning, the medium in magenta boxes was containing Zeatin together with IAA. However, the presence of IAA in the medium inhibited the formation of shoots and this resulted in the overgrowth of calluses (Figure 4.13). To overcome this problem, firstly extra and dead parts of calluses were truncated and then explants were moved into fresh mediums that did not contain IAA. After 5 days first shoots and leaves were formed (Figure 4.14).

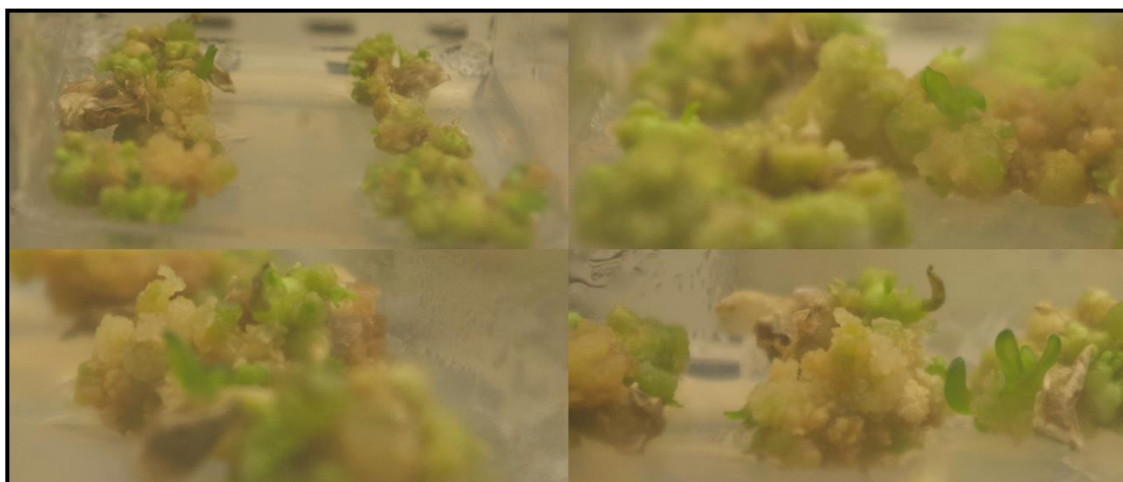


Figure 4.11: Overgrowth of calluses in magenta boxes due to limitation of plant growth by IAA.

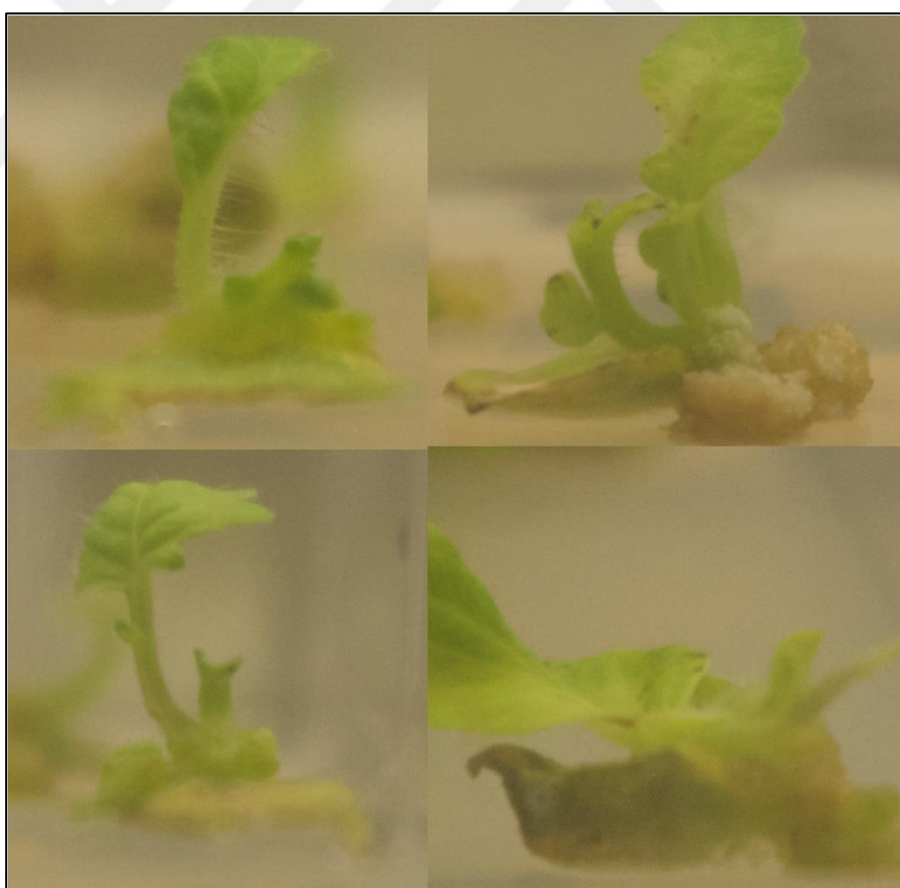


Figure 4.12: Formation of leaves and shoots after the truncation of calluses.

4.5.3. Culturing Explants in Secondary Regeneration Medium (1Z) and Rooting Medium (RM)

After the formation of the shoots, explants were moved to secondary regeneration medium (1Z) (Figure 4.13). Secondary regeneration medium contains Zeatin which promotes shooting in plants. Since calluses were overgrowth, there were multiple shoot formation on single explant. Due to that, so many explants were cut to sections that contain single shoots. Explants cultured on secondary regeneration medium for 4 to 6 weeks. When the explants reach the appropriate size, the shoots were excised from nodal region. Excised newly formed shoots were planted to rooting medium vertically. Approximately nine excised shoots were planted vertically to each rooting medium containing magenta boxes (Figure 4.14).

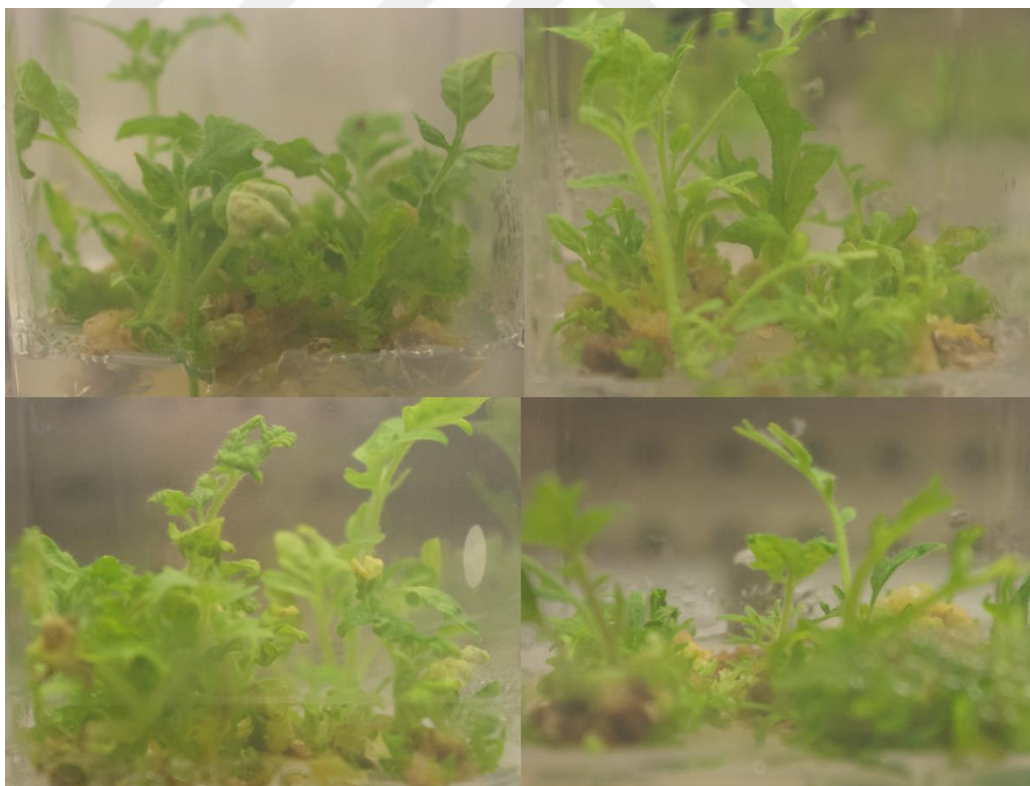


Figure 4.13: Explants on the secondary regeneration medium (1Z). Explants were cultured on this medium for 4-6 weeks and excised for plantation on rooting medium.

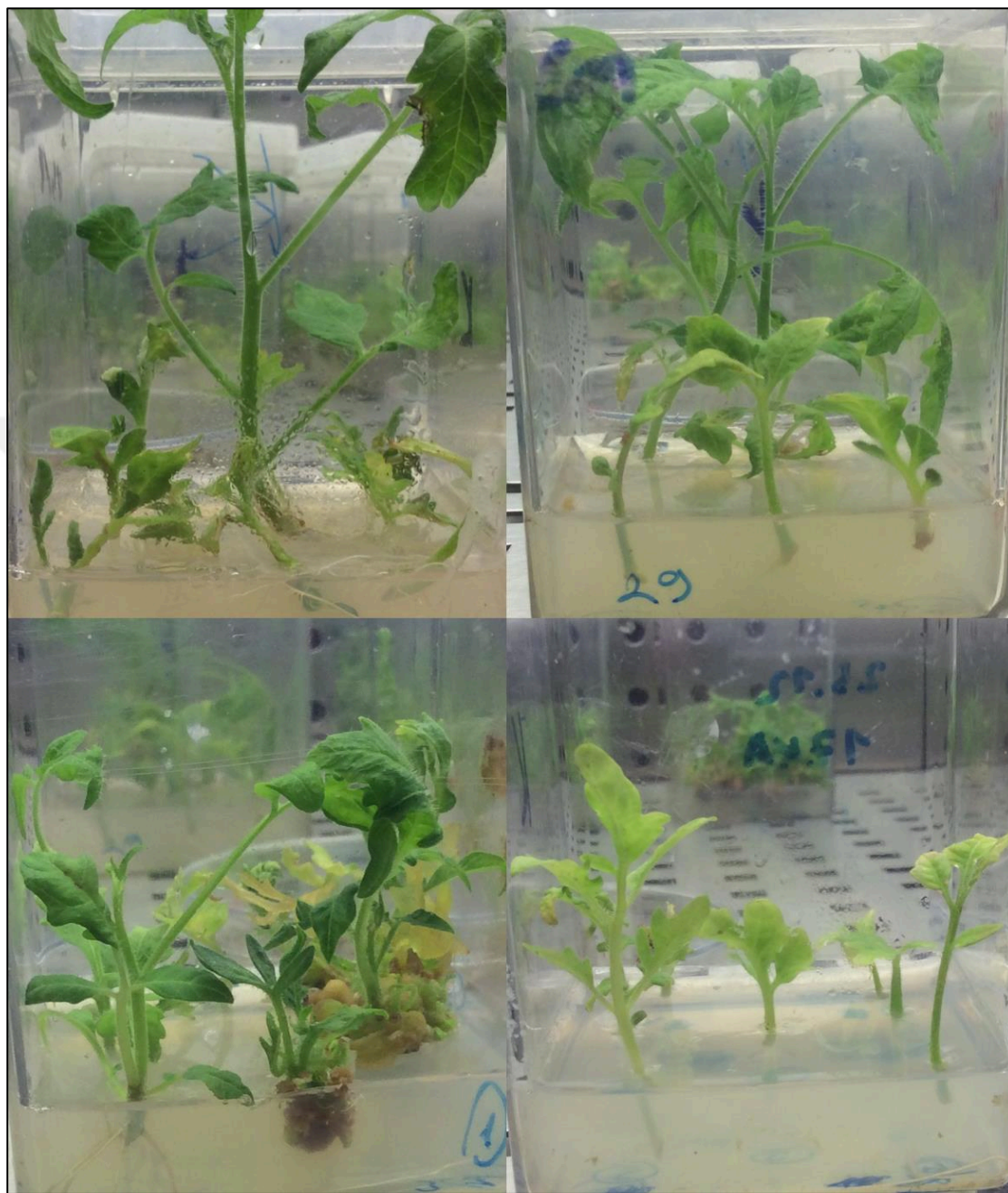


Figure 4.14: Explants on the rooting medium. Each magenta boxes contained 5 to 9 excised shoots. Each shoot was planted vertically into the rooting medium.

4.6. Acclimatization

Acclimatization was done at Plant Biotechnology Laboratory Greenhouse under 25 °C and 16 hours photoperiod conditions. After the rooted plants reached solid shoot lengths, the plants were moved to the greenhouse. To obtain explants with the proper shoot, explants were cultured for 4-6 weeks on the rooting medium. Plants with proper roots and shoots were firstly transferred to small pots with soils (Figure 4.17A). With the continuation of vegetative growth, plants that have grown to a sufficient size were taken into larger pots and suitable conditions for growth were provided (Figure 4.17B). Formation of first flowers was observed between 14 to 21 days (Figure 4.17C). Sometimes this stage occurred earlier due to suboptimal conditions of rooting medium stage of tissue culture process. Complete flowers were appeared 4-5 weeks after the plantation of plants into greenhouse (Figure 4.17D). Pollination of plants with complete flowers was carried out by vibration with a machine toothbrush or telephone. After the pollination formation of fruits were observed from the flowers (Figure 4.17E). During the pollination, some flowers weren't successfully pollinated thus some plants lost flowers before the formation of fruits. Since techniques we used for pollination were not efficient formation of fruits was not significantly high number. Formation of fruits was followed by ripening of fruits. Ripen fruits were observed after 5 months of plantation of plants into greenhouse (Figure 4.17F). Fruits were picked for collecting seeds. 60 explants from tissue culture process were transferred and growth in greenhouse (Figure 4.17G). Approximately 15 of them were lost due to disease and greenhouse conditions. As a result, 40 plants were picked for DNA isolation and detection of transgenic lines. During the acclimatization, dead or old leaves of plants were truncated to promote plants growth. Infected plants were kept away, and infected leaves continuously cut to reduce the effects on the plants.



Figure 4.15: Steps of acclimatization process. A) Plantation of explants to pots, B) Vegetative growth of newly planted plants and transferring them to larger pots, C) Formation of first flowers, D) Complete flowers, E) Formation of fruits, F) Ripe fruit, G) Fully grown tomato plants.

4.7. Identification of Transgenic Lines

In order to identify transgenic plant lines, firstly, genomic DNA was isolated and then PCR reaction was established with RB-F and PI-SceI-R primers designed over the T-DNA region transferred to the plant. To distinguish whether T-DNA is integrated into the genome or not, 2 different PCRs were established. In the second PCR instead of RB-F, Pmel-F1 was used as a forward primer which was located at the outside of T-DNA region (Figure 4.16).

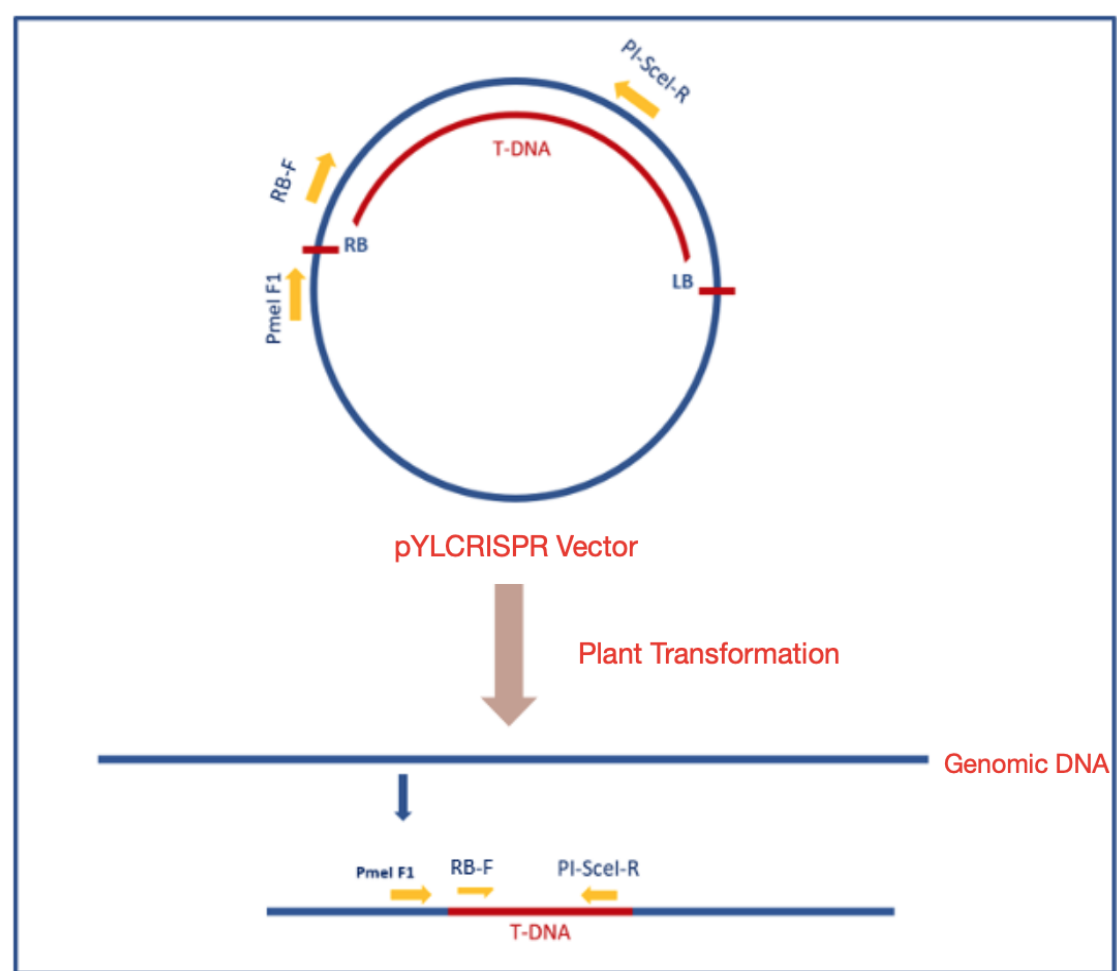


Figure 4.16: Primers and PCR reactions that designed to identify transgenic tomato lines.

Two different PCRs were done simultaneously with a combination of 3 primers mentioned before. After the PCRs, products were run in the gel and to distinguish whether the product is integrated into the plant genome or was detected from *Agrobacterium* plasmid, the gel was carried out with PCR Pmel-F1 and PI-SceI-R on the left and PCR with RB-F and PI-SceI-R on the right (Figure 4.19A). Successfully integrated T-DNA should not have shown a band on the left side of the gel but have shown a band on the right side of the gel. PCR products that have not shown any band on each side of the gel were non-transgenic plants. In total 38 plant samples were used for DNA isolation and the T-DNA identification process. 32 of them were identified as transgenic and 11 of them were sent for the sequencing and mutation detection (Figure 4.19B).

4.8. Genotyping the Sequencing Results and Phenotyping Plants

After identification of transgenic lines, 11 plants were selected for sequencing according to their development and growth. Isolated DNAs of these 11 plants were used as a template for 50 µL PCR reaction. Together with forward primer of miR396b, PCR products were sent to one-direction sequencing. Although some point mutations were detected on the target regions, there were significant amount of miss readings on other regions made this outcome suspicious (Figure 4.20). We claimed that, miss reading have occurred due to poor activity of the Taq polymerase. On the other hand, point mutation detected plants were shown longer shoot elongation (Figure 4.21). Transgenic and mutant plants, Plan T-7 and Plant T-12 shown the longest shoot elongation. Average shoot length of mutant plants was 206 cm. On the other hand, average shoot length of WT plants was 167.5 cm. In addition to that, potentially mutated plants had larger petals and late pollination stage. Because of late pollination or interruption of pollination, some of the potentially mutated plants haven't produced fruits or produced 2-3 weeks later than wild-type plants.

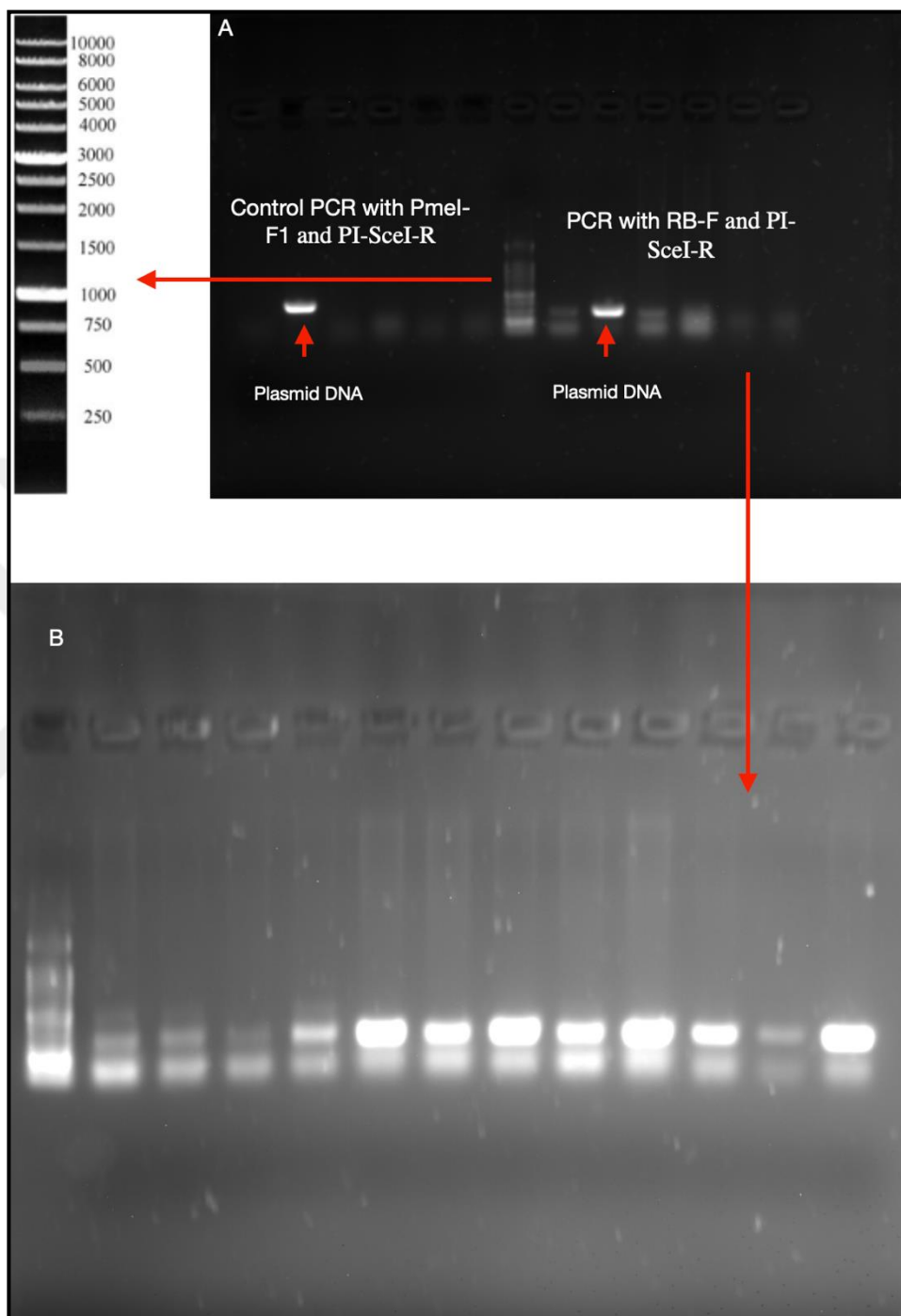


Figure 4.17: Gel image of PCRs with Pmel-F1 and PI-SceI-R primers and PCR with RB-F and PI-SceI-R primers. A) Two simultaneously done PCR to distinguish the integration of T-DNA into genome, B) gel image of transgenic plants. Marker: Intron 1 Kb ladder.

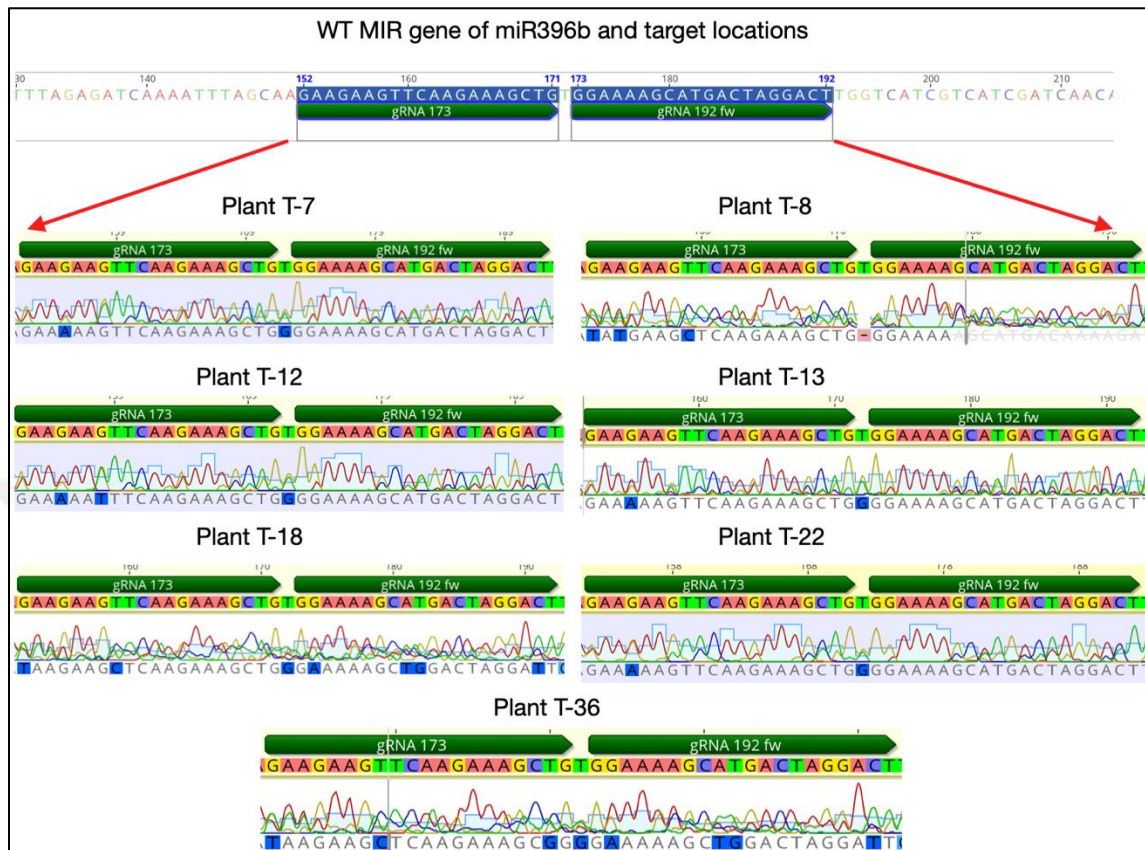


Figure 4.18: Sequencing results of transgenic CRISPR applied tomato plants. In Plant T-7, Plant T-12, Plant T-13, Plant T-18, Plant T-22 and Plant T-36 multiple base exchanges on the target location were observed. One deletion was observed in Plant T-8. However, multiple base deletion hasn't detected in the any of the selected plant samples.

As a conclusion, we obtained 3 mutant plants, plant T-7, Plant T-12 and Plant T-8. The average shoot length of mutant plants is 207 cm, which is almost 40 cm longer than WT plants average shoot length. On the other hand, the average fruit formation of mutant plants was only 1. WT and transgenic mutant free lines had an averagely of 5 fruits on each plant.



Figure 4.19: Comparison of mutant lines with wild type plants. Significant difference in the shoot elongations.

5. DISCUSSION

Until the application of CRISPR/Cas systems, miRNA loss-of-function studies were quite problematic. One of the reasons behind this situation was finding specific and proper KO tools for each miRNA. With CRISPR, now it is possible to target every miRNA or multiple miRNAs simultaneously. Generally, CRISPR/Cas9 technology is preferred to introduce point mutation on the target MIR gene. In this strategy, the aim is to introduce mutation in the exact location of the pre-miRNA duplex forming sequence [Zhou et al., 2017]. Another CRISPR/Cas9 approach is targeting each side of the MIR gene with two sgRNAs to completely remove the MIR gene from the genome [Zhao et al., 2016]. Both methods have critical disadvantages. For example, in the single gRNA approach, it is highly possible to generate a new miRNA that has a completely new target. On the other hand, in the two gRNAs approach efficiency of introducing large deletion (approximately 200-300 bp) in the plant genomes is low. In addition to CRISPR based methods, several other methods have been used to identify miRNAs, such as MIMIC/Inhibitor. In MIMIC inhibitor method, miRNA decoys are used to target and reduce the activity of miRNAs [Wong and Millar, 2019]. However, this method is not applicable to field since all the products that developed by this protocol are transgenic and GMO. The main focus of this thesis was to build up a protocol that can target miRNAs, completely inhibit their biogenesis, and improve plants without side effects. On the other hand, since plants that are developed by CRISPR technology are considered GEO instead of GMO, our newly developed method will also be useful for further applicable plant developments. With our method, it is possible to overcome all the mentioned disadvantages and commercialize ground-breaking results from over-expression studies.

Although this method overcomes various problems of traditional miRNA KO applications, it has some disadvantages. During the guide RNA selection, it is important to pick the first guide RNA that targets the exact location of miRNA forming duplex. However, it is hard to find a guide RNA with optimum attributes. Since the motives of

guide RNAs, their secondary structure with scaffold and GC contents are the attributes that determine the efficiency of the guide RNA [Baloğlu et al., 2022], it is not always possible to pick a guide RNA with high efficiency and score that targets the duplex forming region.

According to our sequencing results, Plant T-7, Plant T-8, Plant T-12, Plant t-18 and Plant T-36 have point mutation on the duplex forming region (Figure 4.18). Since miR396b sequence point of view perfectly matches with target GRF1 domain our potential mutations were enough to disturb miRNA/target formation. Our phenotyping results also confirmed these results.

Within the scope of this thesis, multiple transgenic but mutant free plants were obtained. Since those transgenic plants contains T-DNA together with our constructed CRISPR cassettes in their genome, it is possible that CRISPR system will work and introduce mutation on the target region in the next generation. Especially during the embryogenesis, introducing chance of the mutation on the target region is expected to be higher. We thus planned to collect seeds of mutant free but transgenic lines and genotype their genomes for possible cas9 activity in the further experiments. Also, these plants could be useful asset for further sequential transformation or more efficient CRISPR applications.

Although our phenotyping results are matching perfectly with similar studies [Cao et al., 2016; Liebsch and Palatnik, 2020], pollination problem on the possible miR396b mutant plants is not mentioned in the literature. Since our method is focused on complete inhibition of miR396b biogenesis and enhance the expression of GRFs, it could be side effect of overexpressed GRFs and plant overgrowth.

REFERENCES

- Achard P., Herr A., Baulcombe D. C., Harberd N. P., (2004), "Modulation of floral development by a gibberellin-regulated microRNA", *Development* 131, 3357–3365.
- Albà M., (2017), "Zinc-finger domains in metazoans: evolution gone wild", *Genome biology*, 18(1), 1-3.
- Alonso-Peral M. M., Li J., Li Y., Allen R. S., Schnippenkoetter W., Ohms S., Millar A. A., (2010), "The microRNA159-regulated GAMYB-like genes inhibit growth and promote programmed cell death in Arabidopsis", *Plant physiology*, 154(2), 757-771.
- Aukerman M. J., Sakai H., (2003), "Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes", *The Plant Cell*, 15(11), 2730-2741.
- Baloglu M. C., Çelik Altunoğlu Y., Baloğlu P., Yıldız A. B., Türkölmez N., Özden Çiftçi, Y., (2022) "Gene Editing Technologies and Applications in Legumes: Progress, Evolution and Future Prospects", *Frontiers in Genetics*, 1391.
- Boch J., Scholze H., Schornack S., Landgraf A., Hahn S., Kay S., Bonas U., (2009), "Breaking the code of DNA binding specificity of TAL-type III effectors", *Science*, 326(5959), 1509-1512.
- Bologna N. G., Voinnet O., (2014), "The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis", *Annu Rev Plant Biol*, 65(1), 473-503.
- Cai Z., Wang Y., Zhu L., Tian Y., Chen L., Sun Z., Li X., (2017), "GmTIR1/GmAIF3-based auxin perception regulated by miR393 modulates soybean nodulation", *New Phytologist*, 215(2), 672-686.
- Cao D., Wang J., Ju Z., Liu Q., Li S., Tian H., Zhu B., (2016), "Regulations on growth and development in tomato cotyledon, flower and fruit via destruction of miR396 with short tandem target mimic", *Plant Science*, 247, 1-12.
- Cathomen T., Joung J. K., (2008), "Zinc-finger nucleases: the next generation emerges", *Molecular Therapy*, 16(7), 1200-1207.
- Cebraioğlu N., Yıldız A.B., Akkaya Ö., Özden Çiftçi Y. (2019)., "CRISPR-Cas: Removing Boundaries of the Nature", *European Journal of Biology*, 78(2), 157-164.

Cermak T., Doyle E. L., Christian M., Wang L., Zhang Y., Schmidt C., Voytas D. F., (2011), "Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting", *Nucleic acids research*, 39(12), e82-e82.

Chen H., Li Z., Xiong L., (2012), "A plant microRNA regulates the adaptation of roots to drought stress", *FEBS letters*, 586(12), 1742-1747.

Chen K., Gao C., (2014), "Targeted genome modification technologies and their applications in crop improvements", *Plant cell reports*, 33(4), 575-583.

Chen, X., (2004), "A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development", *Science*, 303(5666), 2022-2025.

Chuck G., Whipple C., Jackson D., Hake S., (2010), "The maize SBP-box transcription factor encoded by tasselsheath4 regulates bract development and the establishment of meristem boundaries", *Development*, 137(8), 1243-1250.

Chyou T. Y., Brown C. M., (2019), "Prediction and diversity of tracrRNAs from type II CRISPR-Cas systems", *RNA biology*, 16(4), 423-434.

Cui Y., Fang X., Qi Y., (2016), "TRANSPORTIN1 promotes the association of microRNA with ARGONAUTE1 in Arabidopsis", *The Plant Cell*, 28(10), 2576-2585.

D'Ario M., Griffiths-Jones S., Kim M., (2017), "Small RNAs: big impact on plant development", *Trends in plant science*, 22(12), 1056-1068.

da Silva E. M., Nogueira F. T., (2021), "Guarding tomato fruit setting in adverse temperatures through the miRNA166-SIHB15A regulatory module", *Molecular Plant*, 14(7), 1046-1048.

Debernardi J. M., Rodriguez R. E., Mecchia M. A., Palatnik J. F., (2012), "Functional specialization of the plant miR396 regulatory network through distinct microRNA-target interactions", *PLoS genetics*, 8(1), e1002419.

Deng F., Zeng F., Shen Q., Abbas A., Cheng J., Jiang W., Chen Z. H., (2022), "Molecular evolution and functional modification of plant miRNAs with CRISPR", *Trends in plant science*.

Dong Z., Han M. H., Fedoroff N., (2008), "The RNA-binding proteins HYL1 and SE promote accurate in vitro processing of pri-miRNA by DCL1", *Proceedings of the National Academy of Sciences*, 105(29), 9970-9975.

Durai S., Mani M., Kandavelou K., Wu J., Porteus M. H., Chandrasegaran S., (2005), "Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells", *Nucleic acids research*, 33(18), 5978-5990.

East-Seletsky A., O'Connell M. R., Knight S. C., Burstein D., Cate J. H., Tjian R., Doudna J. A., (2016), "Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection", *Nature*, 538(7624), 270-273.

Eck J. V., Keen P., Tjahjadi M., (2019), "Agrobacterium tumefaciens-mediated transformation of tomato", In *Transgenic plants* (pp. 225-234), Humana Press, New York, NY.

Faure G., Shmakov S. A., Yan W. X., Cheng D. R., Scott D. A., Peters J. E., ... Koonin E. V., (2019), "CRISPR-Cas in mobile genetic elements: counter-defence and beyond", *Nature Reviews Microbiology*, 17(8), 513-525.

Fonfara I., Richter H., Bratovič M., Le Rhun A., Charpentier E., (2016), "The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA", *Nature*, 532(7600), 517-521.

Fracasso A., Vallino M., Staropoli A., Vinale F., Amaducci S., Carra A., (2021), "Increased water use efficiency in miR396-downregulated tomato plants", *Plant Science*, 303, 110729.

Gandikota M., Birkenbihl R. P., Höhmann S., Cardon G. H., Saedler H., Huijser P., (2007), "The miRNA156/157 recognition element in the 3' UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings", *The Plant Journal*, 49(4), 683-693.

German M. A., Pillay M., Jeong D. H., Hetawal A., Luo S., Janardhanan P., Green, P. J., (2008), "Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends", *Nature biotechnology*, 26(8), 941-946.

Guo H. S., Xie Q., Fei J. F., Chua N. H., (2005), "MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development", *The Plant Cell*, 17(5), 1376-1386.

Gupta S. K., Vishwakarma A., Kenea H. D., Galsurker O., Cohen H., Aharoni A., Arazi T., (2021), "CRISPR/Cas9 mutants of tomato MICRORNA164 genes uncover their functional specialization in development", *Plant Physiology*, 187(3), 1636-1652.

Hamilton A. J., Baulcombe D. C., (1999), "A species of small antisense RNA in posttranscriptional gene silencing in plants" *Science*, 286(5441), 950-952.

Hobecker K. V., Reynoso M. A., Bustos-Sanmamed P., Wen J., Mysore K. S., Crespi M., ... Zanetti M. E., (2017), "The microRNA390/TAS3 pathway mediates symbiotic nodulation and lateral root growth", *Plant physiology*, 174(4), 2469-2486.

Hong Y., Meng J., He X., Zhang Y., Liu Y., Zhang C., Luan Y., (2021), "Editing miR482b and miR482c simultaneously by CRISPR/Cas9 enhanced tomato resistance to *Phytophthora infestans*", *Phytopathology*®, 111(6), 1008-1016.

Huntley R. P., Sitnikov D., Orlic-Milacic M., Balakrishnan R., D'Eustachio P., Gillespie M. E., Lovering R. C., (2016), "Guidelines for the functional annotation of microRNAs using the Gene Ontology", *Rna*, 22(5), 667-676.

Jansen R., Embden J. D. V., Gaastra W., Schouls L. M., (2002), "Identification of genes that are associated with DNA repeats in prokaryotes", *Molecular microbiology*, 43(6), 1565-1575.

Jiang F., Doudna J. A., (2017), "CRISPR-Cas9 structures and mechanisms", *Annu Rev Biophys*, 46(1), 505-529.

Joung J. K., Sander J. D., (2013), "TALENs: a widely applicable technology for targeted genome editing", *Nature reviews Molecular cell biology*, 14(1), 49-55.

Kim J. H., (2019), "Biological roles and an evolutionary sketch of the GRF-GIF transcriptional complex in plants", *BMB reports*, 52(4), 227.

Knauer S., Holt A. L., Rubio-Somoza I., Tucker E. J., Hinze A., Pisch M., Laux T., (2013), "A protodermal miR394 signal defines a region of stem cell competence in the Arabidopsis shoot meristem", *Developmental cell*, 24(2), 125-132.

Kurihara Y., Watanabe Y., (2004), "Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions", *Proceedings of the National Academy of Sciences*, 101(34), 12753-12758.

Lauter N., Kampani A., Carlson S., Goebel M., Moose S. P., (2005), "microRNA172 down-regulates *glossy15* to promote vegetative phase change in maize", *Proceedings of the National Academy of Sciences*, 102(26), 9412-9417.

Lee R. C., Feinbaum R. L., Ambros V., (1993), "The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*" cell, 75(5), 843-854.

Lee Z. H., Yamaguchi N., Ito T., (2018), "Using CRISPR/Cas9 system to introduce targeted mutation in Arabidopsis", In *Plant transcription factors* (pp. 93-108), Humana Press, New York, NY.

Li S., Le B., Ma X., Li S., You C., Yu Y., Chen X., (2016), "Biogenesis of phased siRNAs on membrane-bound polysomes in Arabidopsis", *Elife*, 5, e22750.

Li S., Liu L., Zhuang X., Yu Y., Liu X., Cui X., Chen X., (2013), "MicroRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in Arabidopsis", *Cell*, 153(3), 562-574.

Li X., Wang Y., Chen S., Tian H., Fu D., Zhu B., Zhu H., (2018), "Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing", *Frontiers in plant science*, 9, 559.

Liebsch D., Palatnik JF., (2020), "MicroRNA miR396, GRF transcription factors and GIF co-regulators: a conserved plant growth regulatory module with potential for breeding and biotechnology", *Current opinion in plant biology*, 53, 31-42.

Lin D., Zhu X., Qi B., Gao Z., Tian P., Li Z., Huang T., (2022), "SIMIR164A regulates fruit ripening and quality by controlling SINAM2 and SINAM3 in tomato", *Plant Biotechnology Journal*.

Liu L., Zhang J., Xu J., Li Y., Guo L., Wang Z., Zhang, N. (2020), "CRISPR/Cas9 targeted mutagenesis of SLBD40, a lateral organ boundaries domain transcription factor, enhances drought tolerance in tomato", *Plant Science*, 301, 110683.

Liu N., Wu S., Van Houten J., Wang Y., Ding B., Fei Z., Van Der Knaap E., (2014), "Down-regulation of AUXIN RESPONSE FACTORS 6 and 8 by microRNA 167 leads to floral development defects and female sterility in tomato", *Journal of experimental botany*, 65(9), 2507-2520.

Ma X., Liu Y. G., (2016), "CRISPR/Cas9-based multiplex genome editing in monocot and dicot plants" *Current protocols in molecular biology*, 115(1), 31-6.

Makarova K. S., Wolf Y. I., Iranzo J., Shmakov S. A., Alkhnbashi O. S., Brouns S. J., Koonin E. V., (2020), "Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants", *Nature Reviews Microbiology*, 18(2), 67-83.

Malzahn A., Lowder L., Qi Y., (2017), "Plant genome editing with TALEN and CRISPR", *Cell & bioscience*, 7(1), 1-18.

Miller J., McLachlan A. D., Klug A., (1985), "Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus oocytes*", *The EMBO journal*, 4(6), 1609-1614.

Miyashima S., Koi S., Hashimoto T., Nakajima K., (2011), "Non-cell-autonomous microRNA165 acts in a dose-dependent manner to regulate multiple differentiation status in the Arabidopsis root", *Development*, 138(11), 2303-2313.

Morris E. R., Chevalier D., Walker J. C., (2006), "DAWDLE, a forkhead-associated domain gene, regulates multiple aspects of plant development", *Plant Physiology*, 141(3), 932-941.

Navarro B., Gisel A., Serra P., Chiumenti M., Di Serio F., Flores R., (2021), "Degradome Analysis of Tomato and *Nicotiana benthamiana* Plants Infected with Potato Spindle Tuber Viroid", *International journal of molecular sciences*, 22(7), 3725.

Oraman M. Nail., (1968), "Sebze İlimi", Ankara Üniversitesi Ziraat Fakültesi Yayınları, 323-117.

Özcan A., Pausch P., Linden A., Wulf A., Schühle K., Heider J., Randau L., (2019), "Type IV CRISPR RNA processing and effector complex formation in *Aromatoleum aromaticum*", *Nature microbiology*, 4(1), 89-96.

Park M. Y., Wu G., Gonzalez-Sulser A., Vaucheret H., Poethig R. S., (2005), "Nuclear processing and export of microRNAs in *Arabidopsis*", *Proceedings of the National Academy of Sciences*, 102(10), 3691-3696.

Pillot M., Baroux C., Vazquez M. A., Autran D., Leblanc O., Vielle-Calzada J. P., Grimanelli D., (2010), "Embryo and endosperm inherit distinct chromatin and transcriptional states from the female gametes in *Arabidopsis*", *The Plant Cell*, 22(2), 307-320.

Rahim J., Gulzar S., Zahid R., Rahim K. A., (2021), "Systematic review on the comparison of molecular gene editing tools", *Int. J. Innov. Sci. Res. Tech*, 6, 1-8.

Rajagopalan R., Vaucheret H., Trejo, J., Bartel D. P., (2006), "A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*", *Genes & development*, 20(24), 3407-3425.

Ramachandran V., Chen X., (2008), "Small RNA metabolism in *Arabidopsis*" *Trends in plant science*, 13(7), 368-374.

Rani R., Yadav P., Barbadikar K. M. Baliyan N., Malhotra E. V., Singh B. K., Singh D., (2016), "CRISPR/Cas9: a promising way to exploit genetic variation in plants", *Biotechnology letters*, 38(12), 1991-2006.

Reem N. T., Eck J. V., (2019), "Application of CRISPR/Cas9-mediated gene editing in tomato", In *Plant Genome Editing with CRISPR Systems* (pp. 171-182), Humana Press, New York, NY.

Ren G., Xie M., Dou Y., Zhang S., Zhang C., Yu B., (2012), "Regulation of miRNA abundance by RNA binding protein TOUGH in Arabidopsis", *Proceedings of the National Academy of Sciences*, 109(31), 12817-12821.

Richter C., Chang J. T., Fineran P. C., (2012), "Function and regulation of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) systems", *Viruses*, 4(10), 2291-2311.

Rodriguez R. E., Ercoli M. F., Debernardi J. M., Palatnik J. F., (2016), "Growth-regulating factors, a transcription factor family regulating more than just plant growth", In *Plant Transcription Factors* (pp. 269-280), Academic Press.

Santillán Martínez M. I., Bracuto V., Koseoglou E., Appiano M., Jacobsen E., Visser R. G., Bai Y., (2020), "CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene PMR4 for resistance against powdery mildew", *BMC plant biology*, 20(1), 1-13.

Semenova E., Severinov K., (2017), "Interdependencies between the adaptation and interference modules guide efficient crispr-cas immunity", In *Evolutionary Biology: Self/Nonself Evolution, Species and Complex Traits Evolution, Methods and Concepts* (pp. 51-62). Springer, Cham.

Shah S. A., Alkhnbashi O. S., Behler J., Han W., She Q., Hess W. R., Backofen, R., (2018), "Conserved accessory proteins encoded with archaeal and bacterial Type III CRISPR-Cas gene cassettes that may specifically modulate, complement or extend interference activity", *BioRxiv*, 262675.

Shamshirgaran Y., Liu J., Sumer H., Verma P.J., Taheri-Ghahfarokhi A, (2022), "Tools for Efficient Genome Editing; ZFN, TALEN, and CRISPR", *Applications of Genome Modulation and Editing Methods in Molecular Biology*, vol 2495.

Shmakov S., Smargon A., Scott D., Cox D., Pyzocha N., Yan W., Koonin E. V., (2017), "Diversity and evolution of class 2 CRISPR–Cas systems", *Nature reviews microbiology*, 15(3), 169-182.

Tang J., Chu C., (2017), "MicroRNAs in crop improvement: fine-tuners for complex traits", *Nature plants*, 3(7), 1-11.

Tao H., Jia Z., Gao X., Gui M., Li Y., Liu, Y., (2022), "Analysis of the miRNA expression profile involved in the tomato spotted wilt orthotospovirus–pepper interaction", *Virus Research*, 312, 198710.

Wah D. A., Bitinaite J., Schildkraut I., Aggarwal A. K., (1998), "Structure of Fok I has implications for DNA cleavage", *Proceedings of the National Academy of Sciences*, 95(18), 10564-10569.

Wightman B., Ha I., Ruvkun G., (1993), "Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*", *Cell*, 75(5), 855-862.

Wong G., Millar A A., (2019), "The use of microRNA decoy technologies to inhibit miRNA function in *Arabidopsis*", In *Plant MicroRNAs* (pp. 227-238), Humana Press, New York, NY.

Xu L., Yang L., Huang H., (2007), "Transcriptional, post-transcriptional and post-translational regulations of gene expression during leaf polarity formation", *Cell research*, 17(6), 512-519.

Yamaguchi A., Abe M., (2012), "Regulation of reproductive development by non-coding RNA in *Arabidopsis*: to flower or not to flower", *Journal of plant research*, 125(6), 693-704.

Yoshikawa T., Ozawa S., Sentoku N., Itoh J. I., Nagato Y., Yokoi S., (2013), "Change of shoot architecture during juvenile-to-adult phase transition in soybean", *Planta*, 238(1), 229-237.

Yu B., Yang Z., Li J., Minakhina S., Yang M., Padgett R. W., Chen X., (2005), "Methylation as a crucial step in plant microRNA biogenesis", *Science*, 307(5711), 932-935.

Yu Q. H., Wang B., Li N., Tang Y., Yang S., Yang T., Asmutola P., (2017), "CRISPR/Cas9-induced targeted mutagenesis and gene replacement to generate long-shelf life tomato lines", *Scientific reports*, 7(1), 1-9.

Yu Y., Mo X., Mo B., (2020), "Introduction to plant small RNAs", In *Plant Small RNA* (pp. 3-35), Academic Press.

Zhang S., Liu Y., Yu B., (2014), "PRL1, an RNA-binding protein, positively regulates the accumulation of miRNAs and siRNAs in *Arabidopsis*", *PLoS genetics*, 10(12), e1004841.

Zhang S., Xie M., Ren G., Yu B., (2013), "CDC5, a DNA binding protein, positively regulates posttranscriptional processing and/or transcription of primary microRNA transcripts", *Proceedings of the National Academy of Sciences*, 110(43), 17588-17593.

Zhang YY., Hong YH., Liu R., Cui J., Luan YS., (2021), "Function identification of miR394 in tomato resistance to *Phytophthora infestans*", *Plant Cell Reports*, 40(10), 1831-1844.

Zhao Y., Zhang C., Liu W., Gao W., Liu C., Song G., Xie C., (2016), "An alternative strategy for targeted gene replacement in plants using a dual-sgRNA/Cas9 design" *Scientific reports*, 6(1), 1-11.

Zhou J., Deng K., Cheng Y., Zhong Z., Tian L., Tang X., Zhang Y., (2017), “CRISPR-Cas9 based genome editing reveals new insights into microRNA function and regulation in rice”, *Frontiers in Plant Science*, 8, 1598.



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