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**DEVELOPMENT OF BROOMRAPE (*Orobanche* spp., *Phelipanche* spp.) -  
RESISTANT TOMATO (*Solanum lycopersicum*) USING GENE EDITING  
TECHNOLOGY (CRISPR)**

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## ABSTRACT

### DEVELOPMENT OF BROOMRAPE (*Orobanch* spp., *Phelipanche* spp.) - RESISTANT TOMATO (*Solanum lycopersicum*) USING GENE EDITING TECHNOLOGY (CRISPR)

Cansu BÜLBÜL

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Tomato is one of the main cultivated plants with economic value and extensive production-consumption in the World. The factors limiting production and affecting yields, such as environmental conditions, soil structure, plant nutrition practices, irrigation methods, and pathogens, are also quite restrictive in tomato cultivation. Broomrape (*Orobanch* spp. / *Phelipanche* spp.) is a completely parasitic weed that survives depending on its host and causes yield and quality losses in tomato in the World, including Turkey. Under suitable environmental conditions, broomrape seeds germinate by a "strigolactones" signal, a plant hormone synthesized by the carotenoid pathway and secreted from the host plant's roots.

In this thesis, *SlCCD7* and/or *SlCCD8* (Carotenoid Cleavage Dioxygenase 7 and 8) genes, that are known to play a critical role in strigolactone synthesis in tomato, were manipulated by CRISPR-Cas9 gene-editing technology. Guide RNAs and Cas9 designed in this method were transferred to tomato using the *Agrobacterium*-mediated plant transformation method. Among the many transgenic events, deletion/insertion knockouts and in-frame modifications of *slccd7*, *slccd8*, and *slccd7-slccd8* were generated. The significant homozygous biallelic "in-frame" mutation between Thr42-Val105 in the *SlCCD7*-exon 1 region of *slccd7* T2 mutants has been characterized. The in-frame mutation may negatively affect the strigolactone pathway and provide resistance against *Orobanch* spp. / *Phelipanche* spp. Mutants with this "in-frame" mutation have the potential to be an essential breeding material in terms of their agronomic characteristics and -possible- broomrape resistance genetic source.

**KEYWORDS:** *Agrobacterium tumefaciens*-mediated gene transfer, CCD7, CCD8, CRISPR-Cas9 method, Plant transformation in tomato, Strigolactones

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## ÖZET

# GEN DÜZENLEME TEKNOLOJİSİ (CRISPR) KULLANILARAK CANAVAR OTUNA (*Orobanche* spp., *Phelipanche* spp.) DAYANIKLI DOMATES GELİŞTİRİLMESİ

Cansu BÜLBÜL

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Domates, dünyada yoğun bir şekilde üretimi ve tüketimi yapılan, ekonomik değeri olan başlıca kültür bitkilerdendir. Çevre koşulları, toprak yapısı, bitki besleme uygulamaları, sulama yöntemleri, patojenler gibi üretimi kısıtlayan ve verimi etkileyen etmenler, domates üretiminde de oldukça kısıtlayıcıdır. Dünyada ve ülkemizde domates yetiştiriciliğini kısıtlayan unsurlardan birisi de konukçusuna bağlı olarak hayatını sürdüren tam parazitik yabancı otlardan birisi olan “canavar otları”dır (*Orobanche* spp. / *Phelipanche* spp.). Uygun çevre şartlarında, karotenoid yolağı ile sentezlenen ve konukçu bitki köklerinden salgılanan bir bitki hormonu olan “strigolaktonlar” sayesinde çimlenen canavar otu tohumları domatestе zarar meydana getirmektedir.

Bu tezde domatestе strigolakton üretiminde temel rol oynadıkları bilinen *SlCCD7* ve / veya *SlCCD8* (Carotenoid Cleavage Dioxygenase 7 ve 8) genleri CRISPR-Cas9 gen düzenleme teknolojisi ile manipüle edilmiştir. Bu yöntem dahilinde tasarlanan rehber RNA’lar ve Cas9, *Agrobacterium*-aracılı bitki transformasyonu yöntemi kullanılarak domatese aktarılmıştır. Elde edilen birçok *slccd7*, *slccd8* ve *slccd7-slccd8* mutantının arasında, özellikle *SlCCD7* geninin ekson 1 bölgesinde Thr42-Val105 arasında meydana gelen büyük bir homozigot bialellik “in-frame” mutasyonu taşıyan T2 mutantlarında, büyük olasılıkla strigolakton yolağının negatif yönde etkilediği ve *Orobanche* / *Phelipanche* spp.’ye karşı bir dayanım sağladığı düşünülmektedir. Bu “in-frame” mutasyonunu taşıyan mutantlar, agronomik özellikleri ve -olası- canavar otu dayanımları bakımından önemli birer ıslah materyali olma potansiyeli taşımaktadırlar. Bu çalışmadaki yöntemlerin, bu konu dahilinde yapılacak olan diğer çalışmalara da yol gösterici olması ümit edilmektedir.

**ANAHTAR KELİMELE:** *Agrobacterium tumefaciens*-aracılı gen aktarımı, CCD7, CCD8, CRISPR-Cas9 yöntemi, Domatestе bitki transformasyonu, Strigolaktonlar

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## PREFACE

Thanks to this thesis on CRISPR-Cas9, a new technology for our country, I have sought solutions against parasitic weed “Broomrape (*Orobanch* spp.)”; one of the most critical problems for tomato (*Solanum lycopersicum*), an economically important agricultural product in our country and in the world. Thanks to the experiences I have gained, I am happy to have made a significant contribution to the literature. I owe a debt of gratitude to my esteemed mentor Prof. Dr. Nedim MUTLU, who helped me set out on this path and helped me move to an advanced level with his valuable ideas.

I want to thank my Assoc. Prof. Dr. Hasan PINAR and his hardworking team have always guided me with their experiences on this challenging journey and another valuable teacher on my thesis committee, Asst. Prof. Dr. Münevver AKSOY, for her valuable ideas. Of course, I did not walk alone in this challenging and valuable time course. I want to thank my colleagues for their efforts at every stage.

The enormous thanks to my devoted mother Sebahat ŞİMŞEK, who insisted on my doctorate and allowed me to stand on my own feet and raised me as a strong woman, to my dear husband Tolga BÜLBÜL, who always supported me, but most of my thanks to my first teacher and guiding spirit, my father Kemal ŞİMŞEK, whom I lost in this challenging road. I dedicate this thesis to this precious, beautiful person who has always been proud of me and will always be proud of me, who has always had a great place in my heart.

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## DECLARATION

I state that this study named “Development of Broomrape (*Orobanche* spp., *Phelipanche* spp.) - Resistant Tomato (*Solanum lycopersicum*) Using Gene Editing Technology (CRISPR)”, which I submitted as a doctoral thesis, was written under academic rules and ethical values, and I declare that I have indicated the source of all information that does not belong to me in this thesis.

02/07/2021

Cansu BÜLBÜL



## SYMBOLS AND ABBREVIATIONS

### Symbols

|    |              |
|----|--------------|
| g  | : Gram       |
| X  | : Fold       |
| µg | : Microgram  |
| µl | : Microliter |
| V  | : Volt       |

### Abbreviations

|      |                               |
|------|-------------------------------|
| bp   | : Base Pair                   |
| Cas9 | : CRISPR Associated Protein 9 |
| DNA  | : Deoxyribonucleic Acid       |
| OD   | : Optical Density             |
| PCR  | : Polymerase Chain Reaction   |
| RNA  | : Ribonucleic Acid            |
| RPM  | : Revolutions Per Minute      |
| UV   | : Ultraviolet                 |

Comma (,) is used as decimal separator in the thesis (e.g. “21,01”).

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## 1. INTRODUCTION

The tomato (*Solanum lycopersicum*) has been cultivated in South America since 500 B.C. and still maintains its importance until today. It continues to be the subject of academic studies. Molecular studies in tomato have accelerated in recent years. Considering that tomato production is 180.766.329 million tons worldwide (FAO 2019) and approximately 12.841.990 million tons in our country (FAO 2019), the importance of this study can be better understood. Consumption of tomato containing carotenoids (lycopene) and vitamins and minerals, essential for health, is encountered in almost every country. In addition to production volume, tomato attains Turkey's economy with exportation of 531.243,00 tons (FAO 2019) and has generated \$ 301.649,00 in revenue (FAO 2019). Considering all these numerical values, the importance of tomato breeding comes to the fore worldwide. In retrospect, complex classical breeding methods were used when it was desired to increase the yield of a plant. It has been tried to increase the yield by hybridizing genetically different tomatoes (in terms of desired characteristics) for superior gene combinations. Nowadays, thanks to molecular studies, while the time spent on breeding is reducing, the accuracy of the results is increasing. Gene editing technologies such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) have become one of the most widely used molecular methods to obtain the plant with the best properties.

The CRISPR method, which was chosen by the American Scientific Development Association (AAAS) as the most important scientific development of 2015, allows changes to the targeted DNA region. This discovery has gained molecular significance by integrating the natural functions of bacteria into the eukaryotic system. The CRISPR-Cas9 system, which used Cas9 protein and adapted to eukaryotes by Feng Zang in 2013, is considered the most used tool for genome editing studies in plants. The main element of this system, the "Cas9 protein" has enzymatic cleavage capability on the double helix of the targeted region (gene of interest) via guided by an approximately 20 bases-RNA (guide RNA) fragment designed as a complementary to the relevant gene region. As a result of DNA damage, the cell tries to repair this region, mutations occur and cause genetic changes in this region. These genetic changes can be used for a good purpose for all plants. This CRISPR-Cas9 genome editing technology can be used to contribute to breeding by manipulating gene of interest with important agronomic traits in plants.

Environmental conditions, soil structure, plant nutrition applications, irrigation methods, pathogens, weeds are some of the factors that restrict production and affect the yield of the plant. Lots of money are spent on chemicals every year for chemical control, and these agrochemicals harm the environment and human health. The use of 51,297 tons of pesticides (TUIK 2019) clearly shows Turkey's economic loss and ecosystem damage. While these chemicals negatively affect the economy, plants gradually develop resistance to the active ingredients in pesticides, and a vicious cycle occurs. However, when the reproduction, germination, and development mechanisms of weeds are better understood, more ecology-friendly solutions will be produced instead of chemical control.

Chemical pesticides are used in large quantities against weeds and pathogens in tomato cultivation. Herbicides alone accounted for 12.644 tons of the total amount of

agrochemicals in 2019 (TUIK 2019). Since weeds can germinate in cultivated areas, they cause damage to many species, including tomato, and cause significant yield losses. When mentioning the loss of weeds in tomatoes, "Broomrapes (*Orobanche* spp. and *Phelipanche* spp.)" come into prominence. The broomrapes, where their seeds can maintain vitality in the soil for ages, are a complete parasitic plant in Mediterranean countries, including Turkey. However, it is known that 4 of them have caused significant loss of yield in cultivated areas in Turkey (Aksoy and Pekcan 2014). Approximately 40 species of Broomrape are defined in our country (Bakis et al. 2011). The importance of broomrape damage in tomato, which has approximately 13 million tons production (FAO 2019) in our country, is relatively high.

Broomrapes affect yield in many cultivated agricultural areas, including tomato, potato, lentil, and sunflower. Since agricultural activity is carried out in a large area of 37.753,00 hectares in Turkey (TUIK 2020), the damages in these areas are significant and destructive. Because broomrape causes at least 25% yield loss, if all the tomato-cultivated areas are infected with broomrapes, tomato production will decrease and cause severe financial losses. Moreover, most importantly, a chemical control method that achieves 100% success against weeds has not been developed. Broomrapes with more than 100 species are parasitic plants ordered into two distinctive genera: *Orobanche* spp. and *Phelipanche* spp. These weeds do not have photosynthesis abilities and can survive 10-12 years in soil., and also they live dependent on the host plant (Yoneyama et al. 2010). After seed germination, broomrapes build up a retention organ called "haustorium," and they acquire energy by absorbing the water and nutrition from the host plant instead of photosynthesis. Consequently, physiology, quality, and yield problems appear in the host plant. Interestingly, the germination of broomrape seeds depends on the host plant. Broomrape seeds germinate and begin to cause damage due to a substance compound that spreads from the host plant under its favorable natural conditions. This substances that excudes from the host plant's roots is a group hormone called "strigolactone". Thus, broomrapes are depicted as "root parasites" (Fernández-Aparicio et al. 2020).

Strigolactones are one of the natural hormone groups. These are derived from the carotenoid pathway and restrains the development of shoots and roots in plants. *CCD7* (Carotenoid Cleavage Dioxygenase 7) and *CCD8* (Carotenoid Cleavage Dioxygenase 8) catalysts, associated with the primary phases of the strigolactone pathway, assume an essential function in the synthesis of strigolactone. *CCD7*, *CCD8*, and other genes that are involved in the cytoplasmic pathway produce strigolactones that play many physiological roles in plants. This pathway provides a signal mechanism that triggers the germination of parasitic broomrapes (Gomez-Roldan et al. 2008).

In the light of all this information, this study is aimed to develop resistance to *Orobanche* spp. / *Phelipanche* spp. in tomato with the CRISPR-Cas9 method. With the genetic manipulation of *CCD7* and *CCD8* genes, resistance to parasitic *Orobanche* was provided for the first time in our country using the CRISPR-Cas9 method. Two gRNAs were designed for the gene of interest and cloned into a specific codon-optimized binary vector (for dicotyl plants) harboring the *Cas9* gene. This vector was transferred to the tomato by the *Agrobacterium*-mediated transformation method and mutations were created in the *SlCCD7* and/or *SlCCD8* gene regions. As a result of the dysfunction of these genes, strigolactone production was disrupted. These resulting

possible broomrape-resistant mutants were qualified as valuable potential commercial breeding materials.

One of the other essential aims of the study is to develop sustainable, eco-friendly, and high in success methods instead of complex, overcosting, ecology-damaging, and underachievement methods such as fumigation, manual uproot, chemical control. In brief, it was aimed to provide resistance to broomrape that causes significant yield losses in tomato cultivated areas by using the CRISPR-Cas9 method.



## 2. LITERATURE REVIEW

### 2.1. Tomato (*Solanum lycopersicum*) Taxonomy, Distribution, Genetic and Physiological Features, and Statistical Values

Tomato, known by the scientific name "*Solanum lycopersicum*" in Latin, is classified as a *Solanum* genus species in the *Solanaceae* family (also known as the Nightshade family). It has more than 1500 species taxonomically in the plant kingdom (Darwin et al., 2003). Potatoes (*Solanum tuberosum*), eggplant (*Solanum melongena*), peppers (various *Capsicum* species), black nightshades (*Solanum nigrum*, *Solanum dulcamara*, etc.) are other important taxa of the *Solanaceae* family. Tomato was initially classified as *Lycopersicon esculentum* (*Lycopersicum esculentum* Miller) by Miller in 1754 and later renamed *Solanum lycopersicum* by Child in 1990 as a synonym (Diez and Nuez 2008).

The *Lycopersicon* genus is divided into two different sections (Table 2.1): "*L. hirsutum*-like" and "*L. peruvianum* and *L. chilense*-like" (Hobson and Grierson 1993).

**Table 2.1.** Two different divisions of the *Lycopersicon* genus (edited and quoted from Hobson and Grierson 1993)

| 1) <i>L. hirsutum</i> -like                                      | 2) <i>L. peruvianum</i> and <i>L. chilense</i> -like |
|------------------------------------------------------------------|------------------------------------------------------|
| <i>L. hirsutum</i> and <i>L. hirsutum</i> var <i>glabratum</i> . | <i>L. peruvianum</i>                                 |
| <i>L. pennellii</i>                                              | <i>L. peruvianum</i> var <i>humifusum</i>            |
| <i>L. esculentum</i>                                             | <i>L. chilense</i>                                   |
| <i>L. esculentum</i> var <i>cerasiforme</i>                      |                                                      |
| <i>L. cheesmanii</i>                                             |                                                      |
| <i>L. pimpinellifolium</i>                                       |                                                      |
| <i>L. parviflorum</i>                                            |                                                      |
| <i>L. chmielewskii</i>                                           |                                                      |

The story started with the utilization of tomato by the Aztecs in South America. However, there are numerous reviews of European acquaintance with tomato. One of the most sensible ones, the tomato, was sent by boats to Europe after Christopher Columbus discovered America (Rick 1951). Another view is that Hernán Cortés, a mariner, attacked Mexico for Spain's sake and met with the seeds of the tomato in the sixteenth century. Tomato was brought to Europe in the mid-nineteenth century, and from that point, it spread everywhere in the World (Esquinas-Alcázar and Nuez 1995) (Figure 2.1).

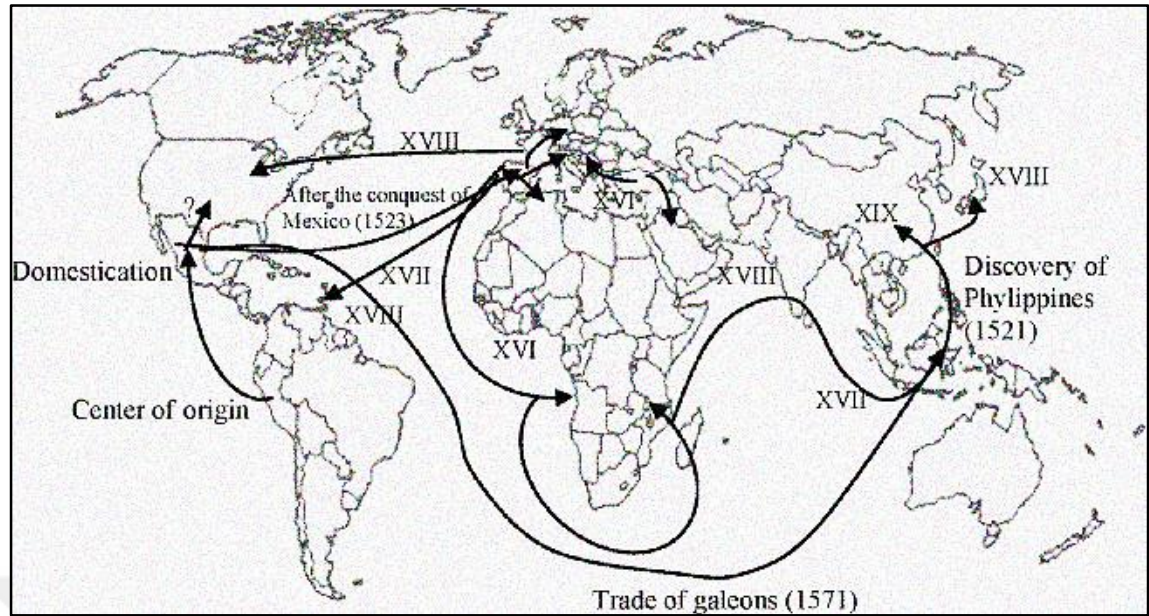


**Figure 2.1.** Potential spread of tomato in the 16th century (edited and quoted from Esquinas-Alcázar and Nuez 1995)

Tomato whose roots can go deep in the soil about 5 meters are dicotyledon (with double cotyledon in embryos), self-fertile (autogamy), annual plant. It has foreign fertilization feature (1-5%) in cases where the stigma and antenna are of different size (alogamy)). Also, it is hermaphrodite (which co-contain the male and female reproductive organs) flowers generally contain one female organ (pistil), five calycul (sepal), petals, male organs (anther) (da Silva et al. 2017). Tomato fruits contain substances that have significant human health benefits, such as carotenoids (lycopene,  $\beta$ -carotene, and lutein), phenolics (flavonoids, phenolic acids, and tannic), and vitamins (C, E, and B) (Ilahy et al. 2011). Tomato, which grows best in pH 5-7 and in light-loamy soil, is a fast-growing plant with an optimum growth time of 90-150 days, with an average daily temperature of 10-20 °C and a night temperature between 18-25 °C (Anonymous 2019). Tomatoes are assessing in various manners in the food business. The processing of tomato varieties due to high dry matter content, color, taste, and aroma are preferred and evaluated in domestic and foreign markets (Costa and Heuvelink 2018).

Tomato has  $2n$  (Diploid) = 24 chromosomes (Poczai et al. 2018). The complete genome sequence of tomato was obtained on the *Solanum lycopersicum* Heinz 1706 (*Solanum lycopersicum* cv. Heinz 1706). The tomato has about 35,000 genes (total 950 megabase pairs genome size and more than 75% of this region is heterochromatin region) were found in 12 chromosomes. They were determined with the "Sanger Sequencing Method" by the International Tomato Genome Sequencing Consortium (100 Tomato Genome Sequencing Consortium, 2014).

Tomato is exported, imported, and grown all over the World (Figure 2.2) (FAO 2019). With a share of 62%, tomato grows mainly in Asia (Figure 2.3) (FAO 2019). Moreover, their products worldwide are increasing day by day. Turkey, mainly located

on the Asian continent, is the third largest tomato production country after China and India (Figure 2.4) (FAO 2019).

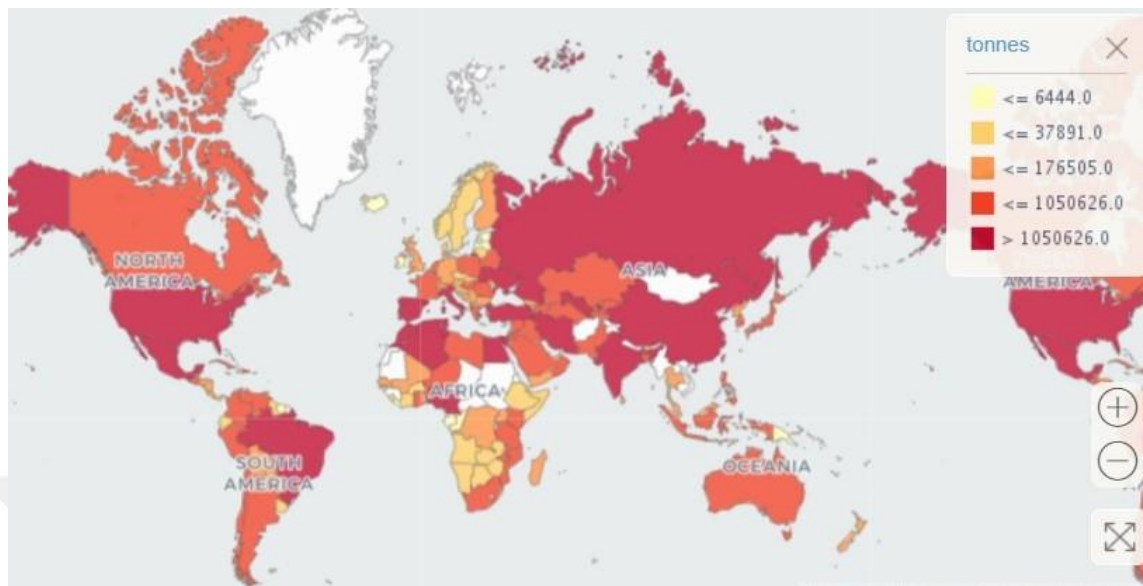


**Figure 2.2.** Places where tomatoes are produced worldwide (FAO 2019)

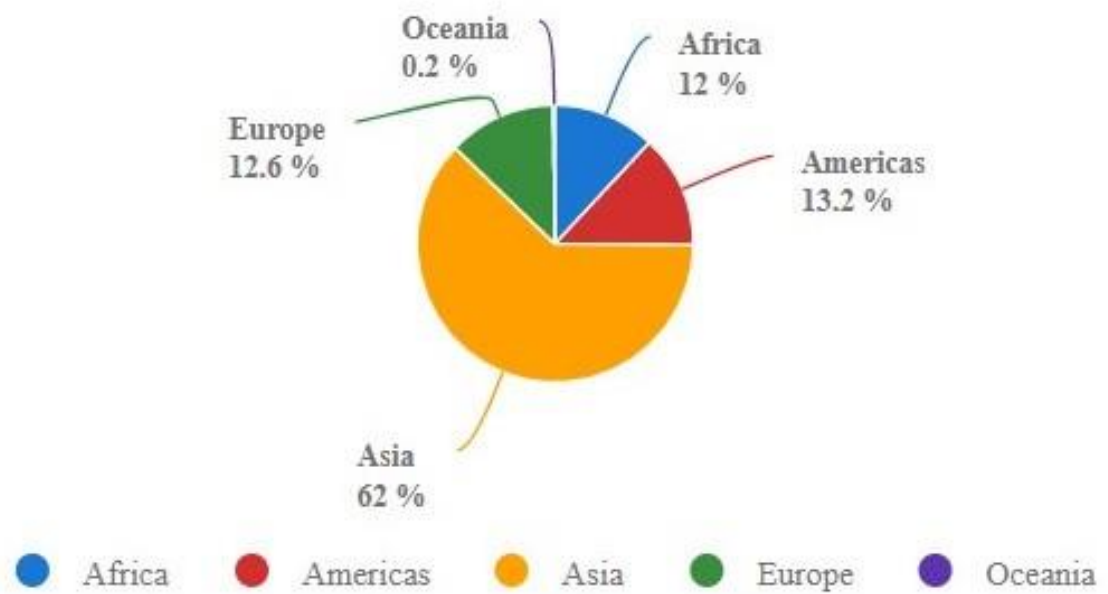


**Figure 2.3.** Continental shares of tomato production (FAO 2019)

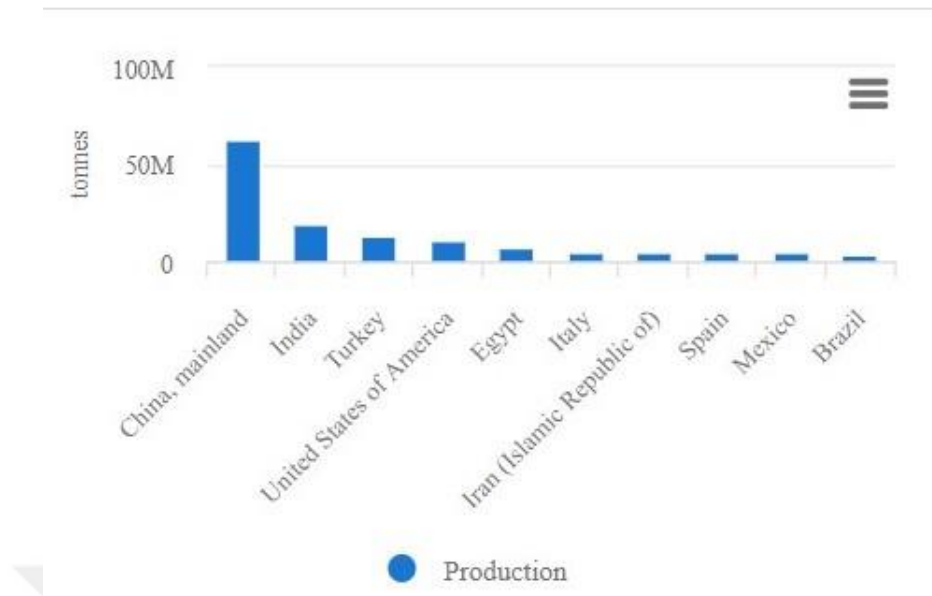


**Figure 2.4.** Ranking of tomato producing countries (FAO 2019)

Agricultural production is carried out in 181.488,00 hectares in Turkey. In this production area, approximately 12.8 million tons of tomatoes are produced, and the amount of production in our country is increasing every year (FAO 2019) (Table 2.2).

**Table 2.2.** Tomato production in Turkey by year (FAO 2019)

| Years                                          | 2004  | 2006  | 2008   | 2010   | 2012   | 2015   | 2016   | 2017   | 2018   | 2019   |
|------------------------------------------------|-------|-------|--------|--------|--------|--------|--------|--------|--------|--------|
| <b>Amount of Tomato Production (1000 Tons)</b> | 9.440 | 9.854 | 10.985 | 10.052 | 11.350 | 12.615 | 12.600 | 12.750 | 12.150 | 12.842 |

As a result of the potential of tomato production in our country, the number of products we export and the income we get from it also contribute to our country's economy (FAO 2019) (Figure 2.5).

| Domain Code | Domain                       | Area Code | Area   | Element Code | Element         | Item Code | Item     | Year Code | Year | Unit      | Value  | Flag | Flag Description |
|-------------|------------------------------|-----------|--------|--------------|-----------------|-----------|----------|-----------|------|-----------|--------|------|------------------|
| TP          | Crops and livestock products | 223       | Turkey | 5910         | Export Quantity | 388       | Tomatoes | 2019      | 2019 | tonnes    | 531243 |      | Official data    |
| TP          | Crops and livestock products | 223       | Turkey | 5922         | Export Value    | 388       | Tomatoes | 2019      | 2019 | 1000 US\$ | 301649 |      | Official data    |

**Figure 2.5.** Tomato exports and income in Turkey (FAO 2019)

In the absence of pesticides in agricultural production, approximately 65% of the yield is lost (Özmen 2004). Particularly in tomato development, loss of yield is caused by numerous factors, for example, viruses, molds, bacteria, mites, whiteflies, thrips, moths, and nematodes (Toksöz et al. 2018). Nevertheless, such methods cannot provide a long-term solution because weeds can develop a resistance mechanism against pesticides used over time (Despres et al. 2007). However, pesticides damage agricultural land, fisheries, fauna, vegetation, and disease, and mortality rates are increasing due to pesticides, especially in agricultural countries (Wilson and Tisdell 2001). In addition, there is evidence of chronic diseases such as various cancer types, diabetes, asthma, etc., in people exposed to pesticides (Mostafalou and Abdollahi 2013).

Pesticides-herbicides are used in all agricultural production areas in our country and the World (Figure 2.6). The herbicides, which have a share of 14.794 tons, are in the 2nd rank among the 600.20 tons of pesticides used in our country (FAO 2018) (Figure 2.7).

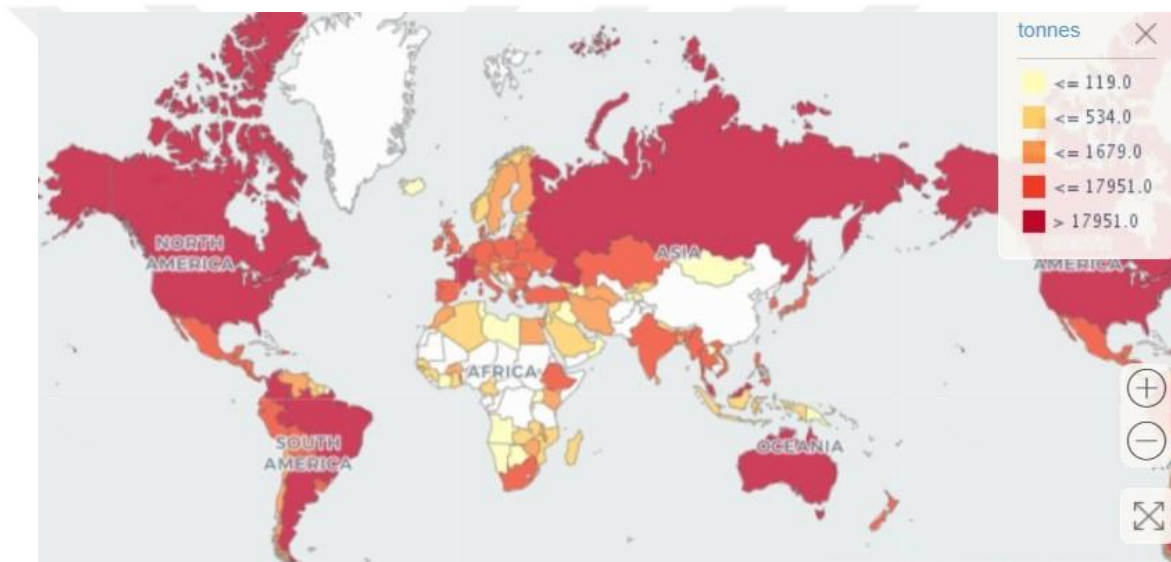


**Figure 2.6.** Distribution of herbicide usage in the worldwide (FAO 2018)

| Domain Code | Domain         | Area Code | Area   | Element Code | Element          | Item Code | Item       | Year Code | Year | Unit   | Value   | Flag |
|-------------|----------------|-----------|--------|--------------|------------------|-----------|------------|-----------|------|--------|---------|------|
| RP          | Pesticides Use | 223       | Turkey | 5157         | Agricultural Use | 1320      | Herbicides | 2018      | 2018 | tonnes | 14794   | Qm   |
| RP          | Pesticides Use | 5000      | World  | 5157         | Agricultural Use | 1320      | Herbicides | 2018      | 2018 | tonnes | 1216330 | A    |

**Figure 2.7.** The amount of herbicides usage in the World and Turkey (FAO 2018)

Turkey is dependent on the active substance, and about 90% of the materials utilized in the production of pesticides are imported (Şentürk 2007). Referring to FAO

(Food and Agriculture Organization) statistics, the pesticide importation value is more than the exportation value in Turkey (FAO, 2018) (Figure 2.8).

| Domain Code | Domain           | Area Code | Area   | Element Code | Element      | Item Code | Item               | Year Code | Year | Unit      | Value      | Flag |
|-------------|------------------|-----------|--------|--------------|--------------|-----------|--------------------|-----------|------|-----------|------------|------|
| RT          | Pesticides Trade | 223       | Turkey | 5622         | Import Value | 1357      | Pesticides (total) | 2018      | 2018 | 1000 US\$ | 465626.012 | I    |
| RT          | Pesticides Trade | 223       | Turkey | 5922         | Export Value | 1357      | Pesticides (total) | 2018      | 2018 | 1000 US\$ | 104145.593 | I    |

**Figure 2.8.** Export and import values of pesticides in Turkey (FAO 2018)

There is no natural resistance mechanism against every pest in agricultural production. For example, one factor that does not have natural resistance in tomato is “Broomrapes,” and it is crucial to develop resistance to prevent yield and quality losses (Kacan and Tursun 2012).

## 2.2. *Orobanche* spp. (Broomrapes)

### 2.2.1. Taxonomy and native range of *Orobanche* spp.

Broomrape is one of the most harmful species of weeds (Wegmann 1998) (Figure 2.9), and it has spread almost all over the World (Figure 2.10). It was first cultivated by Linnaeus in 1753 and named after the Greek Orobus (Linnaeus 1753). However, phylogenetic birthplaces and scientific classifications of Broomrapes, referred to in Turkey with names, for example, "canavar otu, sümbül," are questionable (Aksoy and Pekcan 2014). As a result of molecular characterization studies, the *Orobanche* genus divides into two genera: 1. *Orobanche*, 2. *Phelipanche* (Teryokhin 1997). Distribution of the primary chromosome number (*Phelipanche* group ( $x = 12, 24$ ; *O. sects. Gymnocaulis, Myzorrhiza, and Trionychon*), *Orobanche* group ( $x = 19$ ; *Diphelypaea, O. sect. Orobanche*), *Cistanche* ( $x = 20$ )) and chromosome morphology shows the presence of 3 phylogenetic genealogies. It has additionally been resolved that polyploidization assumes an essential function in the evolution of these groups (Schneeweiss et al. 2004). Broomrapes are taxonomically classified as the order of Laminales, *Orobanche* L. genus found in the *Orobanchaceae* family, and it has been determined that the *Orobanche* genus contains more than 170 holoparasitic species (Uhlich et al. 1995). *Orobanche* is spread all through subtropical and mild districts of the northern side of the equator, with the Mediterranean area one of the main habitats of variety (Plaza et al. 2004).



**Figure 2.9.** Broomrapes (*Orobancha* spp.) and their hosts “tomato”

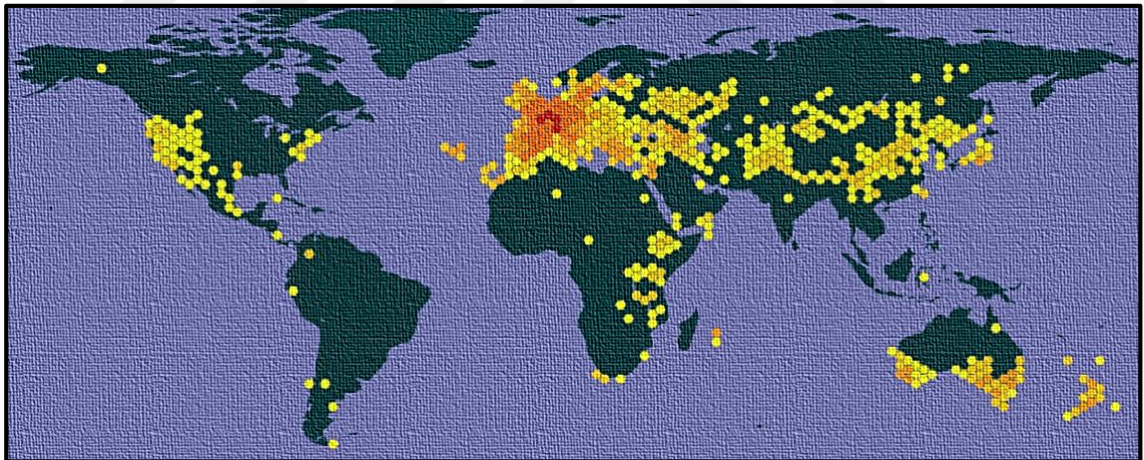


**Figure 2.10.** *Orobancha* distribution areas around the world (edited and quoted from GBIF 2015)

In many parts of the world with a Mediterranean climate, Broomrapes broadly accessible, specifically Turkey, Italy, Spain, and Morocco, are known as the parasite plants' native soils (Sauerborn 1991). The flora of Turkey includes 38 different naturally grown *Orobancha* species (Gilli 1982). There are over 40 known species in Turkey, from around 170 Broomrape species found worldwide, some of which are endemic to Turkey (Bakis et al. 2011) (Table 2.3).

**Table 2.3.** *Orobanch* species in Turkey (edited and quoted from Bakis et al. 2011)

| Taxon Name                        | Local Name                          | Hosts                                                                                                                          | Range in the Turkey                | Endemism       |
|-----------------------------------|-------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|------------------------------------|----------------|
| 1. <i>Orobanch aegyptiaca</i>     | Dinlendiren, Mısırlı Canavar Otu    | <i>Gossypium herbaceum</i> , <i>Solanum melongena</i> , <i>Solanum lycopersicum</i> , <i>Cucumis sativus</i> , <i>Brassica</i> | throughout Turkey                  |                |
| 2. <i>Orobanch alba</i>           | Boğasak                             | <i>Labiatae</i>                                                                                                                | throughout Turkey                  |                |
| 3. <i>Orobanch amethystea</i>     | Yitik Canavarotu                    |                                                                                                                                |                                    |                |
| 4. <i>Orobanch anatolica</i>      | Ana Canavarotu                      | <i>Salvia</i>                                                                                                                  | Anatolia                           |                |
| 5. <i>Orobanch arenaria</i>       | Deli Canavarotu                     | <i>Artemisia</i>                                                                                                               | North and South-West Anatolia      |                |
| 6. <i>Orobanch armena</i>         | Has Canavarotu                      |                                                                                                                                | North-East Anatolia                | <b>Endemic</b> |
| 7. <i>Orobanch bungeana</i>       | Kernek Canavarotu                   | <i>Alyssum</i>                                                                                                                 | North-East and West Anatolia       |                |
| 8. <i>Orobanch caesia</i>         | Mavi Veremotu                       | <i>Artemisia</i>                                                                                                               | East Anatolia                      |                |
| 9. <i>Orobanch caryophyllacea</i> | Kokulu Süpürgeotu                   | <i>Galium</i> , <i>Asperula</i>                                                                                                | North-West and South-East Anatolia |                |
| 10. <i>Orobanch caucasica</i>     | Kaf Canavarotu                      |                                                                                                                                | South Anatolia                     |                |
| 11. <i>Orobanch cernua</i>        | Deli Yergöbeği, Boğumlu Canavar Otu | <i>Compositae</i> , <i>Solanaceae</i> , <i>Polygonum maritimum</i>                                                             | throughout Turkey                  |                |
| 12. <i>Orobanch cilicica</i>      | Toros Veremotu                      |                                                                                                                                | Anatolia                           |                |
| 13. <i>Orobanch coelestis</i>     | Gök Zıpirotu                        |                                                                                                                                | Anatolia                           |                |
| 14. <i>Orobanch coerulescens</i>  | Gök Canavarotu                      |                                                                                                                                |                                    |                |

(Continues on next page)

Continuation of Table 2.3

|                                   |                                     |                                       |                                     |                |
|-----------------------------------|-------------------------------------|---------------------------------------|-------------------------------------|----------------|
| 15. <i>Orobancha crenata</i>      | Zıpirotu, Beyaz Çiçekli Canavar Otu | <i>Leguminosae</i>                    | South and East Anatolia             |                |
| 16. <i>Orobancha elatior</i>      | Boylu Canavarotu                    | <i>Genellikle Centaurea, Echinops</i> | North-East, South and East Anatolia |                |
| 17. <i>Orobancha fuliginosa</i>   | İsli Canavarotu                     |                                       | Anatolia                            |                |
| 18. <i>Orobancha gamosepala</i>   | Yel Canavarotu                      |                                       | North-East Anatolia                 |                |
| 19. <i>Orobancha gracilis</i>     | Yergöbeği                           | <i>Leguminosae</i>                    | South-West Anatolia                 |                |
| 20. <i>Orobancha grisebachii</i>  | Deli Veremotu                       |                                       | North and West Anatolia             |                |
| 21. <i>Orobancha hadroantha</i>   | Has Yergöbeği                       |                                       | Anatolia                            | <b>Endemic</b> |
| 22. <i>Orobancha hederae</i>      | Tez Canavarotu                      | <i>Hedera</i>                         | North-East and West Anatolia        |                |
| 23. <i>Orobancha heldreichii</i>  |                                     |                                       | West Turkey                         |                |
| 24. <i>Orobancha kurdica</i>      | Şark Baklakıranı                    | <i>Labiatae, Compositae</i>           | East Anatolia                       |                |
| 25. <i>Orobancha lavandulacea</i> | Lavantakıran                        |                                       | North and West Anatolia             |                |
| 26. <i>Orobancha lutea</i>        | Sarı Canavarotu                     | <i>Leguminosae</i>                    | North Anatolia                      |                |
| 27. <i>Orobancha minor</i>        | Göveotu                             | <i>Leguminosae</i>                    | North-West Turkey, Anatolia         |                |
| 28. <i>Orobancha mutelii</i>      | Baklakıran                          |                                       | throughout Turkey                   |                |
| 29. <i>Orobancha nana</i>         | Veremotu                            |                                       | throughout Turkey                   |                |
| 30. <i>Orobancha oxyloba</i>      | Kazıkotu                            | <i>Compositae</i>                     | throughout Turkey                   |                |
| 31. <i>Orobancha palaestina</i>   | Alukotu                             |                                       |                                     |                |

(Continues on next page)

Continuation of Table 2.3

|                                   |                                                     |                                                                                      |                                            |                |
|-----------------------------------|-----------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------|----------------|
| 32. <i>Orobanche picridis</i>     | Papatyakıran                                        | <i>Compositae</i> ,<br><i>Umbelliferae</i><br>( <i>Eryngium campestre</i> )          | West Anatolia                              |                |
| 33. <i>Orobanche pubescens</i>    | Tüylü Kazıkotu                                      | <i>Umbelliferae</i><br>( <i>Smyrnum</i> ),<br><i>Compositae</i>                      | West Turkey,<br>South and East<br>Anatolia |                |
| 34. <i>Orobanche purpurea</i>     | Mor Veremotu                                        | <i>Anthemis</i> , <i>Artemisia</i><br><i>Achillea</i>                                | North-West<br>Turkey, Anatolia             |                |
| 35. <i>Orobanche ramosa</i>       | Narin<br>Canavarotu, Mavi<br>Çiçekli Canavar<br>Otu | <i>Cannabis</i> ,<br><i>Nicotiana</i> ,<br><i>Lycopersicon</i> ,<br><i>Armoracia</i> | Thrace and<br>Anatolia                     |                |
| 36. <i>Orobanche rechingeri</i>   | Kır Canavarotu                                      | <i>Alyssum</i> Sect.<br><i>Odontarrhena</i> ,<br><i>Pinus Nigra</i>                  | Anatolia                                   |                |
| 37. <i>Orobanche reticulata</i>   | Kayıp Veremotu                                      |                                                                                      |                                            |                |
| 38. <i>Orobanche schultzei</i>    | Kırk Veremotu                                       |                                                                                      | throughout Turkey                          |                |
| 39. <i>Orobanche serratocalyx</i> | Dişli Yergöbeği                                     |                                                                                      | North-east and<br>West Anatolia            |                |
| 40. <i>Orobanche sideana</i>      | Side Canavarotu                                     | <i>Dunes</i>                                                                         | South Anatolia                             | <b>Endemic</b> |
| 41. <i>Orobanche sintenisii</i>   | Fırat Canavarotu                                    | <i>Centaurea</i> , <i>Saluna</i>                                                     | East Anatolia                              |                |

### 2.2.2. Hosts of *Orobanche* spp. and their control methods

Although broomrapes are parasitic for many plant families worldwide, such as Solanaceae, Asteraceae, Fabaceae, Cucurbitaceae, Apiaceae, etc., some *Orobanche* species infect only certain plant species, while some are parasitic to different species belonging to a particular or different plant family (Parker 2013). Also, although the *Orobanche*-host relationship is dependent on environmental conditions, it can be parasitic for certain plants growing in one season. However, it may not be parasitic for the same host at another time of the same year (Jacobsohn 1984).

Broomrapes cause severe product losses in the Mediterranean climate zone, especially in crops such as broad bean, lentil, and chickpea (Aksoy and Pekcan 2014). *Orobanche ramosa* L. and *Orobanche aegyptiaca* damage advanced plants such as

Persian, lentils, and beans from the Fabaceae family and tomatoes, eggplants, and potatoes from the Solanaceae family. *Orobancha crenata* Fork. damages many vegetables, especially lentils and beans from the Fabaceae family. (Kadioğlu 2009).

Weeds, which caused fundamental problems such as loss of yield, also caused a significant yield loss in tomato in our country, especially 4 *Orobancha* species (*Orobancha aegyptiaca*, *Orobancha ramosa*, *Orobancha crenata*, *Orobancha cernua/cumana*). They cause severe yield losses and cause economic damage, especially in tomato, sunflower, lentils, broad bean, eggplant, and tobacco (Bülbül et al. 2009). The average yield loss occurs because the Broomrapes constitute approximately 30% of the total losses caused by other factors that restrict agricultural production (Hershenhorn et al., 2009). The product loss has occurred worldwide substantially due to the damage of *Orobancha ramosa* (blue-flowered broomrapes). One of the most dangerous broomrape species in the production areas of the tomato and another critical type of broomrape for tomato is *Orobancha/Phelipanche aegyptiaca* (Pers) Pomel (Babaei et al. 2010). As a result of the studies carried out in Turkey, *Orobancha ramosa* had an average yield loss of 24% in tomato. *Orobancha crenata* had an 82% loss in the common bean (Aksoy and Uygur 2008). As the parasite plants absorb/use nutrients such as water, minerals, and organic compounds in the host plant, the growth of the host slows down, and the yield decreases or goes down to zero in severe invasion conditions (Sauerborn 1991). Loss of crop yield in areas without weed control is considered 43% on a global scale (Oerke 2006). The yield losses in host plants generally vary between 5% and 100%, and the average total loss caused by all *Orobancha* species is determined to be around 34% (Linke et al. 1989).

*Helianthus petiolaris*, one of the wild sunflower species, shows natural resistance to *Orobancha*, and natural resistance has not yet been discovered in other host species (Poverene et al. 2014). For this reason, physical, chemical, and biological methods are used in controlling against *Orobancha*. However, since the control methods for *Orobancha* are not economical, they create severe problems in this regard (Dhanapal et al. 1996). Due to the increasing environmental awareness in today's world, the dangers associated with chemicals worry scientists and society. Despite the disadvantages of physical and chemical control methods, such as difficulty, cost, environmental damage, and low chance of success (Figure 2.11), it has become essential to develop permanent methods that are environmentally friendly and successful. Especially in organic agriculture, which has great popularity today, weed control is the biggest obstacle for growers (Abouziena and Haggag 2016). Although there are physical controls in the tomato cultivated areas such as mechanical control methods (hand plucking), submerging, or frequent changing of the tomato cultivation areas, these methods have been reported to have a low chance of success (Bayram and Çıkman 2014). In essence, it is pretty challenging to control parasitic plants by agricultural practices and herbicides, as most of the parasite's life cycle occurs under the ground depending on the host plant (Goldwasser et al. 2003). Therefore, soil solarization method is the most effective in the open areas in the fight against *Orobancha* spp. However, solarization application has limitations, difficulties, and negative aspects such as limited use only in regions and soil conditions where the climate is suitable, expensive for some agricultural products, and ineffective in controlling some diseases (Katan 1981).

Besides, different chemical pesticides are also used with active ingredients such as Imidazolinones in legumes, Glyphosate in Apiaceae and Fabaceae family, Imazapyr, and Acetolactate Synthase in sunflowers (Alonso 2014). Today, the only active-chemical licensed for broomrape is imazamox.

In certain conditions, even though herbicides control the parasite, it is respectably particular for the host. For instance, as in sunflower, imazapyr application does not hurt vegetative development; however, high dose application disturbs generation since it causes harm in the main blossoming stage. In any case, pesticides can be harmful to the climate (Habimana et al., 2014). The results of many studies conducted in our country reveal that there are pesticide residues in many areas, including water resources (Doğan and Karpuzcu 2019). The use of pesticides results in 20,000 deaths each year, and chemical pesticides have many adverse effects on human physiology (WHO 2020). Although biocontrol agents such as *Phytomyza orobanchia* are more reliable than other methods, it has been determined that it shows 30-80% success, therefore it is not a definitive solution method (Klein and Kroschel 2002). Their ability to produce large numbers of seeds that can survive in the soil for a long time even in adverse environmental conditions and their access to the soil surface is much longer than non-parasitic weeds, making the early detection and control of *Orobanche* difficult (Aly 2007).



**Figure 2.11.** Insufficient and unsuccessful control methods against *Orobanche* spp. in tomato cultivated areas

### 2.2.3. Biology of *Orobanche* spp.

Parasitic plants require complete host interactions in the early stages of development compared to other plants (Sürmen et al. 2014). Regarding functional

chloroplasts and photosynthetic activity, parasitic plants are divided into two groups; hemiparasites or holoparasites. In this context, *Orobanche* spp. and *Phelipanche* are holoparasitic, parasitic herbs that are completely dependent on their hosts in terms of water and nutrients (Young and dePamphilis 2000). Due to the absence of chlorophyll in the broomrapes, color occurs with the more pronounced accumulation of anthocyanins (Wheldale 1916). Broomrapes are perennial plants that reproduce via seeds, and their life cycle consists of 3 main phases: propagation of seeds, germination, and parasitic stage (Dhanapal et al. 1996). After the emergence of *Orobanche* shoots, it produces racemes that have hundreds of thousands of “dust-like” seeds challenging to detect in the soil (Joel et al. 2007).

Since broomrapes spend most of their life cycles undersoil, their detection in cultivated areas is quite tricky (Eizenberg et al. 2012). These parasitic plants are characterized by the smallest seeds with a seed length of less than 1 mm in the earth flora (Piwowarczyk and Jankowska-Błaszczuk 2014). Seed morphology is determined as an appropriate criterion to reveal the difference between *Orobanche* and *Phelipanche* species (Deif et al. 2000). It was determined by electron microscopy that *Orobanche* seeds have a granular, fiber-free, and smooth periclinal surface (Krupp et al. 2015) (Figure 2.12).

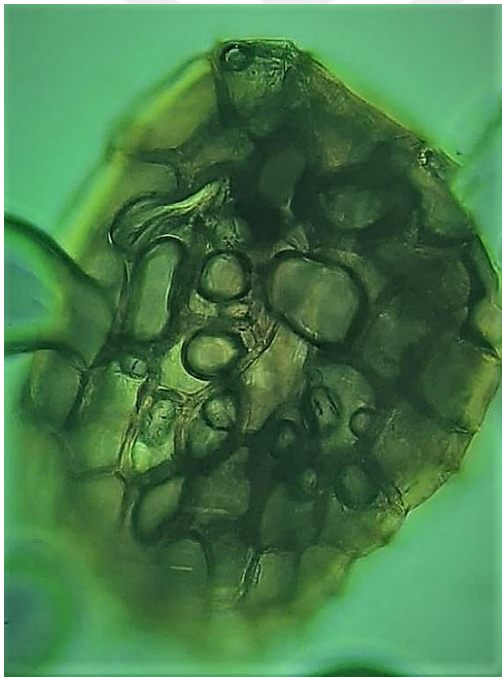


**Figure 2.12.** *Orobanche* spp. seed viewed under microscope

*Orobanche* has bisexual flowers, produces seeds by self-pollination or fertilizing with controlled hybridization (Ephrath and Eizenberg 2010). Their seeds have a brown color and oval shape and are very small in size. Furthermore, they can be easily dispersed by wind, water, or other factors (Dhanapal et al. 1996). Broomrapes can produce more than 50,000 seeds, which can live for more than decades in the soil (Gevezova et al. 2012). Broomrapes are defined as root parasites that cause significant losses in agricultural production worldwide. Because germinating *Orobanche* seeds will

lose their vitality in a short time if they do not attach to the host plant roots in a few days (Kohlen 2011). The seeds of these root parasites are usually dormant under the ground (dormancy) and germinate only when released stimulant from the roots of the host in favorable soil and climatic conditions (Brown et al. 1949). The seed germination occurs thanks to the compound called “strigolactone,” released by the host roots and defined as germination stimulants (Cook et al. 1966). Thanks to the stimulant, germ-tube (radicule) appear first due to germination, haustorium formation and development, attachment to the roots of the host, and vascular tissue penetration, respectively (Musselman 1980). In response to the signals in the roots, the germ tube undergoes a series of cell differentiation processes to create a structure called appressorium (Frank 1883). After the seeds are stimulated, they develop a tiny seedling attached to the root of the host and differentiates from this attachment organ (appressorium). After germination, the seedlings are attached to the roots of their hosts by forming a particular nutrition structure called “haustorium” (Habimana et al. 2014). The haustorium organ, specific to parasitic angiosperms, has three primary functions: binding to host roots, penetration, and water-soluble substance acquisition (Visser and Dorr 1987).

In the *Orobanche* parasite plant, the haustorium comprises the essential haustorium, which has a little tube-like structure for connection with the host root and auxiliary haustorium, wherein there is more contact with the secondary host roots (Rajanna et al. 2005). This organ is known to contain parenchymal cells with organelles such as mitochondria, ribosomes, endoplasmic reticulum (Visser and Dorr 1984). The stage in which the haustorium cells formed by the broomrapes penetrate the hosts' root tissues and attach the parasite plant to the host plant's vascular system (consisting of xylem and phloem) is identified as a critical step in parasitism (Losner-Goshen et al. 1994). When in contact with the host's root, the haustorium forms and penetrates the phloem and xylem to reach growth regulators carried by the host plant (Stewart and Press 1990). The parasite penetrates the roots of the plant's host and transforms it into a flower and seed-producing structure by providing the necessary energy as if it had made photosynthesis (Westwood 2000). About fourteen days after infiltration, the parasite reaches a 5-20 mm diameter, transforming into a tubercle structure with a shoot bud on the root surface, which will later bloom (Hassan et al. 2004). With the formation of tubercles that store most photosynthesis products, the growth and yield of the host plant begin to decrease (Surov et al. 1998). Also, by developing secondary roots on the tubercle, it can communicate separately with the host plant's root system, and after a few weeks, it develops a shoot that can bloom on the soil surface (Díaz et al. 2006). Parasitic plants then quickly form encapsulated flowers containing hundreds of thousands of tiny seeds (Foy et al. 1989).

One of the first stages targeted for genetic control of plant parasitic caused by *Orobanche* and other root parasites is seed germination, a highly complex procedure (Goldwasser and Yoder 2001). Strigolactones are stated as the most potent stimulant that induces germination at  $\leq 10$  pM (Kim et al. 2010).

### 2.3. Strigolactones

*Strigolactones* (SLs) are chemical compounds produced by the plant's roots and are classified as plant hormones or phytohormones due to their mechanism of action (Bouwmeester et al. 2003). The germination stimulant (*Striga lutea* parasite plant seeds)

belonging to strigolactones was identified for the first time by isolating the substance called "Strigol" from the roots of the cotton (*Gossypium hirsutum* L.) (Cook et al. 1966). In the following years, germination stimulants with SL derivatives such as Orobanchol, Alectrol Strigolactone were isolated from the clover broomrape (*Orobanche minor*) (its host red clover (*Trifolium pratense*)) and determined that these stimulate the seed germination of *Orobanche* spp. and *Striga* spp. (Hirayama et al. 1999). Thus, strigolactones are a crucial element in triggering symbiotic interactions with arbuscular mycorrhizal (AM) fungi and germinating root parasitic plant seeds belonging to the Orobanchaceae family. Besides, it performs as a phytohormone to inhibit plant shoot branching by suppressing the growth of axillary buds (Gomez-Roldan et al. 2008).

### 2.3.1. Strigolactone (SL) biosynthesis pathway

Ingredients called "secondary metabolites" (natural products) are produced by plants that are not photosynthesis products, produced by physiological mechanisms, and not necessary for critical physiological events such as photosynthesis or respiration (Thesis and Lerda 2003). Many secondary metabolites are synthesized based on primary, and they can be transported within the plant via xylem, phloem, and apoplast (Akula and Ravishankar 2011). These metabolites play a role in many different ways, such as attractants for animals that play a role in pollination, as a mechanism to protect the plant against pathogens, and chemicals that affect germination, growth, survival, reproduction (Allelopathy) of an organism, etc. (Croteau et al. 2000). *Orobanche* seeds also germinate in the soil in response to germination stimuli, a secondary metabolite, which is synthesized only by the roots of the host under favorable climatic and soil conditions in the rhizosphere region of the host plant (Graves 1995).

Although there is no fixed classification method for secondary metabolites, it is classified based on their biosynthetic origin. These are: (a) Terpenoids (terpenes), (b) Flavonoids and allied phenolic-polyphenolic compounds, (c) Nitrogen-containing alkaloids, and sulfur-containing compounds and terpenes are crucial metabolites that have many types (Crozier et al. 2006). Terpenes are responsible for plant growth/development (phytohormones) and the interaction of plants with the environment. *Terpenes* are an isoprenoid derivative secondary metabolite found in almost all organisms and have numerous essential functions such as structural (cholesterol), functional (carotenoids), etc. (Tholl 2006). Plant terpenoids with a large chemical diversity are produced in the cytosol, plastids, and mitochondria. All terpenoid derivatives are biosynthesized basically from (C<sub>5</sub>H<sub>8</sub>)<sub>n</sub> isoprene (2-methyl-1,3-butadiene) units (substrate) (i.e., isopentenyl diphosphate (IPP) and isomer dimethylallyl diphosphate (DMAPP)) (Mander and Liu 2010). By forming chemical bonds of DMAPP with one or more IPP molecules in different ways, it forms geranyl diphosphate (C<sub>10</sub> / GPP), farnesyl diphosphate (C<sub>15</sub> / FPP), or geranylgeranyl diphosphate (C<sub>20</sub> / GGPP). Also, carotenoid precursors, such as beta-carotene, are formed by the molecular combination of FPP and GGPP (Liang et al. 2002). Carotenoids are characterized as tetraterpenes, having a 40-carbon structure, predominantly combined from mevalonate precursors and arisen from isoprenoid phytoene (Seigler 1998). DMAPP, catalyzed from acetyl CoA or pyruvic acid, formed the colorless carotenoid phytoene with C<sub>40</sub> structure after forming the GPP and GGPP structures, respectively. Phytoene creates lycopene by desaturation thanks to the

phytoene desaturase enzyme and ultimately produces carotenoids ( $\alpha$ -carotene and  $\beta$ -carotene) via the lycopene cyclase enzyme (Maoka 2020). Carotenoids are the only naturally occurring tetraterpen, and approximately 600 carotenoids are found in fungi, bacteria, and algae (Tapiero et al. 2014). Carotenoids are organic pigments found in chloroplasts and chromoplasts in plants and other photosynthetic organisms and play an essential role in synthesizing the hormone abscisic acid in plants (Nambara and Marion-Poll 2005). The most common tetraterpenes in nature; lycopene is  $\alpha$ -  $\beta$ - and  $\gamma$ -carotene, and  $\beta$ -carotene is the most known tetraterpene variety due to its connection to Vitamin A (Weeks 1986).

$\beta$ -Carotene (all-trans- $\beta$ -carotene) is a polyene chain that was first isolated from carrot roots by Wackenroder in 1831. It has a planar structure, all-trans, conjugated double bonds bound to methyl groups, and a  $\beta$ -ionone ring with both ends.  $\beta$ -Carotene is a hydrocarbon with 11 conjugated double bonds and molecular formula  $C_{40}H_{56}$ , a biologically active substance that plays an essential role in the structure of pro-vitamin A (Zabodalova 2009). In addition, many plants in the rhizosphere can have essential functions such as secondary metabolites, chemical signals. Among these, Strigolactones (SLs), which act as phytohormones (endogenic) and signal molecules (exogenic) in the rhizosphere, are plant metabolites that are the most critical carotenoid ( $\beta$ -Carotene (all-trans- $\beta$ -carotene)) derivatives and are classified as terpenoid lactones (sesquiterpene lactones) (Waldie et al. 2014).

The carotenoid-derived compounds such as strigolactones and abscisic acid share common structural similarities (Parry and Horgan 1992). Strigolactones are formed by tricyclic lactone (ABC ring) and a hydroxymethyl butenolide (HMB, D ring) structurally linked by an enol ether bond (Figure 2.13), and this D ring is common to all-natural strigolactones. In addition, while the ABC part of SLs can be modified, the D ring is a critical element for the biological functions of SL, and any change may affect the SL activity (Umehara et al. 2015). While the C and D rings show maximum consistency, the A and B rings with maximum deflection have one or more hydroxyl, acetylonyl, or methoxyl groups (may also contain epoxy- and oxo-) (Xie et al. 2010). Whereas the D ring found in synthetic SL analogs (such as GR24, GR7, and GR5) is modified from the C-2 position, in all-natural SL types, the R configuration at the C-2 'position of the D-ring is required for germination and the AM fungal branching factor (Zwanenburg et al. 2016).

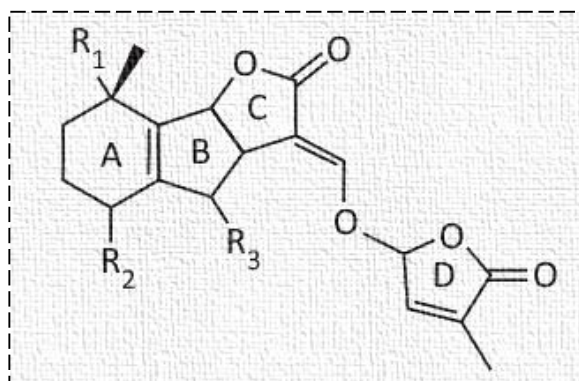


**Figure 2.13.** Basic structure of strigolactone (edited and quoted from Ruyter-Spira et al. 2013)

It was determined for the first time, SLs were derived from a carotenoid precursor in abscisic acid (ABA) deficient maize mutants (Matusova et al. 2005). Zhang et al. (2014) described the SL pathway and mentioned that it was synthesized in several steps as follows: 4 essential enzymes (*D27*, *CCD7*, *CCD8*, *P450*) are involved in the SL biosynthesis pathway, and 3 of them (*CCD7*, *CCD8*, *P450*) have been identified in *Arabidopsis thaliana*. Strigolactones are synthesized by a primary mechanism in all plants with plastid. The following steps can explain this main mechanism: 1. the conversion of all-trans- $\beta$ -carotene to 9-cis- $\beta$ -carotene by the  $\beta$ -carotene isomerase enzyme encoded by the Dwarf27 (*D27*) gene, in the plastid; 2. from the 9-cis- $\beta$ -carotene, 9-cis- $\beta$ -apo-10'-carotenal is formed through the Carotenoid Cleavage Dioxygenases 7 (*CCD7*) enzyme; 3. "Carlactone" (precursor molecule for SLs synthesis) is formed as a result of the processing of this product by Carotenoid Cleavage Dioxygenases 8 (*CCD8*) enzyme (These three stages occur in plastids); 4. various SLs are produced from Carlactone with the aid of the activity of the cytochrome *P450* enzyme in the cytosol (Cheng et al. 2013) (Figure 2.14).

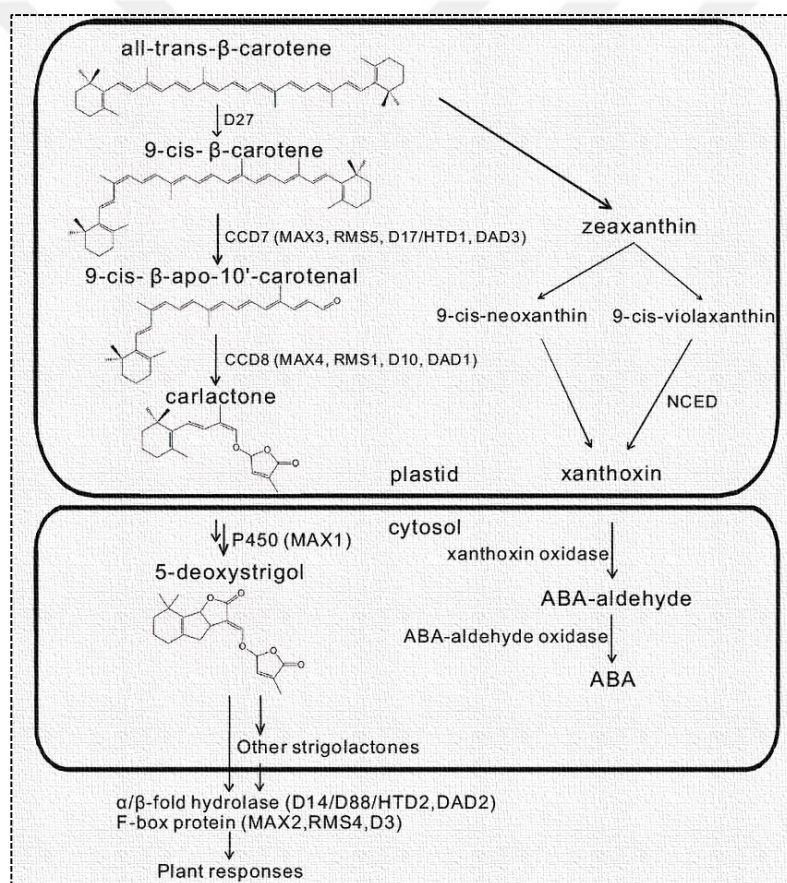


**Figure 2.14.** The pathway of strigolactone biosynthesis (edited and quoted from Cheng et al. 2013)

### 2.3.1.1. Genes involved in strigolactone synthesis

*MAX1*, *MAX3/ RMS5/ D17*, and *MAX4/ RMS1/ D10* genes play a role in the biosynthesis of strigolactones, and *MAX2/ RMS4/ D3* genes play a role in the strigolactone signaling mechanism and it is called "MAX/RMS/D" pathway (Gomez-

Roldan et al. 2008). Furthermore, the *D27* plays a role in early-stage SL biosynthesis due to the reduction of an SL species called 2'-epi-5-deoxystrigol in the root secretions and excessive growth in shoot buds of the *d27* rice (*Oryza sativa*) mutant (Lin et al. 2009).

Bruno and Al-Babili (2016) reported that *D27* is an essential iron-containing polypeptide in the production of SL, and also, in rice, this gene (*OsD27*) is a  $\beta$ -carotene isomerase catalyzing the conversion of all-trans- $\beta$ -carotene to 9-cis- $\beta$ -carotene. Besides, the *OsD27* can also contribute to forming  $\alpha$ -carotene-based strigolactone-like compounds by providing isomerization of the  $\alpha$ -carotene.

In another study conducted by Abauf et al. (2018) with the *AtD27* gene, the *D27* ortholog in *Arabidopsis* proved that *AtD27* catalyzed the all-trans-9-cis- $\beta$ -carotene isomerization but was not able to isomerize 13-cis- or 15-cis-  $\beta$  carotene. Instead, the  $\beta$ -carotene isomerase is isomerized (catalyzed) of all-trans- $\beta$ -carotene into a 9-cis- $\beta$ -carotene reversible manner, and this reaction is particular for the C9-C10 double bond as in the study with rice. Thus, the *AtD27* gene has been proven to function in phosphate starvation and the response mechanism of multiple plant hormones. This context explains the role of *AtD27* in abscisic acid biosynthesis and that the expression of *AtD27* is regulated by auxin and ABA hormones.

Wang et al. (2018) stated that ABA and SL are derived from the same origin and that the *HvD27* isomerase activity differentiates the SL and ABA pathways from the study conducted in *Hordeum vulgare*. It has also been stated that ABA can inhibit the SL biosynthesis pathway by affecting *D27* activity, and as a result, the increase in the all-trans- $\beta$ -carotene precursor can turn the pathway against ABA and achieve internal hormone balance. Two possible consequences: (I) ABA suppressed the SL biosynthesis pathway only by regulating *HvD27*, (II) supported the ABA biosynthesis through higher availability of the precursor, thereby indirectly participating in the ABA regulation (due to the reversible isomerization property of *D27*) (Endo et al. 2014). Furthermore, as a result of another study, it was found that SL biosynthesis increased in response to sulfur deficiency, and this was due to the increase in *D27* gene expression (Shindo et al. 2018).

Catalysis of carotenoids to apocarotenoids is provided by the gene family of CCOs (Carotenoid Cleavage Oxygenases). In this context, two types of carotenoid oxygenase have been described; 9-cis epoxythrotene dioxygenases (NCEDs) and carotenoid cleavage dioxygenases (CCDs). The CCD family is a non-hem iron enzyme that cuts the C-C double bonds and enables the production of carbonyl products by including dioxygen. On the other hand, NCED enzymes catalyze by clearing 9-cis-violaxanthin or 9-cis-neoxanthin, the first stage in abscisic acid (ABA) biosynthesis. Phylogenetic analysis shows that CCDs were found to be distant relative to NCEDs. On the other hand, their substrate specificities and activities are different (Priya and Siva 2014). *CCD1* and *CCD4* play a role in the production of apocarotenoid-derived pigments and the formation of flavor and/or flavor compounds in flowers and various foods by degrading carotenoids (Gonzalez-Jorge et al. 2013).

*CCD7* and *CCD8* proteins which are chloroplastic (localized in plastids), were conserved genes involved in the strigolactone pathway. Exon/intron is preserved in all

the *CCD7* and *CCD8* genes. However, the number of exons in *CCD7* varies from 6 to 9, while *CCD8* has 5 or 6 exons in dicotyledon types and 3 to 5 exons in monocotyledons (NCBI 1988).

The amino acid number of the sequences ranged from 823 to 2002 residues. Besides, it was shown that while *MAX3* (*AtCCD7*), *RMS5* and *HTD1/D17* encode *CCD7*, *MAX4* (*AtCCD8*), *RMS1*, *D10*, and *DAD1* encode *CCD8* in the studies performed in *Arabidopsis* using axillary growth (*max*) mutant, pea *ramosus* (*rms*) mutant, petunia decreased apical dominance (*dad*) mutant, rice dwarf (*d*) mutant (Wang et al. 2011).

The 9-cis- $\beta$ -carotene produced by *D27* activation is first converted into a 9-cis-configured aldehyde by the *CCD7* enzyme activity. *CCD7* enzymes are stereospecific for the 9-cis configuration and are responsible for removing the C9'-C10' double bond found in the trans particle of the 9-cis- $\beta$ -carotene. As a result of this process, 9-cis- $\beta$ -apo-10'-carotenal (C27) and  $\beta$ -ionone (C13) are obtained from 9-cis- $\beta$ -carotene. Following this process,  $\beta$ -apo-13-carotene (C18 ketone), which is involved in root growth, is formed by cleaving the C13'-C14' double bond of 9-cis- $\beta$ -apo-10'-carotenal via the *CCD8* enzyme. However, it is also possible to produce a strigolactone-like product (which includes the A, D ring, and the enol ether bridge of the strigolactones) called "carlactone" (CL) (Alder et al. 2012; Bruno et al. 2016).

Based on tomato genome information obtained from SGN (<http://solgenomics.net/>), *CCD* genes were detected in tomato using a systematic computational approach method and by comparing homology with *Arabidopsis* genes. A total of 7 *CCD* genes were identified in the tomato genome, namely *SlCCD1a*, *SlCCD1b*, *SlCCD4a*, *SlCCD4b*, *SlCCD7*, *SlCCD8*, and *SlCCDlike*. Of them, *SlCCD7* (Gene ID: Solyc01g090660) was located on chromosome 1, has 3726 bp gene length, and seven exons in tomato (*Solanum lycopersicum*), while *SlCCD8* (Gene ID: Solyc08g066650) was found on chromosome 8, 3075 bp gene length and six exons. Since the amino acid sequence of *SlCCD7* protein has a chloroplast targeting peptide at the N terminal, this enzyme is determined to be localized in the chloroplast, while this signal could not be detected in *SlCCD8*. Although they are expressed in all plant parts, while the *SlCCD7* gene mostly expressed in orange fruit from the plant parts, *SlCCD8* has the highest expression in the tomato root and then in the stem and old leaves (Wei et al. 2016). Besides, in the tomato, two gene regions called *LeCCD1A* and *LeCCD1B*, were determined to show homology with *AtCCD1*, which is part of the formation of  $\beta$ -ionone and geranyl acetone in *Arabidopsis* using EST database (Simkin et al. 2004).

After determining the presence of the putative *CCD7* gene in *Phelipanche ramosa*, a broomrape species, using the VIGS (Virus-Induced Gene Silencing) method in *Phelipanche aegyptiaca*, the presence of *PaCCD7* and *PaCCD8* genes, the ortholog of *CCD7* and *CCD8* genes, has been discovered (Aly et al. 2014). As a result of the review of existing databases, it was determined that *PaCCD7* protein showed sequence similarity with *CCD7* found in *Nicotiana tabacum* and *Petunia x hybrida* with 69%, *SlCCD7* in *Solanum lycopersicum* with 65%, and *AtCCD7* in *Arabidopsis thaliana* with 59%. Besides, *PaCCD8* protein showed similarity to *Dad1/CCD8* in *Petunia x hybrida* with 85%, to *SlCCD8* in *Solanum*

*lycopersicum* with 84% *NtCCD8* in *Nicotiana tabacum* with 84%, and to *AtCCD8* in *Arabidopsis thaliana* with 66% similarity (Aly et al. 2014).

Cytochrome P450 (*CYP*) genes (cytosol localized) that play a role in another important step for obtaining strigolactone varieties from carlactone are monooxygenases containing heme are involved in the oxidation of the carlactone. *CYPs* catalyze different reactions, but *CYP* 711 clade (*CYP711A1*) is involved in strigolactone biosynthesis, and also further enzymes can modify SLs. This clade was identified in the *Arabidopsis max1* mutant, and this enzyme was found to be encoded by the *MAX1* gene. Studies have proven that this gene is located upstream of *CCD7* and *CCD8* in the pathway. In the pathway, carlactone as a substrate for the enzyme *MAX1* and *MAX1* type enzymes are involved in the diversity of SLs found in plants (Jia et al. 2019).

The resulting carlactone is subjected to oxidation, BC ring closures catalyzed by *MAX1* after transported to the cytoplasm. Since the carlactone contains the A and D rings and the enol ether bridge, additional steps are required to obtain the strigolactones, such as adding the B and C rings and converting the carlactone into 5-DS (5-deoxystrigol) and/or ent-2'-epi-5-DS (4-deoxyorobanchol, 4-DO) precursors via activities of *MAX1* homologs which catalyze CL oxidation (Seto et al. 2014). However, not always 5-DS and 4-DO are precursors for strigol and orobanchol. There are exceptions in some plants. For example, the Moonseed plant cannot convert to 5-DS when CLA to Strigol, and likewise, 4-DO has not converted an orobanchol precursor in the cowpea. The carlactone is converted into strigolactones in plants (Iseki et al. 2018). Both orobanchol and strigol type SLs are found in the tobacco plant, and it has been discovered that both types are derived from 5-deoxystrigol (Xie et al. 2013).

A result of a study conducted to investigate the *MAX1* enzymatic function homologs in *Arabidopsis*, rice, maize, and tomato, shows that the conversion of CL to CLA is a common reaction catalyzed by *MAX1* and its homologs, besides, *MAX1* can be divided into three classes: (A1) CL to CLA, (A2) CL to 4-DO via CLA and (A3) CL to CLA and 4-DO to orobanchol conversions (Yoneyama et al. 2018). CL is converted to CLA via *MAX1* (and also methylated to methyl carlactonoate (MeCLA)) in *Arabidopsis* whereas in rice with five different *OsMAX1* homologs, while catalyzes the conversion from CL to CLA-mediated 4-DO by *MAX1* homolog *Os900* (Carlactone oxidase) (*CYP711A2*), a second *Os1400* (*Orobanchol synthase*) (*CYP711A3*) converts 4-DO to orobanchol with SL activity (Zhang et al. 2014).

### 2.3.1.2. Strigolactone types

To date, more than 20 types of strigolactone have been identified in the collection from various plant species, and their natural species consists of a tricyclic part (ABC ring) and a butenolide ring (D ring) attached to this part by an enol ether bridge. All these rings, but showing stereochemical differences in the B-C ring combination and called "canonical strigolactones," are divided into two basic classes: strigol and orobanchol (Monohydroxy-SLs) derivative SLs. Recently, a total of 3 basic classes have emerged, with the identification of "non-canonical" SLs without A, B, or C rings (usually without B-C ring closure) but have the enol ether-D ring, such as carlactone, methyl carlactonoate (MeCLA), avenaol, heliolactone, and zelactone. Non-canonical

SLs, including CLA, were found to be more common than canonical SLs as rhizosphere signals. The carlactone does not have the C ring, but the seed germination stimulant suggests that the C ring is not essential for SL activity. Especially for the production of strigol ( $\beta$ -oriented C ring) and orobanchol ( $\alpha$ -oriented C ring), carlactonic acid (CLA) precursor produced by C19 oxidation from carlactone must first be formed, which is catalyzed by MAX1 activity (core step) (Flematti et al. 2016; Yoneyama et al. 2018; Zwanenburg et al. 2018) (Figure 2.15).

Of the two stimulants with oxidized A-B rings, strigol stimulates *Striga* spp. seed germination while orobanchol stimulates *Orobanche* spp. seed germination. Generally, other SLs derived from these two strigolactone families are obtained by different B-C ring stereochemistry (Al-Babili and Bouwmeester 2015).

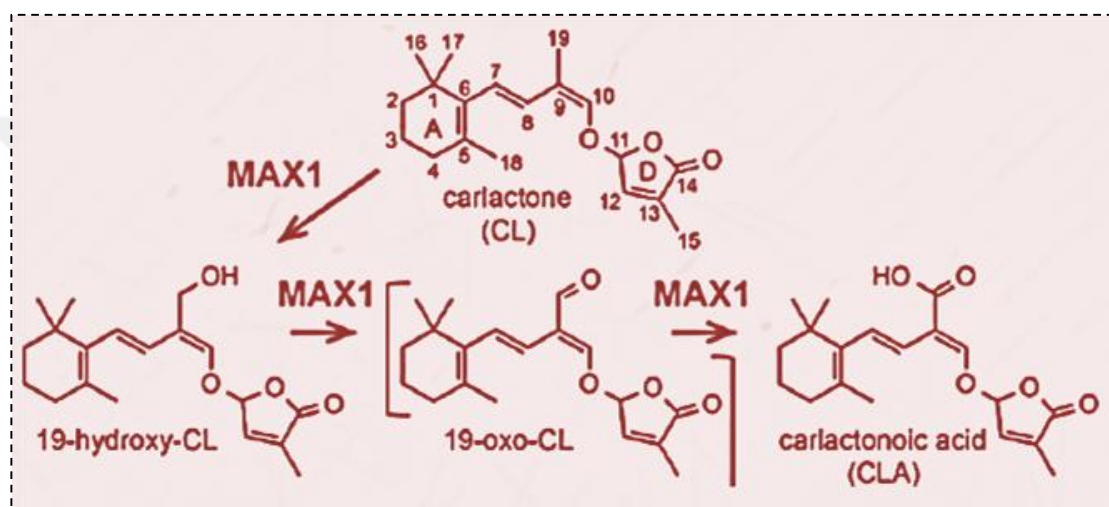


**Figure 2.15.** The conversion from CL to CLA by MAX1 (edited and quoted from Abe 2014)

Different SL compounds emerge with the occurrence of different oxidations. Some of these; a) in C-19 and C-18: CL was converted to 5-DS or 4-DO and subsequently modified independently, b) in C-5 and C-4: 5-DS strigol and 4-hydroxy-5DS, c) in C-9: sorgomol, d) C-4 of 4-DO: orobanchol, e) in C-7 of orobanchol: 7-oxoorobanchol is formed (and 7-hydroxorobanchol is formed with further oxidation), f) 7-hydroxy-orobanchol is converted to solanacol through dehydration, oxidation, and migration of a methyl group. Also, orobanchol can transform into fabacol by epoxidation, and hydroxy-SLs can be acetylated to acetoxy-SLs (Xie 2016) (Figure 2.16).

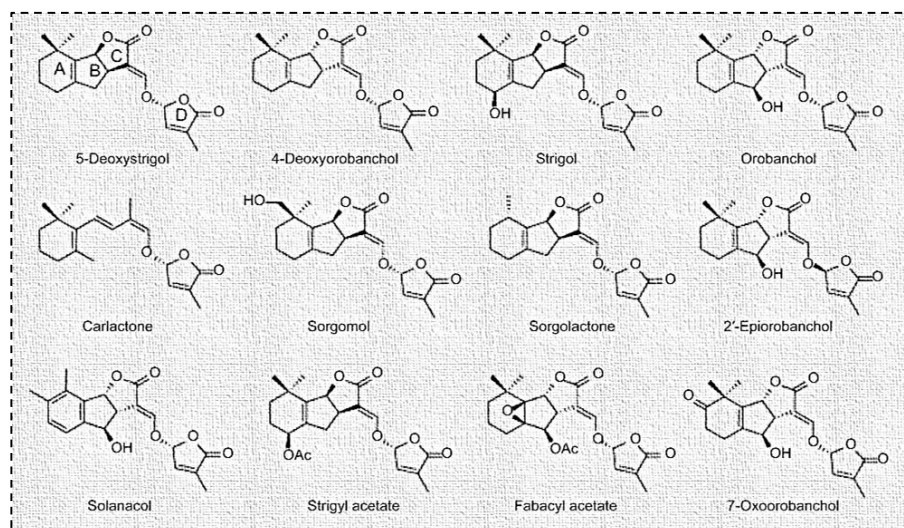


**Figure 2.16.** Examples of naturally occurring SLs (edited and quoted from Wang et al. 2017)

*SIMAX1*, the tomato *MAX1* homolog, was determined with *Slmax1* mutants, which shows strongly reduced orobanchol, solanacol, and didehydro-orobanchol (DDH) isomers (1 and 2) amounts from orobanchol derivatives. As a result of the transient expression of *SIMAX1* in the tobacco (*Nicotiana benthamiana*) made with the genes *SID27*, *SICCD7*, and *SICCD8*, *SIMAX1* also catalyzed the formation of carlactonic acid from carlactone, which is a common reaction. Moreover, the solanacol first identified in the *Nicotiana benthamiana* is the only SL with an aromatic A-ring. It is thought to be derived from orobanchol by changing a methyl group, double bonds, and hydroxylation-dehydroxylation (Zhang et al. 2018).

Another study conducted in tomato, spectrophotometric methods that orobanchyl acetate, found in xylem sap in previous studies, was also found in tomato roots. Furthermore, 7-oxoorobanchol and two 7-hydroxyorobanchol isomers were also determined in tomato exudate, although they were minor compared to the amount of orobanchyl acetate. Therefore, it was thought that it could not be detected in previous studies due to its low levels. Besides, with the discovery of two new didehydroorobanchol isomers, in addition to the didehydroorobanchol detected in the previous study, the number of SL detected in tomato increased to 10 in total. Thus, many of the strigolactones defined in tomato are thought to be intermediate products in the pathway (Kohlen et al. 2013).

### 2.3.2. Strigolactone transportation, signaling and perception

Studies have shown that most of the SLs released into the rhizosphere are produced in plant roots and can form symbiosis between plant and AM fungi and play a role in parasitic plant seed germination. SLs transported to the shoots via the xylem are also responsible for regulating "branching" (Yang et al. 2019). SL biosynthesis and signaling mechanisms have been revealed thanks to studies conducted using SL-deficient and SL-insensitive mutants. Two genes have been identified for the strigolactone signaling mechanism: 1. *MAX2* encodes LRR/F-box proteins. These proteins provide protein-protein interaction by participating in *SKP*, *CULLIN*, and an *F-*

box protein (SCF) complex of *E3 ubiquitin ligase*. This complex mediates SL-induced protein degradation (Kohlen 2011); 2. *MAX2* interferes with  $\alpha/\beta$ -hydrolase superfamily protein "*D14*" for the SL signaling mechanism. *D14* has a conserved "Ser-Her-Asp catalytic triad" required for SL hydrolysis and signalization. During SL hydrolysis, the D ring of SL is cleaved, and an intermediate molecule is formed that covalently binds to each residue of the catalytic triad of *D14*. (This intermediate-product has no function as shoot branching suppression signals). Signal transduction of SLs is allowed, thanks to the removal of various transcriptional repressors. This process is called the "standard *D14*-*MAX2*-*D53* signaling mechanism" (Waters et al. 2017).

However, the mechanism by which SLs play a role as rhizosphere signaling molecules in root-parasitic plants seed germination and AM fungal colonization has not been fully elucidated. It is known that parasitic seeds need a preconditioning period (stratification for dormancy) before being triggered by germination stimuli such as SLs. However, parasitic plant seeds begin to germinate after this conditioning process when exposed to SL, which are specific compounds produced by the host plant. Also, spontaneous germination is rarely (1–3%) seen in and *Striga* spp, *Orobanche* spp. and *Phelipanche* spp. species. (Cardoso et al. 2011). On the other hand, the picomolar concentrations of strigolactones provide 50% germination in parasitic weed seeds. Therefore, it is thought to be the germination of parasitic weed seeds are triggered by a receptor-mediated mechanism, which is due to the addition of a nucleophilic species located in the enol ether carbon double bond of a transient molecular mechanism, and then the removal of the SLs' D ring (Akiyama and Hayashi 2006).

Besides, ATP-binding cassette (ABC) transporters, which are members of the ABC gene family and have transmembrane domains, have played a role in the transport of various phytohormones abscisic acid (ABA), cytokine, strigolactone, and auxin derivatives. These transmembrane domains act as a way for unidirectional transport of the substrate by forming a pore for transporting the substrate. In this context, a total of 154 -putative- ABC proteins were determined in the tomato genome (Ofori et al. 2018).

### 2.3.3. The role of strigolactones

SLs have many beneficial functions in rhizosphere communication and plant growth and development, especially by symbiotic arbuscular roles by mycorrhizal fungi in preventing root colonization and inhibition of shoot branching (Xie et al. 2010).

A study with the synthetic SL analog GR24 induces hyphal branching in a fungus symbiote that forms arbuscular mycorrhizae (AM) in the host plant. Conversely, AM formation decreases in SL mutants. Arbuscules are complex structures that occur in cortical cells in plant roots, and thanks to the development of hyphal induced by SL, it is thought that the fungus forms arbuscular by colonizing the host root. Although the mechanism that triggers this symbiotic relationship is not fully known, *D14* type proteins that play a role in the signaling mechanism have not been found in fungi (Smith 2014).

In addition to the roles of the strigolactones in the rhizosphere, they have an essential role in preventing shoot branching (Gomez-Roldan et al. 2008). Due to the observation that SL-deficient mutants have a branched phenotype, a hypothesis has

emerged that SLs act as negative regulators of shoot branching (Figure 2.17). Two mechanisms are possible in this regard: (I) The signal mechanism occurs due to the transcriptional regulation of specific genes in the *TEOSINTE BRANCHED1*, *CYCLOIDEA*, and *PROLIFERATING CELL FACTORS 1-2 (TCP)* family in the buds. Auxin acting in the stem has been found to inhibit cytokinin levels, promoting the expression of strigolactone biosynthesis genes and strigolactones regulate the *BRC1* gene. The *BRC1* gene encodes a transcription factor required for branching inhibition in the plant. Cytokinins are plant hormones antagonistic to strigolactones, but they promote bud growth by suppressing *BRC1* expression.

On the other hand, (II) Mechanism by which SL triggers systematic removal of *PIN-FORMED (PIN)* family of auxin “efflux carrier proteins” from the plasma membrane *PIN* proteins are the main components of the auxin flow in the cell. Strigolactones regulate the auxin flux by affecting the plasma membrane localization of the auxin carrier *PIN*. In this way, strigolactones can disrupt the transport of auxin from the apical meristem to the main stem. As a result, the absence of auxin necessary for the growth of the shoot bud occurs because the auxin moves down from the main stem and inhibits the bud branching activity by preventing the export of auxin from axillary buds. Thus, the competition between the axillary branch is increased due to decreased auxin transport in the presence of SLs (Koltai 2014; Waldie et al. 2014; Brewer et al. 2015). Besides, phosphate deficiency (Pi starvation) in the rhizosphere is related to SL biosynthesis and its AM fungi interaction increments (Czarnecki et al. 2013).

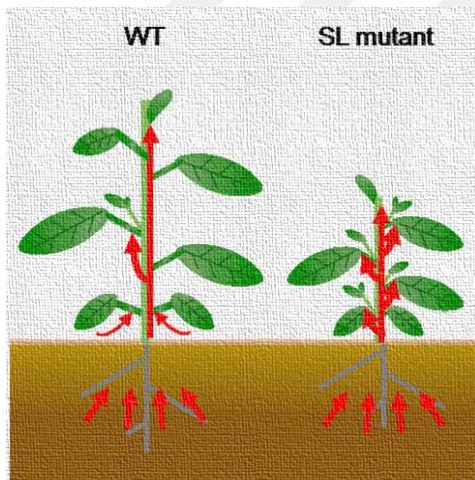


**Figure 2.17.** Strigolactones and shoot branching (edited and quoted from Yamada and Umehara 2015)

There are several gene-editing studies in the literature for strigolactone. Among the gene manipulations for the SL mechanism in plants, methods such as TILLING (Targeting Induced Local Lesions in Genomes), RNAi, FNM (Fast Neutron Mutagenesis), Chemical Mutagenesis (with ethyl methanesulfonate), and CRISPR (mentioned in CRISPR section (2.4)) were used, and these studies were carried out with tomato (Kostov et al. 2007; Dor et al. 2010; Dor et al. 2011; Kohlen et al. 2012; Hasegawa et al. 2018; Zhang et al. 2018; Bari et al. 2019a; Bari et al. 2019b), rice (Yang et al. 2017; Butt et al. 2018), tobacco (Gao et al. 2018).

As a result of the study carried out by Kostov et al. (2007), tomato mutants resistant to *Phelipanche ramosa* were produced by chemical mutagenesis of tomato seeds using ethyl methanesulfonate (EMS). As a result of the pot experiments, 16 plants resistant to *P. ramosa* were selected, and M2 progenies were obtained, and 6 of them were found to show significant resistance.

Through the fast neutron mutagenesis treatment applied to the M82 tomato line by Dor et al. (2010), the random mutation was created in the recessive *ort1* allele. These mutants (*Sl-ORT1*) showed resistance against *Phelipanche ramosa*, *Orobancha cernua*, and *Orobancha crenata*, especially *Phelipanche aegyptiaca*. Therefore, the mutant tomato can be included in the breeding program. In a second study conducted by Dor et al. (2011), which is a continuation of the previous study, *SL-ORT1* mutant exudates did not contain strigolactone compounds such as orobanchol, solanacol, and didehydro-orobanchol isomer, and it was determined that the *SL-ORT1* resistance was caused by the inability to produce and secrete these natural germinating stimuli to the rhizosphere.

Transgenic *SlCCD8* tomatoes were obtained due to the treatment of the *CCD8* gene by the RNAi method by Kohlen et al. (2012). Phenotypic characters such as expanded shoot branching, diminished plant stature, an increased number of nodes were observed in lines with low *SlCCD8* levels. Also, smaller flowers and fruits were formed and fewer and smaller seeds per fruit. No significant changes were observed, except for reducing the root amino acid content in the transgenic lines. Compared to the wild type, the germination of *Orobancha ramosa* seeds were reduced by up to 90% in the *SlCCD8* lines. As a result of the study, it was shown that the *CCD8* gene region is essential for strigolactone biosynthesis, and even a slight decrease in the level of strigolactone has an essential effect on the development of *O. ramosa*.

In the study performed by the TILLING method by Hasegawa et al. (2018), the *CCD8* gene was targeted in the most miniature tomato “Micro-Tom.” It was concluded that strigolactone deficiency caused by manipulation did not critically affect the fruit characteristics of the transgenic Micro-Tom/*SlCCD8* line. As a result of the resulting mutation, it was concluded that *SlCCD8* mutants show resistance to *P. aegyptiaca* due to decreased orobanchol and solanacol levels and can be used as new tomato lines in breeding studies.

In another study conducted by Zhang et al. (2018) using the TILLING method, *SlMAX1*, the homolog of the *MAX1* gene, was characterized in tomato. In this study, the TILLING method was used to obtain *Slmax1* mutants in which strigolactone production was inhibited, such as orobanchol, solanacol, and didehydro-orobanchol (DDH). Transient expression of *SlMAX1* made in *N. benthamiana* plant - together with *SlD27*, *SlCCD7*, and *SlCCD8*. Furthermore, it was shown that *SlMAX1* catalyzes the formation of carlactonoic acid from the carlactone in the pathway. Furthermore, as a result of plant-feeding experiments, it was determined that the natural precursor of orobanchol is not 4-DO but a carlactone. The orobanchol biosynthesis from carlactone is catalyzed by *SlMAX1* in tomato.

## 2.4. CRISPR-Cas9 System and Targeted Genome-Editing

Thanks to recent advances in genome editing technologies, it has become possible to make fundamental changes to the genomes of eukaryotic cells. In genome editing, methods such as random mutagenesis, meganuclease, transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) still have been used today. However, they were replaced by new methods due to the high probability of creating off-target changes in the genome. Later, the genome editing strategy began to focus against small RNA species, including microRNA (miRNA) and small interfering RNA (siRNA) (Khan 2019). ZFNs are costly and time-consuming because they require unique methods and optimization difficulties (Chandrasegaran 2017). In the TALEN method, difficulties arise due to the need for a new TALEN dimer for each new target region (Gersbach et al. 2014). With the discovery of a new gene-editing method called CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), fundamental problems were resolved, such as size (RNA, protein), precision (complementarity from RNA to DNA, for precise recognition of the target), and price (Iancu 2018).

For the first time in 1987 at the University of Osaka, by Yoshizumi Ishino and colleagues, the accidental cloning of a section of the CRISPR sequence in *E. coli* and its original targets, the *iap* gene (Ishino et al. 1987), is a step into the CRISPR system, whose function was not yet known at the time. After the determination of a type of repetitive element (Short Regularly Spaced Repeats (SRSRs) in different prokaryotes thanks to genomic sequence (Mojica et al. 2000) in 2002. This mechanism has been better illuminated by proving that naming a new repetitive DNA sequence present in archaea and bacteria but not found in eukaryotes or viruses as CRISPR and Cas (CRISPR-associated) proteins is a functional component of CRISPR systems (Jansen et al. 2002). By 2005, different research groups showed that some CRISPR spacers are derived from phage DNA and non-chromosomal DNA, such as plasmids (Pourcel et al. 2005; Mojica et al. 2005; Bolotin et al. 2005). CRISPR, a distinctive feature of most bacteria and archaea genomes, has been proven to integrate new spacers derived from the phage genomic sequences of bacteria after the viral challenge. CRISPR has been found to resist phages with associated cas genes and is determined by the spacer-phage sequence similarity. Thus, in 2007, it was determined for the first time that CRISPR played a role in the cas genes in the defense mechanism of bacteria against viruses (Barrangou et al. 2007). The CRISPR/Cas immune system has been proven to be rapidly adapted to cleave invading DNA and separates the double helix DNA of the plasmid and bacteriophage in the proto-spacer. The spacer sequence guides cas proteins for target DNA cleavage (Garneau et al. 2010). The tracrRNA directs the maturation of crRNAs, which is associated with cas proteins. With activities of the RNase III, for bacterial RNA-mediated immunity against invaders like eukaryotic RNA interference (Deltcheva et al. 2011).

In this context, CRISPR-Cas systems are divided into three main classes (1-3) and a total of 11 subtypes (1-A to F, 2-A to C, and 3-A, and B). There are specific proteins specific to each type of system, such as the Cas3 protein found in type 1, the Cas9 protein found in type 2, and the Cas10 protein found only in the type 3 system, and Cas1 and Cas2 proteins are genetically available in all types. Although type 2 systems are defined in bacteria, type 1 systems are seen in bacteria and archaea, and type 3 systems are primarily seen in archaea (Shabbir et al. 2016). While multiple

subunit effector complexes (multiple protein complexes) are used in type 1 of CRISPR-Cas systems, single protein effectors such as Cas9 and Cpf1 (also referred to as type V) are used in type 2 (Shmakov et al. 2015). Cas9 can cleave a target DNA which is complementary to mature crRNA (Chylinski et al. 2013). However, later on, the CRISPR system is in two main classes, and these classes have different types. These classes are: type 1, type 3, type 4 (putative type) for Class 1, type 2, type 5, and type 6 for Class 2, and each type has different subtypes (Makarova et al. 2015).

Endonucleases process RNA transcripts into short gRNAs in RNA-based defense systems such as RNA interference (eukaryotes) or CRISPR (bacteria (found in half) and archaea (found in nearly all) (Gottesman 2011). Clusters of short DNA repeats/direct repeats with nonhomologous viral originated spacers with similar lengths are known as CRISPRs (Haft et al. 2005). CRISPR array plays in replicon dividing, DNA repair, control, and chromosomal modification (Kunin et al. 2007). CRISPRs are complex biological mechanisms that have played an essential role in the evolution of Archaea and Bacteria's defense mechanism against foreign DNA. It contains these spacers and repeats that can target and degrade this invading DNA in the CRISPR array, similar to the RNA interference mechanism. Spacer sizes range from 26 to 72 bp compared to repeat sizes ranging from 24 to 47 bp, and the number of repeats in a CRISPR locus varies widely within. Among organisms, repeat sequences within the CRISPR locus are highly conserved but repeat at distinctive CRISPR loci may change in length (Grissa et al. 2007).

The CRISPR-Cas9 consists of 3 primary processes: *acquisition*, *expression*, and *interference* (Makarova et al. 2011). Spacer acquisition is the first step in the structuring of the immune system of bacteria and archaea. Next, CRISPR-derived RNAs (crRNAs) must be formed to protect from invading DNA. In other words, CRISPR must be transcribed and processed. Spacers are derived from fragments of invasive genetic elements called protospacers and are included in CRISPR loci in repeats adjacent to CRISPR leaders. At this stage, called immunity, the protospacer sequence of a foreign DNA acquired by a viral infection is recognized by the Cas proteins complex called Cas1-Cas2 and joins in the host CRISPR sequence, which creates a new spacer. The choice of protospacer in type 1 and type 2 CRISPR is due to 2-5 bp protected sequences on the protospacer, defined as the protospacer adjacent motif (PAM) that is not required for type 3 systems. Therefore, any sequence harboring the correct PAM sequence has the chance to integrate into the CRISPR array in the host genome. The triggering of CRISPR immunity is usually performed by transcribing a long precursor-crRNA (pre-crRNA) that forms the crRNA molecules of the CRISPR array. (Plagens et al. 2015).

In the expression phase in the CRISPR-Cas9 mechanism, CRISPR repeat-spacer arrays are transcribed to produce small, non-coding CRISPR RNAs (crRNAs). First, long and primary precursor crRNA (pre-crRNA) molecules are produced that will go through the maturation steps to generate mature crRNA. Another signature of the type 2 systems is the trans-activating CRISPR RNA (tracrRNA). TracrRNA is fundamental for its handling into mature crRNA, catalyzed by RNase III recognizing the repeat-anti-repeat duplex. TracrRNA hybridizes with pre-crRNA to generate duplex RNAs that are the substrate for RNase III activity. This activity may also require Cas9. TracrRNA also forms a secondary structure that mediates the association with the Cas9 protein (Deltcheva et al. 2011; Chylinski et al. 2013).

The secondary structure of tracrRNA:crRNA occurs by base pairing between the 22 nucleotides at the 3' end of the crRNA and the nucleotides around the 5' end of the tracrRNA (Figure 2.18). As a result of this secondary structure, a structure with 20 nucleotides is formed in the 5' terminal region of the crRNA that allows it to bind to the target DNA (Jinek et al. 2012).

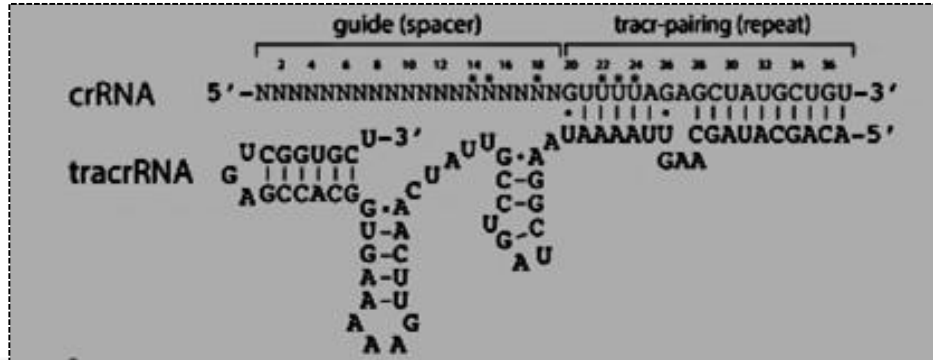


**Figure 2.18.** crRNA and tracrRNA base pairing (edited and quoted from O'Reilly et al. 2019)

There are other components required for the proper working of the CRISPR system in a CRISPR loci. For example, the CRISPR array is preceded by a leader region (100-500 bp) rich in Adenine-Thymine bases and containing a promoter region. Also, cas genes (locus of cas genes) located close to the CRISPR array encode Cas proteins that play a role in cell protection and different immune stages (Sternberg et al. 2016) (Figure 2.19).

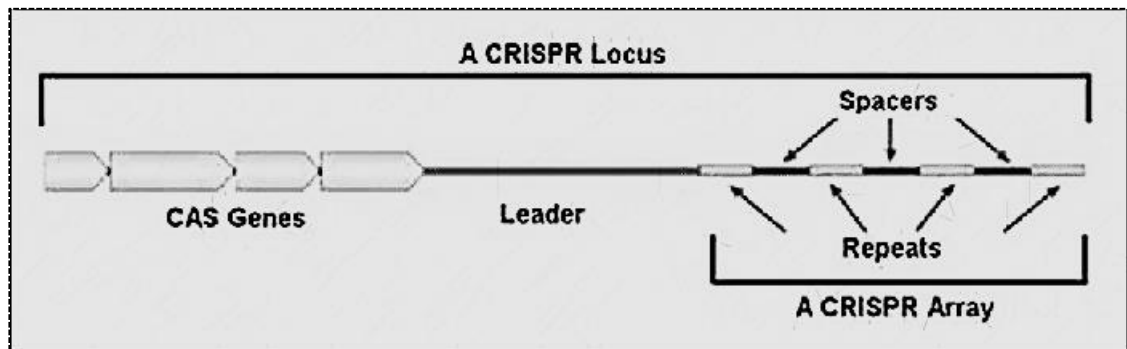


**Figure 2.19.** The CRISPR locus (edited and quoted from Mahmud et al. 2015)

#### 2.4.1. Cas proteins

Cas genes are involved in processing transcripts and produced by the CRISPR locus, cleavage of target DNA/RNA, and the integration of new spacers. The Cas genes are found near the CRISPR array. This suggests that the cas genes and the CRISPR array have a tight functional relationship. There are four Cas1, Cas2, Cas3, and Cas4, are found in CRISPR negative prokaryotes. The four cas genes were not found in all species with CRISPR locus, but Cas proteins found in closely related species were found to show more remarkable amino acid sequence similarity than non-closely related species (Jansen et al. 2002).

In the immune process, while *Cas1* and *Cas2* proteins are involved in CRISPR spacer integration by forming a complex structure, the endonuclease activity of *Cas1* protein is required for the spacer integration, while *Cas2* protein is determined to perform a non-enzymatic function (Nuñez et al. 2014). Especially *Cas1* and *Cas2* conservative proteins in the information processing part of the adaptation phase and other cas proteins produced from different cas loci in the expression and intervention phase differ significantly between different organisms (Makarova et al. 2011). Afterward, discoveries of *Cas5* and *Cas6* proteins total of six conserved (core) *Cas* genes were discovered. *Cas6* was found in both bacteria and archaea. While not every cas core gene is associated with all CRISPR loci, they contain multiple subtypes (Haft et al. 2005).

The type 2 CRISPR-Cas system identified in the *Streptococcus pyogenes* genome consists of several *Cas* genes: *Cas1* and *Cas2* (these are core proteins for spacer acquisition in all CRISPR types), *Csn2* and *Cas4* (play a role in adaptation), and *Cas9* (formerly called *Csn1*) (Wiedenheft et al. 2012). The type 2 systems are also known by different names such as the “HNH” systems, Streptococcus-like or Nmeni subtypes. The signature gene of type 2 CRISPR-Cas systems is *Cas9*, and in this type of system, in addition to the *Cas9* gene, it contains ubiquitous *Cas1* and *Cas2* genes. The *Cas9* protein, consisting of about 800-1400 amino acids, contains two nuclease domains: RuvC-like nuclease (RNase H fold) and HNH (McrA-like), which are responsible for cleavage of the non-complementary and complementary strand of the target DNA, respectively, in the crRNA–target DNA complex (Makarova and Koonin 2015). Unlike *Cas9*, *Cpf1* is a putative type V (class II) CRISPR effector that does not need additional tracrRNA for target cleavage, and it uses different PAM sequences (Koonin et al. 2017). Although there are many orthologs (approximately 75) of the *Cas9* protein, they contain only a few identical amino acids, especially the length of the inter-domain regions. Moreover, the orthologous sequences of the *Cas9* protein are directly related to the variation in CRISPR repeat length (Chylinski et al. 2013). *Cas9* protein consists of two lobes: 1. REC lobe (recognition) and 2. NUC (for nuclease activity). Among these lobes, REC contains bridge helix, REC1, and REC2 domains, while the NUC lobe contains RuvC, HNH nuclease domains, and more variable C-terminal domain (CTD), which contains PAM interacting (PI) domain. REC lobe and PI domains are also *Cas9*-specific functional domains. Recognition of the tracrRNA and crRNA duplex by the REC1 domain is essential for the *Cas9* protein function (Nishimasu et al. 2014).

Both the protospacer sequence and the adjacent protospacer motif (PAM) must be found and recognized on the target DNA for the adaptation phase. PAM is not present in the crRNA sequence to protect the autoimmunity and cleavage of the host genome but needs to be downstream of the target site in the genomic DNA. Even though *Cas9* is linked to a tracrRNA-crRNA complex that allows directing to the invader protospacer sequence, it will not leave the protospacer sequence unless there is a PAM sequence at the 3' end of target DNA. In other words, PAM and protospacer sequences (for crRNA complementarity) are required for double-strand DNA cleavage by the *Cas9*–crRNA complex (Gasiunas et al. 2012).

Although PAM sequences recognized by *Cas9* are a conserved construct, they show a significant relationship with CRISPR repeat sequences (Kunin et al. 2007).

PAM sequence depends on two main molecular processes: motif recognition by the spacer acquisition protein complex and the selection of protospacers for targeting (Shah et al. 2013). In type 1 systems, PAM sequences upstream of the protospacer and type 2 systems, the PAM sequences and positions downstream of the protospacer sequence vary in different CRISPR system types. In type 3 systems, it was determined that there is no need for the PAM sequence. (Makarova et al. 2011). PAMs (2-5 bp, conserved sequence) recognized by *Cas9* differ in sequence, between different organisms such as in the type 2 system of *S. pyogenes*, the PAM sequence is NGG (or NAG at a low rate) (Shah et al. 2013).

*Cas9*-mediated DNA cleavage requires the 5'-NGG-3' sequence in the non-target DNA strand, but not in the complementary target strand. Thanks to the lack of interaction with the target strand, incompatibilities in PAM can be tolerated, provided that the GG dinucleotide in the non-target strand is present. Arginine residues found in *Cas9* proteins play a crucial role in recognizing PAM, and non-target strands are read out base-specific hydrogen-bonding interactions with Arg1333 and Arg1335 (R1333 and R1335). These arginine residues in the PAM Interacting (PI) domain bind to their major groove in the guanine bases in PAM. Base mapping of the 8-12 bp region (seed region) disrupts the stabilization of the target DNA region, leading to the formation of the heteroduplex structure. (Anders et al. 2014).

In the interference phase in the CRISPR-*Cas9* mechanism, thanks to this PAM sequence recognized by *Cas* proteins, the target double-stranded DNA helix begins to unwind. This process is necessary to destabilize the *Cas9*-RNA heteroduplex structure and provide base matching between the target DNA sequence and the crRNA guide sequence. This exposed DNA creates a triple-stranded R-loop structure (RNA-DNA hybrids), enabling hybridization with crRNA (Gleditzsch et al. 2019). PAM recognition triggers the R-loop formation without ATP-dependent helicase activity, and *Cas9* interacts with the ends of the open DNA helix to provide a 30° helix angle that provides the structural distortion required for R-loop stabilization (Jiang et al. 2015). PAM interactions trigger *Cas9* catalytic activity and base pairing of the guide RNA with the target double-stranded DNA (20 nt), allowing cleavage between the third and fourth nucleotides upstream of PAM HNH and RuvC nuclease domain of the dsDNA (Sternberg et al. 2014). The HNH domain, one of the two nuclease domains of *Cas9*, cleaves the target strand matched with the guide RNA region of crRNA, and the non-target strand is cleaved by the RuvC domain (Double-Strand Break) (Gasiunas et al. 2012).

*Cas9* can be used as an RNA-guided DNA binding platform to target and transcribe effector domains. This system, in which crRNA and tracrRNA are used together, is facilitated using a chimeric single-guide RNA (sgRNA) with *Cas9* protein. The sgRNA is sufficient for binding and cleavage to the targeted DNA. The *Cas9* protein can be programmed by creating a hairpin structure that preserves base pairing between tracrRNA:crRNA and the chimeric RNA containing the target recognition sequence at the 5' end. It effectively fuses the 3' end of the crRNA to the 5' end of the tracrRNA, mimicking the crRNA: tracrRNA construct required by *Cas9* to direct the region-specific DNA cleavage. This structure is also in the form that will provide a binding to the *Cas9* protein by 3' (Jinek et al. 2012).

The sgRNA consists of different components (Figure 2.20): 1. crRNA sequence is complementary to target DNA. It is divided into two parts: a) 17-20 nt guide region (12 nt seed is more sensitive to mismatches and eight nt non-seed regions)), b) 12 nt repeat region; 2. tracrRNA sequence serves as a binding scaffold for the Cas nuclease. It is divided into two parts: a) 14 nt anti-repeat region, b) three stem-loops. Stem-loop 1 is necessary for forming the functional Cas9-sgRNA complex, and stem-loop 2 and 3 provide stable complex formation and increase the stability of sgRNA (Liang et al. 2016).

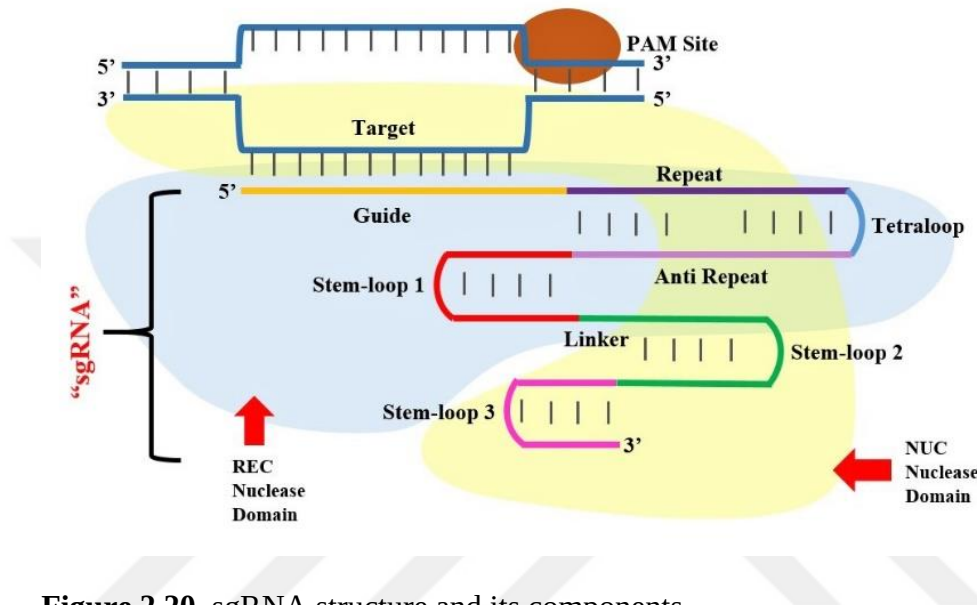


**Figure 2.20.** sgRNA structure and its components

Cas9 recognizes the PAM-proximal guide region, and the repeat:anti-repeat heteroduplex of sgRNA and 20 bp guide region is primarily recognized by the REC lobe. The backbone phosphate groups of the target DNA interact with the REC1, RuvC, and PI domains. The gRNA locates in the positively charged part between the REC and NUC lobes and positions the phosphate group of the target DNA for the cleavage by the HNH domain. Finally, the HNH domain of the Cas9, which makes a blunt cut between the 17th and 18th bases in the target sequence protein, cleaves the complementary strand in the guide: target heteroduplex, whereas the RuvC domain cleaves the non-complementary strand (Nishimasu et al. 2014).

#### 2.4.2. DNA repair mechanisms

Following damage to the DNA, cellular repair mechanisms are triggered by error-prone non-homologous joining (NHEJ) or template-mediated homology-directed repair (HDR) pathways. NHEJ repairing mechanism is more efficient than HDR, occurs at the DSBs region, and resulting in insertions and deletions (indels) of nucleotides at the target locus. In the presence of a separate DNA template containing homologous sequences to the regions surrounding the DSB, the HDR mechanism can be triggered, and the repair template can be added to the genomic DNA. Even though HDR creates very sensitive changes, the desired recombination is rare (106 to 109/1 cells) (Komor et al. 2017). Unlike the NHEJ mechanism is the main DSB repair path in G0/G1 phase,

cell repair can occur at all stages of the cell cycle. HDR requires homologous DNA sequences, occurs only at the S or G2 stages of the cell cycle when a sister chromatid is available. For DSB repair, homologous recombination must be performed for precise genome editing, which occurs with the presence of a homologous duplex template. Therefore, HDR must have the repair template in the right place and at the right time for the DSB mechanism to process correctly. Thanks to the HDR mechanism, DSB can be repaired without errors (Kakarougkas and Jeggo 2014). The NHEJ mechanism uses a nuclease to remove damaged DNA, polymerases to fill the new DNA formed, and a ligase to restore integrity to the DNA chains. In NHEJ repairing pathway, Pol  $\mu$  and Pol  $\lambda$  polymerases have capabilities that enable them to incorporate nucleotides to blunt dsDNA ends and fill ssDNA gaps (Lieber 2010).

Today, the CRISPR-Cas9 (type 2) system has created a cost-effective and easy-to-use base for many processes such as targeting, editing, and modifying the genomes of various cells and organisms more precisely and effectively. The guide RNA-mediated Cas9 method has significant potential for gene targeting and genome editing studies. Cleavage of targeted regions in dsDNA with the CRISPR-Cas9 method has been determined as a valuable strategy for genome engineering. The success of the CRISPR-Cas9 mediated editing method has 80% more efficiency depending on the target than the methods using ZFN and TALEN (Doudna and Charpentier 2014). CRISPR-Cas9 genome editing technology depends on creating double-stranded DNA breaking and repairing this gene disruption by generating mutation (insertions and deletions (indels)) at a target locus through DNA repair mechanisms. In this regard, both error-prone NHEJ or HDR pathways are methods that can be used to achieve the desired gene editing (Jinek et al. 2012). Studies have shown that this system can also be used in eukaryotic cells, and the mutation to be produced in the target DNA is accomplished by the expression of Cas9 protein (endonuclease) and guide RNA in eukaryotic cells (Jinek et al. 2013). While the HDR mechanism is used for "knock-in or knock-out" studies that require insertion of a specific DNA fragment, the NHEJ mechanism creates a loss of function in the target gene subsequent frameshift mutations due to adding or deleting bases that result in "gene knock-out" (Rozov et al. 2019). For effective genome editing to occur, it is necessary to target the specific genomic loci with high efficiency while operating nuclease (Cas9) with high sensitivity (Cong et al. 2013). As a result of indels in exons, frameshift mutation occurs, and when they stop codons are found in the second and third reading frames premature stop codons (PSC) are occurred, and the reading frame of the mRNA is disturbed (Lalonde et al. 2017).

To create Cas9/gRNA constructs, practical to clone the approximately 20 bp spacer sequence with a scaffold of the gRNA, which is necessary for Cas9 binding. Besides, multiple gRNAs with a different promoter can also be used to target several loci at once (Wu et al. 2014). The gRNA expression cassette consists of the Pol III promoter, the gRNA, and the Pol III terminator, and several sgRNAs can be co-expressed by sequentially linking the gRNAs expression cassette to modify multiple targets (Ma et al. 2015) simultaneously. The selection of sgRNAs with an efficient function such as (1) 30% to 80% G/C content, (2) intact secondary structures, (3) maximum seven consecutive bases, 12 total base pairs, six internal base pairs between guide sequence and other sequences (Liang et al. 2016). Although the PAM number is directly proportional to the size of the genome, it is determined that there are more

specific PAMs in the monocot genomes than the dicot plant genomes. Also, mutation efficiency and cleavage specificity depend on the ability of gRNAs to be expressed under the control of many promoters, including those recognized by RNA polymerase II and III. sgRNA can be functionally expressed in monocot and dicot plants by U6 or U3 promoters. Besides, *Cas9* is usually fused with a nuclear localization signal (NLS) and expressed with cauliflower mosaic virus (*CaMV*) 35S promoter or gene-specific promoters such as the maize ubiquitin promoter, which are *RNA polymerase II (Pol II)* promoters in plants (Li et al. 2013).

#### 2.4.3. Genome editing in plants with CRISPR-Cas9

Since *Agrobacterium*-mediated transformation is the most effective method for genome editing in many plants, CRISPR binary vectors are widely used to efficiently express *Cas9* and gRNA cassettes (Yun et al. 2019). Creating the binary vector for CRISPR-Cas9 for targeted mutations of one or more plant genes has several advantages: it is efficient, inexpensive, time-saving, user-friendly, and versatile (Xing et al. 2014).

Binary vectors are suitable for *Agrobacterium tumefaciens*-mediated transformation. They consist of basic parts such as T-DNA borders, multiple cloning sites, replication origins for *Escherichia coli*, and *A. tumefaciens*, selectable marker genes, reporter genes. An artificial T-DNA is created in a plasmid that can be replicated in both *A. tumefaciens* and *E. coli* and made available for plant transformation. The binary vector backbone contains essential components for bacterial selectable marker genes, plasmid replication functions, plasmid mobilization functions. The T-DNA part has T-DNA borders, multiple cloning sites (MCS), selectable marker gene cassette (promoter-selectable marker gene-terminator) for plant transformation (Komari et al. 2006) (Figure 2.21).

*Agrobacterium tumefaciens* can transfer a DNA (T-DNA) region containing the tumor-forming loci in its tumor-inducing (Ti) plasmid to plant genomes. However, this can be done thanks to cis-effect T-DNA borders sequences, and trans-acting virulence (vir) functions encoded by the *Agrobacterium* chromosome with the help of chromosomal genes. The functions of these vir genes remain active for transfer, even when placed in separate replicons from T-DNA in *Agrobacterium*. In this way, binary vector usage has become possible for *Agrobacterium tumefaciens*-mediated studies (An et al. 1989).

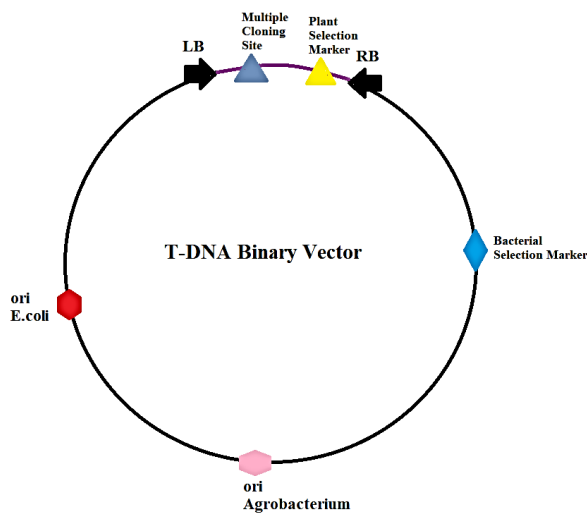


**Figure 2.21.** T-DNA binary vector

*A. tumefaciens* is a soil bacterium used extensively to transfer foreign genes. It is a bacterium that causes the formation of "crown gall tumors" by infecting plants from wound tissues under natural conditions. Also, it is known that the formation of this tumor structure occurs by integrating the T-DNA region (specific DNA segment) harboring on the tumor-inducing (Ti) plasmid (200-800 kb) of *A. tumefaciens* into the infected plant cell. T-DNA regions have 25 bp long homologous double-stranded border sequences flanking the T-DNA region. These borders are called "right border (RB) and left border (LB)." This T-DNA region contains two types of genes: 1. Oncogenic genes (They are involved in auxin and cytokine synthesis and encode the synthesis of enzymes responsible for tumor formation; 2. Genes encoding the synthesis of opines. Ti plasmids are classified according to the types of opins produced and secreted by the tumors they induced. In this context, there are 3 basic Ti plasmids: Agropine-type (pEHA105), Octopine-type (pAL4404), Nopaline-type (pTiC58). A series of processes are required to transfer the T-DNA region to the plant. These processes take place thanks to the activity of the virulence (*vir*) genes (*VirA*, *VirB*, *VirC*, *VirD*, *VirE*, *VirG*, and two non-essential (*VirF* and *VirH*)) on the Ti plasmid and the integration of *Agrobacterium* chromosomal genes into the plant cell. These *Vir* genes have different functions. Also, activation of *Vir* genes is highly dependent on external factors such as temperature. For example, a temperature higher than 32°C causes a structural change in *VirA* folding. Therefore, it causes gene inactivation. For this reason, a temperature of 28°C is optimal for the growth of *A. tumefaciens*.

The transfer of the T-DNA region to the plant genome can be summarized as follows: 1. Activation occurs through *VirA*, which acts as a TF regulating the expression of *Vir* genes, followed by the production of single-stranded molecules representing the copy of the bottom-strand of the T-DNA. For this reason, the T-DNA region transferred to the plant cell is technically transferred as a single strand; 2. *VirD1* and *VirD2* proteins recognize RB and LB regions located in the T-DNA and separate the bottom-strand of T-DNA by endonuclease activity; 3. *VirD2* protects against exonucleolytic attacks by staying covalently attached to the 5' end of the single-stranded T-DNA; 4. While *VirE2* functions as plant nuclear localization signals, *VirB*, which has ATPase

activity required for the transmission of T-DNA, mediates the transfer of T-DNA between bacteria and plant cells with the help of *VirD4*; 5. T-DNA in a single-stranded state is processed in the nucleus into a double-stranded form and is randomly integrated into the host genome by recombination. Small deletions are likely to occur at the recombination sites. From this system, in the binary vector method, plasmids have the potential to be easily transformed into a suitable *Agrobacterium* strain containing a helper region by transformation (De La Riva et al. 1998; Gelvin 2003; Lee et al. 2008).

In transformation with *A. tumefaciens*, virulence proteins encoded by the helper Ti plasmid play a role in transferring T-DNA on the binary vector from bacteria to plants. *A. tumefaciens* contains two plasmids except for its chromosome: (I) Ti plasmid, lacking its native T-DNA but containing a virulence region, (II) one binary vector containing T-DNA region (Michielse et al. 2008). The Ti plasmid types of *A. tumefaciens* strains plays role in the bacterial host range and transformation efficiency of plant species. Among the most widely used transformation of these disarmed strains, AGL1, EHA101, EHA105, and GV3101 (pMP90) are derived from the nopaline type C58 chromosomal background and have been modified from the pTiBo542 / pTiC58 plasmids, while the LBA4404 strain has the plasmid pAL4404, derived from octopine type *Ach5* and modified from the pTiAch5 plasmid. Antibiotic resistance of strains may differ according to the Ti plasmids they have (Figure 2.22) (Hwang et al. 2017).

| Strain Name             | Chromosome background | Ti-plasmid types                                                             | Antibiotic Resistance <sup>1</sup>                  | Reference                    |
|-------------------------|-----------------------|------------------------------------------------------------------------------|-----------------------------------------------------|------------------------------|
| LBA4404                 | Ach5                  | a disarmed octopine-type Ti plasmid pAL4404                                  | Rm <sup>R</sup>                                     | Hoekema et al., 1983         |
| EHA101                  | C58                   | a disarmed agropine-type Ti plasmid pEHA101 (pTiBo542ΔT-DNA)                 | Rm <sup>R</sup> , Km <sup>R</sup>                   | Hood et al., 1986            |
| EHA105                  | C58                   | a disarmed agropine-type Ti plasmid pEHA105 (pTiBo542ΔT-DNA)                 | Rm <sup>R</sup>                                     | Hood et al., 1993            |
| C58C1                   | C58                   | Cured of Ti plasmid                                                          | Rm <sup>R</sup>                                     | Deblaere et al., 1985        |
| C58C1(pTiB6S3ΔT, pCH32) | C58                   | a disarmed octopine-type Ti plasmid pTiB6S3ΔT-DNA and a helper plasmid pCH32 | Rm <sup>R</sup> , Cb <sup>R</sup> , Tc <sup>R</sup> | McBride and Summerfelt, 1990 |
| GV3101                  | C58                   | Cured of Ti plasmid                                                          | Rm <sup>R</sup>                                     | Holsters et al., 1980        |
| GV3101(pMP90)           | C58                   | a disarmed nopaline-type pTiC58ΔT-DNA                                        | Rm <sup>R</sup> , Gm <sup>R</sup>                   | Koncz and Schell, 1986       |
| A136                    | C58                   | Cured of Ti plasmid                                                          | Rm <sup>R</sup>                                     | Watson et al., 1975          |
| AGL-1                   | C58                   | a disarmed pTiBo542ΔT-DNA                                                    | Rm <sup>R</sup> , Cb <sup>R</sup>                   | Lazo et al., 1991            |
| C58-Z707                | C58                   | a disarmed nopaline-type pTiC58-Z707                                         | Km <sup>R</sup>                                     | Hepburn et al., 1985         |
| NtL4(pKPSF2)            | C58                   | a disarmed chrysopine-type pTiChry5ΔT-DNA                                    | Em <sup>R</sup>                                     | Palanichelvam et al., 2000   |

<sup>1</sup>Cb, carbenicillin; Em, erythromycin; Gm, gentamicin; Km, kanamycin; Rm, rifampicin; Tc, tetracycline.

**Figure 2.22.** *Agrobacterium* strains and their features (edited and quoted from Hwang et al. 2017)

Cloning with binary vectors is done in *E. coli* since the binary vectors are broad host plasmids containing an artificial T-DNA region in which both *E. coli* and *A. tumefaciens* can clone related genes. This vector obtained is then transferred to an *A. tumefaciens* strain to deliver T-DNA to plant cells (Samal et al. 2018). Thanks to *Agrobacterium*-mediated transformation, more stable transgene expression over generations, a lower copy, and high expression of transgenes are obtained compared to other transformation methods such as particle bombardment or polyethylene glycol-mediated transformation (Shou et al. 2004). Also, the Agroinfiltration method is more often used to provide transient (the DNA does not integrate into the host genome)

transformation to observe the functions of specific genes in a short time (Zhao et al. 2017).

For stable transformation, T-DNA is integrated into the host cell genome and transferred to the next generation. For this purpose, methods that require tissue culture techniques are used to promote whole-plant growth from transformed cells or tissues. This method requires the target gene to be delivered to plant cells by *A. tumefaciens*-mediated transformation, followed by selecting and regenerating transgenic plants from transformed cells by tissue culture techniques (Hwang et al. 2017).

Some other additions have been identified to increase the transformation efficiency of agroinfiltration. For example, plant species have developed a post-transcriptional gene silencing (PTGS) system that produces small RNAs that interfere with silencing unnatural genes for defense against viral attack. However, many viruses have developed a system that can interfere with the plant. An example of this system is tomato bushy stunt virus (TBSV) p19 (Reducing the ss siRNA level). Therefore, to increase transformation efficiency, p19 is transferred to the plant cell and *Agrobacterium* cells bearing the structure of the gene of interest (Zhao et al. 2017). In some wounded dicotyledonous plants, it is known to secrete many different phenolic compounds, including acetosyringone, which enable the activating genes in the Ti plasmid's virulence (Vir) region to play a role in promoting DNA transfer from bacteria to plant cells. A pre-treatment with the synthetic version of acetosyringone, a natural wound response molecule of *Agrobacterium*, enhances the transformation ability of the *Agrobacterium* (Sheikholeslam and Weeks 1987).

An *Agrobacterium*-mediated transformation method is widely used today. It was successfully used in 1986 by McCormick et al. for tomato (*Solanum lycopersicum*). This method includes six main stages. These are “(I) Seed growing, (II) Cotyledon cut (explant preparation), (III) Infection of cotyledons with *Agrobacterium* (Co-cultivation), (IV) Transfer of explants to regeneration medium, (V) Transfer of explants to rooting medium, (VI) Transfer of the plant to soil (acclimatization)” (McCormick 1991). Today, there are quite effective and well-established methods of transformation of tomato (Kimura and Sinha 2008; Van Eck et al. 2019).

Many factors affect *Agrobacterium*-mediated transformation efficiency and plant regeneration, such as explant type, plasmid vector, bacterial strain, the composition of the culture medium (composition-pH), selection markers, and the antibiotic type suppresses or eliminates *Agrobacterium* infection after cocultivation (Ziemienowicz 2014).

One of the critical aspects of the transformation is choosing a suitable selection marker to regenerate the plant from correct transformed cells with the corresponding resistance gene. Selectable marker genes are dominant genes that are toxic to plant cells in natural conditions or inhibit plant growth, such as antibiotics, herbicides. Concomitant use of nptII with antibiotic kanamycin is the most commonly used selectable marker gene system in dicotyledonous plants (Rosellini 2011). Herbicide markers are also widely used in the selection of transformant plants. The *bar* (or *pat*) gene is expressed under the control of Nos or CaMV 35S promoter from *Streptomyces hygroscopicus*. It encodes the enzyme phosphinothricin acetyltransferase and provides

resistance to herbicides containing phosphinothricin (glufosinate) (Leason et al. 1982). This substance is an inhibitor of glutamine synthetase, a central enzyme for nitrogen metabolism in plants, and the active ingredient of the commercial herbicide Basta is a non-toxic herbicide in plant transformation at specific concentrations (Khuong et al. 2013).

In order to obtain a transgenic plant, it is necessary to regenerate with tissue culture methods (treatment with appropriate phytohormones) from totipotent transformed cells selected from appropriate selection media. While the high concentration of auxins generally plays a role in root formation, the high concentration of cytokines affects shoot renewal (Hussain et al. 2021). In general, balanced use of the ratio of auxin and cytokine induces callus formation (Su et al. 2011). One of the studies in tomato suggests that the best callus induction can be obtained with a higher cytokinin concentration than auxins (Skoog and Miller 1957). In addition, MS+IAA (0,1 mg/L) + Zeatin (2,0 mg/L) medium is determined to be the best shoot elongation medium with 89.4% shoot regeneration success (Sharma et al. 2009). The same concentration of auxin-cytokinin (MS+IAA (0,1 mg/L) + Zeatin (2,0 mg/L)) was determined as the best callus induction and shoot regeneration from cotyledon explant in five different tomato genotypes (Park et al. 2003).

Single copy T-DNA integration is desirable in transgenic plant production. Because, thanks to segregation, "marker-free" plants can be obtained by getting rid of selection markers (eliminates vector backbone sequences in transgenic plants). If the exogenous DNA fragment is added only once in a single location in the genome, a single copy of the transgene is obtained. Likewise, if a single exogenous DNA is present as two identical alleles on homologous chromosomes found in the organism, homozygous transgenic with a single transgene copy is present in only one homologous chromosome. It is called hemizygous. T0 plants are usually heterozygous or hemizygous states in terms of the transgene (Yuan et al. 2007).

*A. tumefaciens* strains differ in the transformation ability and the number of transgene copies in which these strains are integrated into the tomato genome. Although the highest conversion rate (65%) was achieved with the *Agrobacterium* strain GV3101, the EHA105 strain was found to have an optimal combination of high conversion efficiency (40%) and the ability to produce single-copy transgenic plants. The mortality rate of cotyledons due to *Agrobacterium* overgrowth was lowest in the GV3101 strain, and it was found that EHA105 was more effective than GV3101 in single T-DNA insertions transfer. 35% transformation success was achieved with the AGL1 strain and 15% with the C58pMP90 strain, and the C58pMP90 was determined to be the most effective strain in the transfer of single transgene insertions (Koncz et al. 1992; Chetty et al. 2013).

In gene editing studies, different mutation possibilities can appear in a diploid organism: "(I) absence of mutation (no detected mutations like a wild type), (II) heterozygous mutation (only one allele is mutated, or both alleles are mutated, but the sequence of each allele is different (biallelic mutation), (III) a homozygous mutation (both alleles have the same mutation)" and an allele can be utilized as a template for cleavage repairing in the other allele by HDR system can be triggered (Zischewski et al. 2017). With the indels formed as a result of DSB repair with NHEJ, the different repair

of the two alleles result in a biallelic and consequently "complete gene function loss," or in alleles without frameshift (in-frame), the present loss can be tolerated by the preservation of partial gene function (Schoonenberg et al. 2018).

The selection of transgenic plants in which the T-DNA integration occurs as a single copy simplifies the procedure for obtaining plants without T-DNA due to the monogenic inheritance model (showing 3: 1 segregation). Progenies have lost their T-DNA by segregation but still maintain the target mutations (Koncz et al. 1992). In this context, when 3:1 (1:2:1; 1 transgene in homozygous, two transgenes in hemizygous condition and one without transgene) Mendelian ratio in T1 generation shows that T-DNA integration occurs in one locus and two locations in 15:1 ratio (Cortina and Culiáñez-Macià 2004).

Also, the monoallelic/biallelic state of the mutation produced by sequencing PCR amplification with specific primers to cover the target region was determined. The preferred biallelic mutation for an effective knockout shows the "1 (homozygous for mutation 1): 2 (biallelic): 1 (homozygous for mutation 2)" segregation ratio in T1 progenies. Homozygous and biallelic mutations (gene modifications) can be stably maintained during regeneration in T0 transgenic plants (Pan et al. 2016).

In the literature, there are studies for strigolactones in *Solanum lycopersicum* (Bari et al. 2019a; Bari et al. 2019b), *Oryza sativa* (Yang et al. 2017; Butt et al. 2018), and *Nicotiana benthamiana* (Gao et al. 2018) plants using CRISPR-Cas9 system. In the study conducted by Yang et al. (2017), the knockout was performed in five carotenoid catabolic genes (*OsCYP97A4*, *OsDSM2*, *OsCCD4a*, *OsCCD4b*, and *OsCCD7*) found in rice (*Oryza sativa*) with the CRISPR-Cas9 method. Although the main target in this study was to increase the accumulation of  $\beta$ -carotene in the rice endosperm, carotenoid accumulation was not observed due to mono-allelic / bi-allelic knockout mutations in these five genes. However, a similar phenotype with the SL-deficient plant phenotype occurred in the mutant lines in which the mutation occurred in the *OsCCD7* gene. In a similar study by Butt et al. (2018), SL-deficient mutants were obtained by generating a mutation in the *OsCCD7* gene in rice through CRISPR-Cas9. It was determined by liquid chromatography/mass spectrometry analysis that SL amounts decreased in root extracts of these mutants. Consistent with previous studies, features such as increased tillering and plant height reduction, which can be recovered by applying the synthetic SL analog GR24, have been observed.

With the study carried out in tobacco (*Nicotiana tabacum* L.) by Gao et al. (2018), the mutation was created in 2 homologs of the *CCD8* gene (*NtCCD8A* and *NtCCD8B*) via CRISPR-Cas9. SL-deficient mutant plants were obtained by producing premature stop codons resulting from mutations caused by the deletion of three nucleotides in the *ntccd8a* mutant allele and insertion of a nucleotide in the *ntccd8b* mutant allele. Also, consistently with SL-deficient phenotype, in these mutant plants, more shoot branching, reduced plant height, increased leaf and node number, reduced total plant biomass, shorter primary root, and more lateral roots have occurred compared to the wild type.

In another study by Bari et al. (2019a), two homologs in tomato (*Solyc08g067610* and *Solyc08g067620*) of the ATP-binding cassette (ABC) protein

were mutated through the CRISPR-Cas9 system, and germination of the seeds of *P. aegyptiaca* weed was significantly reduced. The mutation was inherited as a result of the genotype analysis of T1 plants. In mutant plants, phenotypes consistent with SL deficiency have occurred, and a decrease in the expression of *CCD8* and *MAX1* genes were detected. However, the level of orobanchol does not change in the root extracts of mutant plants. Thanks to this study, ABC protein homologs can develop an effective method to reduce parasitic weed germination.

In the study conducted by Bari et al. (2019b), a single exon region in the *SICCD8* gene (in tomato) was targeted with a single gRNA using the CRISPR-Cas9 system, and this gene was knockout. As a result, the *SICCD8*-mutant line was resistant to *P. aegyptiaca* and carried out in the T1 generation, the mutation in the *SICCD8* gene was inherited. It was determined that the mutant line exhibits a phenotype with features such as dwarf, extreme shoot branching, and adventitious root. Also, orobanchol level and parasite infestation significantly reduced compared to the unmutated tomato.

Kumar et al. (2021) mutated the third exon of the *SIMAX1* gene (in tomato) with a single gRNA designed with the CRISPR-Cas9 method. As a result of the mutation, nine nt (3 amino acids (Lys252-Arg253-Iso254)) were deleted and inherited in the next generations. Compared to WT plants, *Simax1*-Cas9 heterozygous mutant T1 plants show phenotypic changes (dwarfism, increased shoot branching, more growth in axillary buds, and excessive adventitious root formation), which were milder than homozygous plants. However, resistance was obtained against *P. aegyptiaca* due to the decrease in strigolactone level in mutant lines (homozygous mutants showed higher resistance compared to heterozygous mutants), increased carotenoid level in all mutants. In addition, the mutant plants were observed to have a significant difference in dry root weights compared to the control plant (WT).

### 3. MATERIAL AND METHOD

#### 3.1. Bioinformatics Studies

The sequence information for the *SICCD7* and *SICCD8* gene regions was obtained from the National Center for Biotechnology Information (NCBI 1988) "Gene" database. The tomato genome in this database is Tomato Genome version SL3.0, which stakeholders cure the International Tomato Genome Sequencing Project. The exon (protein-coding region) and intron (non-coding) regions were determined by checking the resulting sequence information.

CRISPRDirect (Natio et al. 2015) program was used to rationalize sgRNAs required for the CRISPR-Cas9 system to perform double-strand cleavage in the desired gene region and create a mutation. All components of the gRNA to be used in the CRISPR-Cas9 system are available from this software as a whole. In addition, this software determines gRNA sequences according to PAM sequences (NGG). There are two main reasons why this program was chosen among many software. These are (I) detailed off-target analysis and (II) to present the entire sgRNA sequence as an output. In addition to these two essential features, the other reasons behind choosing the CRISPRDirect program are; the program provides the user with the GC rate (an important performance criterion increases the success rate with GC > 40%). Also, this software provides the restriction enzymes that cut in the selected region in detail to make identification of any mutation easy. In order to create mutations in the desired genes, the first and third exon regions of *SICCD7* and the first and second exon regions of *SICCD8* genes were targeted. In this way, more mutations can be created in the sequences of both genes that will produce protein, and the chance of success of the silencing process will be increased. For this purpose, two gRNAs were designed for the different exon regions of *the SICCD7* and *SICCD8* genes, respectively.

With the ExPASy/Translate tool (Gasteiger et al. 2003), the nucleotide sequence data of the genes were converted to mRNA sequences, and the intron parts of the genes were removed, and the actual sequence of the protein to be formed was determined. The correctness of the protein sequence (amino acid sequence) data obtained was also checked with the Sol Genomics Network program (Fernandez-Pozo et al. 2015), containing the sequence information of the previous studies mentioned in the literature. With this bioinformatics study, it was possible to accurately determine the gRNA sequence information used in the CRISPR method. Finally, the "off-target" possibilities of gRNA sequences have been checked. gRNAs was not match any sequence other than the target gene region. The CRISPRDirect program performs a detailed off-target analysis using the output of different genome projects. CRISPRDirect performs off-target analysis on more than 200 different genomes (including SL3.0). Exons determined for *SICCD7* and *SICCD8* are presented to the program, and possible gRNAs are evaluated not only 20mer but also for 12mer within the scope of off-target.

A universal sgRNA scaffold sequence (76 bp) was added to the selected gRNAs (20 bp without PAM sequences), and also, codon-optimized *A. thaliana* AtU6-26p RNA Pol III promoter (424 bp) and AtU6-26t terminator (192 bp) were added for the most effective expression. In addition, a cassette was created by adding the appropriate cut-regions (sequences) to combine gRNA 1 and 2, which were designed separately

for *SICCD7* and *SICCD8*, and integrated into the vector to be used in cloning (Figure 3.1) and sent to Sentebiolab Biotechnology (Ankara) company for their synthesis.

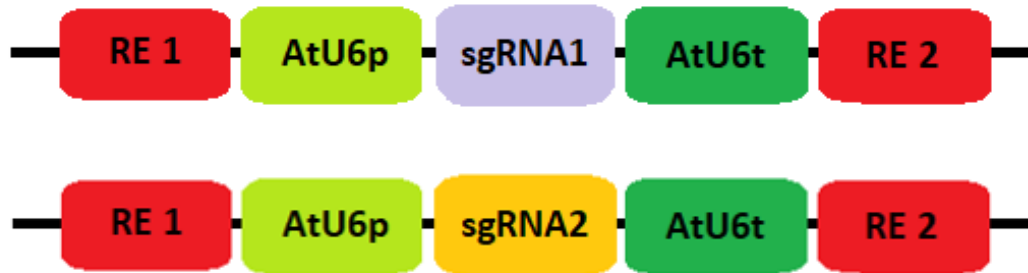


**Figure 3.1.** Representative image of created sgRNA 1 and sgRNA 2 cassettes (RE 1: PacI (5'...TTAAT/TAA...3'), RE 2: AclI (5'...AA/CGTT...3'))

### 3.1.1. Confirmation of the *SICCD7* and *SICCD8* gRNA targets on *Solanum lycopersicum* genome

In order to test whether the gRNAs designed with the CRISPRDirect program target the correct region in tomato, a PCR reaction was set up with tomato DNA extracted with 2% CTAB protocol (Doyle 1991). Because two exon regions were targeted for each gene (*SICCD7* and *SICCD8*) region, four separate primer pairs were designed with the Primer3Plus database (Untergasser et al. 2012) (Table 3.1) to amplify the targeted exon regions in the *SICCD7* and *SICCD8* genes (Figure 3.2a, b).

**Table 3.1.** Features of *SICCD7* gRNA1-2 and *SICCD8* gRNA1-2 primer pairs

| Primer Name            | Sequence (5' → 3')       | Length (bp) | Tm (°C) |
|------------------------|--------------------------|-------------|---------|
| <i>SICCD7</i> -gRNA1-F | GGCCAAAGCTTGCCATAATA     | 20          | 56      |
| <i>SICCD7</i> -gRNA1-R | AGTCTGAGCCTCCGTTTCAA     | 20          | 56      |
| <i>SICCD7</i> -gRNA2-F | AAGAGTAGAGGGTCCAGTGATAAA | 24          | 56      |
| <i>SICCD7</i> -gRNA2-R | TGGAGATGTTGGTTTGCTTG     | 20          | 55      |
| <i>SICCD8</i> -gRNA1-F | CAAGATCCTTCCTGACATGTTTGA | 24          | 56      |
| <i>SICCD8</i> -gRNA1-R | TAAGCTAACTCGGCCGAAAA     | 20          | 56      |
| <i>SICCD8</i> -gRNA2-F | GGTGCATCCCTAACCGATAA     | 20          | 56      |
| <i>SICCD8</i> -gRNA2-R | GTTGCCACTGGCTTTACACA     | 20          | 59      |



All PCR amplifications were performed with Bio-Rad T100™ thermocycler according to the optimized conditions presented in Table 3.2 and Table 3.3. The 20 µl of PCR products were mixed with 5 µl of loading dye (15 ml of Glycerol, 35 ml of H<sub>2</sub>O, 10 mg of Bromophenol Blue (Sigma-Aldrich)) and was run in 100 ml 1X TBE buffer solution containing 1.5 % agarose gel for 1 hour at 100 V. Gel imaging is done under UV (DNR, MiniLumi). For the preparation of the 1X TBE buffer solution used, 108 g Tris-base (Merck) (890 mM), 55 g Boric acid (Merck) (890 mM), 40 ml 0.5 M EDTA (Merck) are added to 1 liter of H<sub>2</sub>O, and the pH is adjusted to 8.0. 10X TBE is diluted with water in a ratio of 1:9 and made into 1X. Then, 1 g of agarose (Invitrogen) was added to 100 ml of 1X TBE, and it was left in the microwave oven for approximately 3 minutes to dissolve. When the cooling process begins, 5 µl of Ethidium Bromide (EtBr - C<sub>21</sub>H<sub>20</sub>BrN<sub>3</sub>) (Sigma-Aldrich) was added. After gelation, products were subjected to electrophoresis.

| Component    | Quantity | Brand |
|--------------|----------|-------|
| DNA Template | 2 µl     |       |

45

**Continuation of Table 3.2**

|                                                                                      |                                      |                          |
|--------------------------------------------------------------------------------------|--------------------------------------|--------------------------|
| ddH <sub>2</sub> O                                                                   | 75 µl                                |                          |
| 10X Taq Buffer ((NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> -MgCl <sub>2</sub> ) | 10 µl                                | Thermo Fisher Scientific |
| MgCl <sub>2</sub> (25 mM)                                                            | 8 µl                                 | Thermo Fisher Scientific |
| Taq DNA Polymerase (5 U/µl)                                                          | 1 µl                                 | Thermo Fisher Scientific |
| dNTP (10 mM each)                                                                    | 2 µl                                 | abm                      |
| Primer-Forward (10 µM)                                                               | 2 µl                                 | Sentegen                 |
| Primer-Reverse (10 µM)                                                               | 2 µl                                 | Sentegen                 |
| TOTAL                                                                                | approximately 100 µl (for 5 samples) |                          |

**Table 3.3.** PCR cycles used in DNA amplification

|                                           |            |           |                   |
|-------------------------------------------|------------|-----------|-------------------|
| 95°C                                      | 5 minutes  | 1 cycle   | Denaturation (I)  |
| 95°C                                      | 45 seconds | 35 cycles | Denaturation (II) |
| 57°C (optimized for these 4 primer pairs) | 45 seconds |           | Annealing         |
| 72°C                                      | 1 minute   |           | Extension (I)     |
| 72°C                                      | 10 minutes | 1 cycle   | Extension (II)    |

In consequence of products' electroporation, single bands of 448 bp for SICCD7 gRNA1, 562 bp for SICCD7 gRNA2, 594 bp for SICCD8 gRNA1, 483 bp for SICCD8 gRNA2 are expected. These single bands belonging to each gRNA region were cut with a scalpel on a low-beam intensity UV device (Transilluminator, UVP). GeneJet Gel Extraction Kit (Thermo Fisher Scientific) was used to purify the cut pieces from agarose gel. The products removed from the agarose gel were eluted to 20 µl and then sent to the BMLabosis (Ankara) company for Sanger sequencing.

### 3.1.2 Design of cloning vector

As the cloning vector, the binary pFGC-pcoCas9 (Addgene, plasmid #52256) vector was determined as the most suitable vector for this study. This binary vector is one of the essential elements of the CRISPR system, in the presence of the appropriate promoter (35PPDK, hybrid promoter consisting of the cauliflower mosaic virus 35S

enhancer fused to the maize C4PPDK basal promoter (Yoo et al. 2007) and terminator (NOS). It contains an optimized Cas9 construct (pcoCas9), but it contains MCS that allows easy integration of gRNA cassettes. Because this vector is binary, it contains two replication origins; pVS1 for *Agrobacterium* (origin of replication for the *Pseudomonas* plasmid pVS1, (Heeb et al. 2000)) and pBR322 for *Escherichia coli* (*E. coli*). Kanamycin resistance plays a role in the bacterial selection of the vector with the aphA3 gene. Thus, in the presence of Kanamycin, which is the antibiotic suitable for bacterial selection, *E. coli* (e.g., DH5 $\alpha$  strain) can be used as the replication line of the vector. Also, because it contains the *Agrobacterium* replication origin, the *Agrobacterium* strains can be used for plant transformation. BASTA (Glufosinate Ammonium) is used for plant selection due to the presence of the bar gene, which is an extra element in the binary vector.

### 3.2. Cloning of Plasmids Harboring gRNAs and the pFGC-pcoCas9 Vector

The gRNAs were arrived in plasmids with a total volume of 35  $\mu$ l (having a 2887 bp long pTZ57R backbone), and after centrifugation at 4 °C, 6000 x g for 1 minute, 100  $\mu$ l DNase / RNase-Free dH<sub>2</sub>O was added, and their concentrations were optimized. After the process, plasmids were stored at -20°C in 2 ml microtubes. These plasmids needed to be transformed into *E.coli* first to increase the number of copies available to carry out further analysis.

#### 3.2.1. Preparation of media

In order to create the living environment of bacterial cultures, 25 g of LB Broth Miller (Sigma-Aldrich) mixture containing 10 g/L Tryptone, 10 g/L NaCl, 5 g/L Yeast Extract was weighed and added to the 1-liter bottle. 1 liter of ddH<sub>2</sub>O and 15 g of agar (Sigma-Aldrich) were added (pH: 7.0  $\pm$  0.2 at 25°C). Three of them were prepared in order to prepare plates with different antibiotic content from this mixture. The mixtures were autoclaved at 121°C for 30 minutes and cooled in the incubator until the temperature dropped to 55°C. At the same time, an agar-free version of the same medium (without antibiotics) was prepared with the same ingredients for use as a liquid medium. The first of the cooling mixtures were added to the final concentration 100  $\mu$ g/ml from ampicillin antibiotic, which has 100 mg/ml stock concentration for use in the bacterial selection of the plasmids, which have gRNA cassettes were synthesized by Sentebiolab Biotechnology. The final concentration was added to 50  $\mu$ g/ml from the kanamycin antibiotic to the second mixture, which has a stock concentration of 50 mg/ml, for use in the transformation of the Addgene pFGC-pcoCas9 (#52256) vector. The final mixture was divided into 25 ml volumes without adding antibiotics. After the media solidified in the petri dish, the petri dishes were covered with parafilm and kept at 4°C until use.

#### 3.2.2. Preparation of competent cells of *E. coli* - DH5 $\alpha$ strain

In order to increase the number of clones by utilizing the division of a bacterial cell of plasmid DNA, the bacteria used must first be competent. The competent cell should be prepared according to the method which is used in transformation. Bacteria cells (e.g., *E. coli* DH5 $\alpha$  strain) for this purpose are treated with positively charged ions such as CaCl<sub>2</sub> (CaCl<sub>2</sub> treatment), and pores are opened in the cell membranes with the

cold/hot process to be applied during the heat-shock transformation method. This process involves several stages:

- i. On the antibiotic-free plates, the stock *E. coli DH5α* strain culture from -80°C were streaked.
- ii. It was placed in the incubator and kept overnight (O/N (overnight)) to ensure that *E. coli* reproduces at 37°C, the optimum growing temperature.
- iii. After 1 night, 3 bacterial colonies were selected from the plates where reproduction was observed, transferred to 15 ml falcon tubes containing 10 ml of medium (agar-free LB Broth Miller) with the help of the spreader. Moreover, it was left in the shaker for O/N (about 18 hours) at a rate of 225 rpm and 37°C to allow it to reproduce.
- iv. To determine the Optical Density (OD) of the cells grown in the falcon tubes the next day, measurements were performed on the spectrophotometer device by placing 900 µl dH<sub>2</sub>O and 100 µl cell culture in spectrophotometer tubes. The device calibration was made with a “calibration sample” (cell non-cultural) prepared with 900 µl dH<sub>2</sub>O and 100 µl pure liquid LB Broth Miller. After the measurement, a value of 0.78 OD was obtained.
- v. In order to achieve the optimum 0,5 OD value in the preparation of the competent cell, 100 X dilution was made by taken from the cultural environment and transferred to 100 ml pure liquid medium. In this way, only living cells are reproduced in cell culture, and an optimum 0.5 OD value is obtained. The *E. coli* cell divides every 20-30 minutes, doubling the number of cells. Therefore, OD measurement with certain intervals (60 minutes later - 0.016, 120 minutes later - 0.295, 140 minutes later - 0.341, 170 minutes later - 0.509) 0,5 OD value is achieved.
- vi. When the value of O.D 0,509 was obtained in the 170th minute, 100 ml of cell culture was taken into two pre-cooled falcon tubes, 50 ml – 50 ml, and the cells were incubated on ice for 10 minutes.
- vii. After incubation, liquid cell cultures were centrifuged at 4°C and 5000 rpm for 10 minutes, the supernatant part was discarded, and the pellet was slowly dissolved with 0.1M cold CaCl<sub>2</sub> (Merck). For each falcon, 5 mL of solution was used. Since the salt formed pores in the cell wall, the cells were sensitive, so precision was treated during dissolving. Cell culture with CaCl<sub>2</sub> containing dissolved pellets was incubated on ice for 30 minutes.
- viii. After incubation and centrifugation for 10 minutes at 4°C and 5000rpm, the supernatant part was discarded, and the pellet was slowly dissolved with 0.1M CaCl<sub>2</sub> + 15% Glycerol (Isolab) solution. 2.5 mL of solution was used for each falcon. A total of 5 mL of competent cells were obtained, and the cells were divided into volumes of 100 µl and streaked to the agar-free media of the prepared, competent cell before transfer to -80°C. Simultaneously, a control Petri was created by streaking them on the agar medium containing Ampicillin (NeoFroxx). Thanks to this test, the purity of *E. coli DH5α* competent cells was checked.

### 3.2.3. Transformation and plasmid purification of pTZ57R-gRNA1/2 plasmids harboring gRNAs

The process of transferring the plasmid DNAs to be cloned into competent cells prepared to increase the number of clones by utilizing the division of a bacterial cell of the plasmid DNA is called "Bacterial transformation". For this purpose, the heat-shock method was used as the transformation method. The hot-cold treatment to be applied consecutively to the competent cell and plasmid DNA mixture, and electrostatic repulsion created by the CaCl<sub>2</sub> in the medium between the competent cell and the plasmid DNA, the plasmid DNA is transferred into these cells by forming pores in the membrane of the cells. In this context:

- i. The competent cells taken from -80°C were melted on ice. In this study, 100 µl of competent cells and 3 µl of plasmids were used for each transformation. The competent cell and plasmid DNA (separately for each DNA) were mixed in a 1,5 ml microtube and incubated on ice for 30 minutes.
- ii. To perform transformation with the heat-shock method, the tubes incubated on the ice were kept for 2 minutes at 42°C, which is the optimum transformation temperature where the cells can maintain their viability. After 2 minutes, the cells were retaken on the ice and left for another 2 minutes.
- iii. After 2 minutes, 900 µl of antibiotic-free liquid LB Broth Miller was added into the tubes and incubated at 37°C, 225 rpm, and 1 hour in a shaker. In this way, the cells are allowed to perform cell division within 1 hour without antibiotic pressure.
- iv. At the end of 1 hour, 100 µl products containing pTZ57R-SICCD7/8-gRNA1/2 plasmid DNA were streaked in antibiotic (Ampicillin) petri dishes for bacterial selection. The petri dishes were inverted and left in an incubator at 37°C and O/N, without parafilm.
- vi. It was checked whether there were colonies in petri dishes. Three of the colonies were selected and transferred to 15 ml falcon with 5 ml of liquid LB and 5 µl antibiotics (Ampicillin) and left the O/N at 37°C by leaving an opening to provide an oxygen inlet and outlet from the covers.

The cell culture, which was left at 37°C and 225 rpm for 1 night, was allowed to precipitate at 4°C, 5000 rpm, and 15 minutes by centrifugation. By pouring the supernatant phase formed, the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) was used for plasmid isolation from the remaining pellet, and the manufacturer's procedure was used. DNA control was performed by loading 5 µl of the obtained plasmids to 1% agarose gel. The remaining parts were transferred to -20°C.

### 3.2.4. Determination of the accuracy of purified pTZ57R-gRNA1/2 plasmid DNAs

The forward primer-gRNAc/F (5'-AGAAGAGAAGCAGGCCCAT-3') was designed over the AtU6-26p promoter (typical for pTZ57R-gRNA1/2) and the reverse primer-gRNAc/R (5'-CGGTGCCCACTTTTCAAGTT-3') over the gRNA scaffold (also typical for pTZ57R-gRNA1/2) through Primer3Plus database and were

synthesized in Sentebiolab Biotechnology. The reaction using the purified 3 pTZ57R-gRNA1 / 2 plasmid DNAs as a template was prepared under the PCR conditions in Table 3.2 and Table 3.3 ( $T_m$  was used as 57°C).

### 3.2.5. Cloning the pFGC-pcoCas9 vector and determining the accuracy of the purified plasmid DNAs

The pFGC-pcoCas9 vector (ordered from Addgene) arrived as a cell culture in the medium. The bacterial cell culture was streaked on agar media harboring selection (Kanamycin) to form a colony. The next day, 5 of the colonies were selected and left on O/N incubation (colonies transferred to 5 ml agar-free LB medium containing 5 µl antibiotics (50 mg/ml Kanamycin (NeoFroxx) stock) and incubated in a shaker at 37°C, at 225 rpm). Plasmids were purified with GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) from these liquid cell cultures. According to the heat-shock protocol, the transformation was done using 100 µl competent cells and 3 µl from one selected Miniprep product. In this way, while increasing the number of copies of the pFGC-pcoCas9 plasmid, its accuracy and transformation ability was also checked. Simultaneously, *E. coli* DH5α competent cells were tested to control the selection medium containing 50 µg/ml Kanamycin. The cell culture obtained after transformation was incubated at 37°C, O/N in an agar medium containing Kanamycin to form a colony. The next day, 2 colonies were selected from the selection medium and transferred into 5 ml agar-free LB and 5 µl antibiotics (added from 50 mg/ml Kanamycin stock) media for O/N shaking at 37°C, 225 rpm. Afterward, plasmid purification was done with GeneJET Plasmid Miniprep Kit and kept at -20°C.

In order to determine the correctness of the two pFGC-pcoCas9 plasmid DNAs purified, a digestion reaction was performed by selecting two enzyme cut regions. For this reaction, a total of 20 µl reaction mix was created, with 1X EcoRI (Thermo Fisher Scientific) and 1X NcoI (Thermo Fisher Scientific) enzymes and 2X Tango Buffer (Thermo Fisher Scientific), 1 µl plasmid DNA, and it was incubated at 37°C for 2.5 hours. The resulting digestion products were run in 1.5% agarose gel for 1 hour at 100 V and visualized under UV. As a result of the digestion process, based on the map information of the pFGC-pcoCas9 plasmid, two bands of size 4931 bp and 8399 bp are expected.

### 3.3. Combining sgRNA1<sub>SlCC7/8</sub> and sgRNA2<sub>SlCCD7/8</sub> and Ligation to pFGC-pcoCas9 Vector

Plasmids with pTZ57R backbones harboring gRNAs (pTZ57R-SlCCD7-gRNA1, pTZ57R-SlCCD7-gRNA2, pTZ57R-SlCCD8-gRNA1, pTZ57R-SlCCD8-gRNA2) were synthesized separately because of their long sequences. Enzyme sites added to the ends of gRNAs (Figure 3.3) were used to drop the desired gRNA fragments from the backbone.

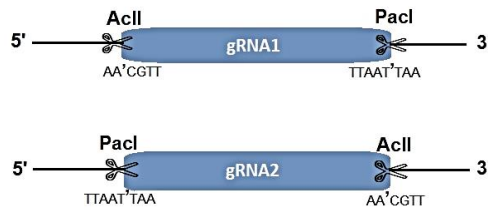


**Figure 3.3.** Enzyme restriction sites at the head and end of gRNAs in the plasmids with pTZ57R backbone

5 µl of pTZ57R plasmids containing gRNAs were digested for 2 hours at 37°C. Final concentrations were adjusted as 1X AclI (NEB), 1X PacI (NEB) enzymes, and 1X CutSmart Buffer (NEB). The size of the expected fragment containing the gRNA region was determined from the plasmid map since there is one PacI cut region on the plasmids but more than one AclI cut region. Band lengths of 730 bp for SlCCD7-gRNA1, 717 bp for SlCCD7-gRNA2, 726 bp for SlCCD8-gRNA1, and 721 bp for SlCCD8-gRNA2 are expected after digestion. The digested products were run on 0.8% agarose gel and 75 V for 4 hours. When enough separation of the bands is completed according to their size, correct-sized bands were cut on a UV transilluminator (to prevent a possible mutation) and purified. To determine the accuracy of digestion fragments, using gRNAc-F / R primers and purified products as template thermocycler reaction set up according to the conditions in Table 3.2 and Table 3.3. The plasmid pFGC-pcoCas9 (#52256), where gRNAs will be ligated and used as a vector, was digested from the PacI digestion region in only one location (base position 78) (Figure 3.4), allowing the plasmid to open (become linear). Using 2 µl (2X) of pFGC-pcoCas9 plasmid DNA as a template, a total of 20 µl digestion reaction with the final concentration was 1X PacI-Fast Digest (Thermo Fisher Scientific) enzyme, and 2X Fast Digest Buffer (Thermo Fisher Scientific) was created and digested at 37°C for 1 hour (then 65°C - 10 minutes for enzyme inactivation). After the digestion products were run in 0,8% agarose gel and 75 V for 3 hours, 13,330 bp single band (the main size of the linear plasmid) was cut on UV transilluminator and purified.

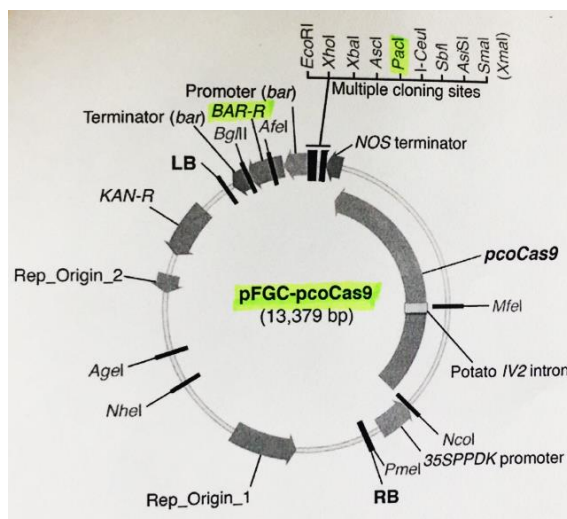


**Figure 3.4.** Representative plasmid map of the pFGC-pcoCas9 vector

Before ligating the gRNA regions to the pFGC-pcoCas9 vector linearized with the PacI restriction enzyme via the T4 ligase enzyme, rSAP (Shrimp Alkaline Phosphatase (rSAP), NEB) was applied to prevent self-ligation of the pFGC-pcoCas9 plasmid (digested from a single point (base position 78)). With rSAP treatment, "self-ligation" is prevented by catalyzing the phosphorylation of the 5' and 3' ends of the DNA and RNA phosphomonoesters. For this purpose, a 10 µl reaction is prepared with the final volume of 1X Cut Smart Buffer (NEB), 1X rSAP enzyme (NEB), and 8X pFGC-pcoCas9 gel purification product for 2.5 hours at 37°C and then incubated for 5 minutes at 65°C for enzyme inactivation.

T4 Ligase enzyme was used to ligate gRNA1 and gRNA2 isolated in separate fragments (for *SICCD7* and *SICCD8* genes) to pFGC-pcoCas9 vectors. For the ligation process with T4 ligase enzyme (Thermo Fisher Scientific), a total of 10 µl reaction was created with 1X pFGC-pcoCas9 (#52256) rSAP product, 1X T4 Ligase Buffer, 1X T4 Ligase Enzyme, 3X gRNA1, and 3X gRNA2. The mixture was incubated at 16°C for O/N (about 18 hours) and then for 10 minutes at 65°C for enzyme inactivation. According to the heat-shock method, the transformation was performed using 8 µl ligated products and 100 µl *E. coli* competent cell (*DH5α* strain). Transformation products were taken into media containing 50 µg/ml Kanamycin-LB plates and incubated at 37°C for O/N (approximately 20 hours). The next day, five colonies were transferred into liquid LB medium containing 50 µg/ml kanamycin, and colony PCRs were done with gRNAc-F/R primers for verification simultaneously. The correct plasmids were purified and sent to Sentegen for Sanger sequencing.

At the same time, using the Primer3Plus program, a new primer was designed (Table 3.4) to cover the post-ligated combination of gRNAs (gRNA1-gRNA2) designed separately for the *SICCD7* and *SICCD8* genes. As a result of DNA amplification, 754 bp product is expected for *SICCD7* and 755 bp product for *SICCD8* to verify gRNA regions.

**Table 3.4.** Features of designed *SICCD7* - F/R and *SICCD8* - F/R primers

| Primer Name      | Sequence (5' → 3')       | Length (bp) | % GC | Hairpin Tm (°C) | Self Dimer Tm (°C) | Tm (°C) |
|------------------|--------------------------|-------------|------|-----------------|--------------------|---------|
| <i>SICCD7</i> /F | TCTTGGCTTTGTAGTAGCGTTTT  | 23          | 41,7 | 45,4            | 0,8                | 57,9    |
| <i>SICCD7</i> /R | CTCTAAAACCGTCCCCAACTC    | 21          | 50,0 | 42,0            | -                  | 57,6    |
| <i>SICCD8</i> /F | CAACGACGAGTTCTCCTTCA     | 20          | 52,4 | -               | -                  | 58,3    |
| <i>SICCD8</i> /R | GTAGTGATTATTCTCACCACCGTA | 24          | 39,1 | 39,5            | -                  | 58,9    |

### 3.4. Transformation of pFGC-pcoCas9<sub>SICCD7/8</sub>-gRNA1+2 Plasmids into *Agrobacterium tumefaciens* Strain

The *Agrobacterium tumefaciens* strain, which plays an essential role in the next stage of plant transformation, was selected by looking at the studies in the literature, and

for this purpose, the EHA105 strain, which is one of the most effective strains, was chosen for a plant transformation.

#### **3.4.1. Preparation of competent cells of *Agrobacterium tumefaciens* EHA105 strain**

As in *E. coli* cell, *Agrobacterium tumefaciens* competent cell preparation was made with the protocol determined for the transformation method. In the “electroporation” method for transformation, plasmid transport to the cells is provided by the electrical field to be created from the pores in the *Agrobacterium* cell membrane. Therefore, to avoid problems in the electrical field, competent cells must be purified from charged ions from the medium.

i. *Agrobacterium tumefaciens* EHA105 strain (provided from Assoc. Professor Amit MITRA, University of Nebraska-Lincoln) was streaked on agar medium containing the final concentration of 40 µg/ml Rifampicin (AppliChem) antibiotic and left at 28°C, the optimum growth temperature for *Agrobacterium* for two days. Rifampicin resistance is due to a mutation in the *Agrobacterium* genome and is used to produce the correct bacterial colony against possible contaminations.

ii. At the end of 2 days, one of the colonies observed in petri dish was selected and transferred to 100 ml liquid YEP medium (Sigma-Aldrich) (10 g Bacto-peptone, 5 g NaCl, 10 g Yeast extract, 15 g agar) with a final concentration of 25 µg/ml Rifampicin antibiotic and incubated overnight by shaking in a 28°C.

iii. The next day, measurement was made until the optimum OD (600 nm) value of 0,5 was obtained for competent cell preparation. The processes after this stage were continued on ice.

iv. After adjusting the OD value, the liquid culture was divided into 50 ml falcons and centrifuged at 5000 rpm for 15 minutes.

vi. After centrifugation, the supernatant was discarded, and the pellet settled to the bottom was dissolved with 50 ml of cold and sterile ddH<sub>2</sub>O, again centrifuged at 5000 rpm for 15 minutes.

vi. After centrifugation, the supernatant was discarded, and the pellet settled to the bottom was dissolved with 25 ml of cold and sterile ddH<sub>2</sub>O, again centrifuged at 5000 rpm for 15 minutes.

vii. After centrifugation, the supernatant was discarded, and the pellet settled to the bottom was dissolved with 12.5 ml cold and sterile ddH<sub>2</sub>O, again centrifuged at 5000 rpm for 15 minutes.

viii. After centrifugation, the supernatant was discarded, and the pellet settled to the bottom was dissolved with 10 ml of cold and sterile ddH<sub>2</sub>O, again centrifuged at 5000 rpm for 15 minutes.

ix. After centrifugation, the supernatant was discarded, and the pellet accumulated to the bottom was dissolved with 5 ml of cold and sterile 10% glycerol, again centrifuged at 5000 rpm for 15 minutes.

x. After centrifugation, the supernatant was discarded, and the pellet settled to the bottom was dissolved with 1 ml of cold and sterile 10% glycerol and divided into microtubes, and the prepared, competent cell was kept at -80°C.

### 3.4.2. Transformation of pFGC-pcoCas9 plasmids harboring gRNAs into *Agrobacterium tumefaciens* - EHA105 strain

The electroporation method is one of the most effective methods in bacterial transformation with *Agrobacterium* competent cells. This method was used to transfer post-ligated miniprep products (pFGC-pcoCas9<sub>SICCD7/8</sub>-gRNA1+2) to *A. tumefaciens* (EHA105 strain) competent cells. The electroporation method transfers the desired plasmid DNA from the membrane of the competent cell, creating an electrical field with high voltage. This process is done with the help of special equipment (Figure 3.5).



**Figure 3.5.** Electroporation device used in transformation with *Agrobacterium* competent cell

- i. After concentrations of pFGC-pcoCas9 (# 52256), plasmids harboring gRNAs were optimized to be maximum 80 ng/μl, 1 μl was taken for each of them, 100 μl of EHA105 electro-competent cell was mixed slowly by pipetting on ice.
- ii. This mixture was transferred to 0,2 cm ice-cold electroporation cuvettes, and the water droplets on the outer wall of the cuvette were cleaned.
- iii. After placing the cuvette in the part of the electroporation device where the electric current will be created, 2,5 kV current was given to transfer the plasmids into the competent cells. Immediately after the transfer, 1 ml of liquid LB medium was added to the cuvettes, and the cells were kept alive. The cell culture in the cuvette was taken into a microcentrifuge tube and left to grow for 2 hours at 28°C without antibiotics.
- iv. 100 μl of cells with increased cell density were grown in YEP medium containing agar antibiotics containing 50 μg/ml Kanamycin (bacterial selection of pFGC-pcoCas9 plasmid) + 25 μg/ml Rifampicin (to prevent possible bacterial contamination). It was left to reproduce at 28°C for two days. As a control, the empty competent EHA105

strain was also cultivated in an agar YEP medium containing 50 µg/ml Kanamycin + 25 µg/ml Rifampicin. The competent cell without construct should not grow in Kanamycin selection medium.

vi. At the end of 2 days, 5 of the colonies observed in petri were selected. 5 ml of liquid LB containing 50 µg/ml Kanamycin + 25 µg/ml Rifampicin antibiotics were transferred to LB medium, and the O/N was shaken at 28°C. Simultaneously, colony PCR was performed with the selected colonies and newly designed primers according to the optimized conditions in Table 3.2 and Table 3.3 (T<sub>m</sub>: 53°C) and miniprep stocks of the ligation products used as positive control and the water as a negative control. The products were visualized under 1,5% agarose gel under UV.

vi. Cell cultures containing confirmed colonies were mixed with 80% sterile glycerol in a 1: 1 ratio and the stock was prepared and kept at -80°C.

### 3.5. Plant Transformation

Transferring the designed CRISPR construct to the tomato through the *A. tumefaciens* EHA105 strain is called plant transformation. As mentioned in the literature summary, thanks to this process, the plant selection marker and the region of gRNAs and Cas9 gene sequences between the right and left border on the binary pFGC-pcoCas9SlCCD7/8-gRNA1 + 2 vectors are transferred to the plant genome as a single strand with the help of Vir genes owned by *A. tumefaciens*.

#### 3.5.1. Analyzing the plant transformation potential of *Agrobacterium tumefaciens* EHA105 strain

For plant transformation, pCambia1303<sub>*A.tumefaciens*</sub>-EHA105 (Bacterial Kanamycin resistance) vector (Figure 3.6), which has the *GFP* (*mgfp5*) gene (Green Fluorescence Protein), were transferred to the tomato by vacuum infiltration method in order to get information about expression efficiency of *A. tumefaciens* EHA105 strain.

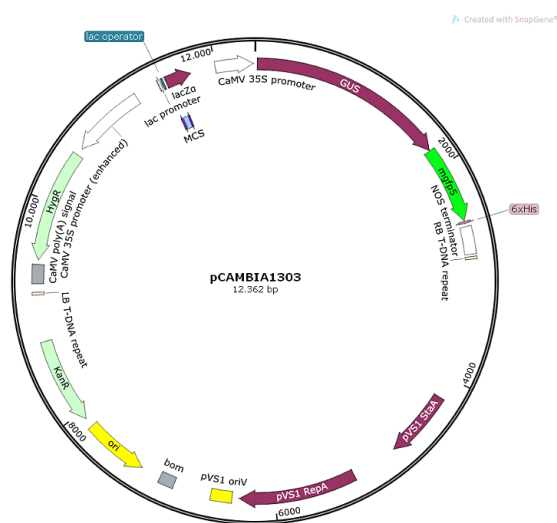


**Figure 3.6.** Representative map of the pCambia1303 vector

During the period which the *GFP* begins to be expressed transiently in the plant genome, and expression cannot be observed observationally (approximately 12 days), sections from the plant's leaf will be examined with a fluorescence microscope (488 nm excitation). In this way, it is aimed to gather estimated information about the expression of the construct transformed into *A. tumefaciens* EHA105 strain in the tomato genome. Accordingly,

i. The EHA105 strain containing the binary pCAMBIA1303 vector harboring the *GFP* gene (*mgfp5* under CaMV 35s promoter control) was grown on LB medium containing 25 µg/ml Rifampicin and 50 µg/L Kanamycin and at 28°C for two days.

ii. One of the colonies was transferred to a 50 ml BBL (5 g NaCl, 5 g Yeast Extract, 10 g Soy Hydrolysate (pH: 7.0)) liquid medium containing 25 µg/ml Rifampicin and 50 µg/L Kanamycin antibiotics and incubated by shaking O/N in 28°C. Likewise, the glycerol stock, including the tomato bushy stunt virus (TBSV) P19, was transferred to the liquid medium containing 10 ml of BBL and 50 µg/L Kanamycin antibiotics and shaken in a 28°C. In this way, it acts as reducing the single strand siRNA level and increase transformation efficiency.

iii. The next day, the liquid culture, whose OD value was measured as 2,0, was centrifuged at 5000 g for 5 minutes (at +4°C). After pouring the supernatant portion, the pellet was diluted with MES buffer (2- (N-Morpholino)ethane sulfonic acid) (Goldbio) in a 250 ml beaker with final OD 0,5. Acetosyringone (Sigma) (200 mM stock) was added to a 1:1 ratio and was stirred at room temperature and 225 rpm for 2 hours. Likewise, P19 cell culture was diluted with MES to final OD 0,5, and 1:1 Acetosyringone was added and mixed at 225 rpm for 2 hours.

iv. 10 µl of the surfactant Tween20 and P19 mixture was added in 1:9 ratios to the pCAMBIA1303-MES mixture, ready after 2 hours. Tomato seedlings were fixed so that they were upside down in the beaker, and the entire leaf surface was covered with this mixture (Figure 3.7). The plant was vacuumed at about 200 mmHg (0,26 bar) for 6 minutes in the desiccator, and the process was repeated twice. As a control, only the MES buffer solution was infiltrated. While the negative pressure increases during the vacuum process, the plant sap between the tissue gaps of the plant emerges, and when the vacuum is removed, the plant transformation process is completed within a short period by replacing these spaces with the liquid cell culture. After transformation, the plants were taken in viols containing perlite: peat mixture and preserved in greenhouse conditions.



**Figure 3.7.** Desiccator equipments for basic vacuum infiltration

### 3.5.2. *Agrobacterium*-mediated plant transformation of pFGC-pcoCas9 vectors harboring CRISPR constructs

267 F1 (indeterminate variety) and Micro-Tom (the model cultivar of tomato) genotypes were used in plant transformation with tomato, and two different methods were used: a modified version of the basic tomato transformation protocol created by Van Eck (2019) (Protocol A) and the optimized transformation protocol of Assoc. Prof. Dr. Hasan Pınar (Protocol B). Protocol A was used for the Micro-Tom with dwarf and short life cycle genotype, while protocol B was used for the 267 F1 genotypes. For *Agrobacterium*-mediated plant transformation, the EHA105 strain was used, which contains pFGC-pcoCas9 vectors harboring CRISPR constructs and whose success in plant transformation was confirmed by a preliminary study. For plant transformation and regeneration, the media given in Table 3.5 and Table 3.6 have been used, and studies have been carried out under controlled conditions such as growing cabins adjusted for optimum conditions for the growth of the tomato.

**Table 3.5.** Contents of the protocol A

|                                                                   | <b>Germination<br/>Medium<br/>(GM)</b> | <b>Pre-<br/>Induction<br/>Medium<br/>(PM)</b> | <b>Co-<br/>cultivation<br/>Medium<br/>(CM)</b> | <b>Regeneration<br/>Medium I<br/>(SM)</b> | <b>Regeneration<br/>Medium II<br/>(SM)</b> | <b>Rooting<br/>Medium<br/>(RM)</b> |
|-------------------------------------------------------------------|----------------------------------------|-----------------------------------------------|------------------------------------------------|-------------------------------------------|--------------------------------------------|------------------------------------|
| <b>Ingredients<br/>and Stock<br/>Concentrations</b>               | For 1<br>liter                         | For 1<br>liter                                | For 1<br>liter                                 | For 1<br>liter                            | For 1<br>liter                             | For 1<br>liter                     |
| MS<br>(Murashige and<br>Skoog 1962)<br>with vitamins<br>(Duchefa) | 4,3 g                                  |                                               |                                                |                                           |                                            |                                    |

(Continues on next page)

**Continuation of Table 3.5**

|                                                                  |      |       |       |        |               |        |
|------------------------------------------------------------------|------|-------|-------|--------|---------------|--------|
| MS Basal Salt Mixture<br>(Murashige and Skoog 1962)<br>(Duchefa) |      | 4,3 g | 4,3 g | 4,3 g  | 4,3 g         | 4,3 g  |
| Sucrose<br>(Isolab)                                              | 10 g | 20 g  | 20 g  | 20 g   | 20 g          | 30 g   |
| Nitsch & Nitsch Vitamin<br>(1000x)                               |      | 1 ml  | 1 ml  | 1 ml   | 1 ml          | 1 ml   |
| pH                                                               | 5,8  | 6,0   | 6,0   | 5,8    | 5,8           | 5,8    |
| Plant Agar<br>Type I<br>(Caisson)                                | 7 g  | 6 g   | 6 g   | 7 g    | 7 g           | 6 g    |
| Zeatin<br>(Caisson)<br>(1 mg/ml)                                 |      | 2 ml  | 2 ml  | 2 ml   | 1 ml          |        |
| Acetosyringone<br>(Sigma-Aldrich)<br>(200 µM)                    |      |       | 1 ml  |        |               |        |
| Timentin<br>(Goldbio)<br>(200 mg/ml)                             |      |       |       | 1 ml   | 1 ml          | 1 ml   |
| Glufosinate-Ammonium<br>(Basta)<br>(Sigma-Aldrich)<br>(1 mg/ml)  |      |       |       | 2 ml   | 1 ml / 0,5 ml |        |
| IAA (Caisson)<br>(1 mg/ml)                                       |      |       |       | 0,1 ml |               | 0,1 ml |
| IBA (Caisson)<br>(1 mg/ml)                                       |      |       |       |        |               | 0,1 ml |

**Table 3.6.** Content of the protocol B

|                                                                           | <b>Germination<br/>Medium<br/>(GM)</b> | <b>Pre-<br/>Induction<br/>Medium<br/>(PM)</b> | <b>Co-cultivation<br/>Medium (CM)</b> | <b>Shoot<br/>Induction<br/>Medium I<br/>(SM)</b> | <b>Shoot<br/>Induction<br/>Medium<br/>II (SM)</b> | <b>Rooting<br/>Medium<br/>(RM)</b> |
|---------------------------------------------------------------------------|----------------------------------------|-----------------------------------------------|---------------------------------------|--------------------------------------------------|---------------------------------------------------|------------------------------------|
| <b>Ingredients<br/>and Stock<br/>Concentrations</b>                       | For 1<br>liter                         | For 1<br>liter                                | For 1<br>liter                        | For 1<br>liter                                   | For 1<br>liter                                    | For 1<br>liter                     |
| MS<br>(Murashige and<br>Skoog 1962)<br>basal salt<br>mixture<br>(Duchefa) | 4,3 g                                  | 4,3 g                                         | 4,3 g                                 | 4,3 g                                            | 4,3 g                                             | 4,3 g                              |
| Nitsch & Nitsch<br>Vitamin<br>(1000x)                                     |                                        | 10 ml                                         | 10 ml                                 | 10 ml                                            | 10 ml                                             | 10 ml                              |
| Tiamin<br>(0.3 mg/ml)                                                     |                                        | 1 ml                                          | 1 ml                                  |                                                  |                                                   |                                    |
| Kinetin<br>(1 mg/ml)                                                      |                                        | 100 µl                                        |                                       |                                                  |                                                   |                                    |
| pCPA<br>(1 mg/ml)                                                         |                                        | 8 µl                                          |                                       |                                                  |                                                   |                                    |
| Sucrose (Isolab)                                                          | 30 g                                   | 30 g                                          | 30 g                                  | 30 g                                             | 30 g                                              | 30 g                               |
| MES free acid<br>(Goldbio) (3<br>mM)                                      | 0,56 g                                 | 0,56 g                                        | 0,56 g                                | 0,56 g                                           | 0,56 g                                            | 0,56 g                             |
| pH                                                                        | 5,8                                    | 5,8                                           | 5,8                                   | 5,8                                              | 5,8                                               | 5,8                                |
| Plant Agar<br>Type I<br>(Caisson)                                         | 7 g                                    | 7 g                                           | 7 g                                   | 8 g                                              | 6 g                                               | 8 g                                |
| Zeatin<br>(Caisson)<br>(1 mg/ml)                                          |                                        | 2 ml                                          | 2 ml                                  | 2 ml                                             | 1 ml                                              |                                    |
| Acetosyringone<br>(Sigma-<br>Aldrich)<br>(200 µM)                         |                                        |                                               | 1 ml                                  |                                                  |                                                   |                                    |

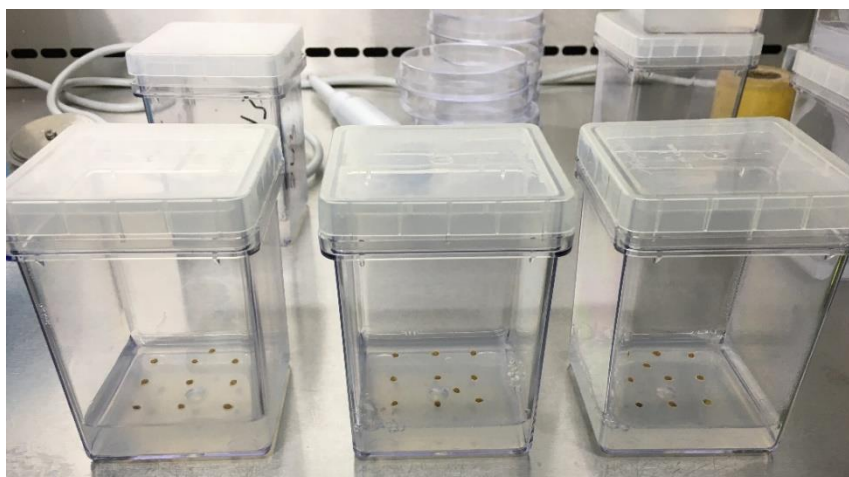
(Continues on next page)

**Continuation of Table 3.6**

|                                                              |  |  |       |       |        |        |
|--------------------------------------------------------------|--|--|-------|-------|--------|--------|
| Timentin (Goldbio)<br>(200 mg/ml)                            |  |  |       | 1 ml  | 1 ml   | 1 ml   |
| Glufosinate-Ammonium<br>(Basta) (Sigma-Aldrich)<br>(1 mg/ml) |  |  |       | 1 ml  | 0,5 ml | 0,5 ml |
| IAA (Caisson)<br>(10 mg/ml)                                  |  |  | 10 µl | 10 µl | 10 µl  | 10 µl  |

While preparing all mediums, the main components; MS (containing macro-micro nutrients that the plant needs for development), Nitsch & Nitsch Vitamin (Biotin, Folic acid, Glycine, Nicotinic acid, Pyridoxine HCl, Thiamine HCl), sucrose (carbon source), and MES (buffer with MES + MgCl<sub>2</sub>, pH: 5,8-6,0) were dissolved in water to half the final volume and pH was adjusted to 5,8-6,0. Then, plant agar was added to it, and after completion with sterile water to the final volume, it was sterilized by autoclaving at 121°C for 30 minutes. The materials to be added after the autoclave was added after cooling the media to 55°C so that the effect of temperature does not denature them since they are mainly protein. Due to the agar in it, the medium was quickly transferred to the petri dishes (approximately 25 ml) without solidification in the bottle, and the agar medium was left to solidify.

For this purpose, 300 each of 267-F1 and Micro-Tom seeds were kept in the sterile cabin for 30 minutes in 70% ethanol and 30% in 30% hypochlorite +100 µl Tween-20 for 30 minutes. Then it was rinsed six times with sterile distilled water and made sterile. Sterilized ten seeds per Magenta box were transferred to the seed germination medium (GM) (Figure 3.8) and its contents in Table 3.5 and 3.6, and then at 25°C for four days in the dark environment 16:8-h photoperiods for 7-10 days until cotyledons grow.

**Figure 3.8.** Sterile tomato seeds planted in germination medium in Magenta boxes

The cotyledon leaves obtained from about 7-10 days old plants were taken from the tips of the cotyledon leaves (Figure 3.9a, b) and were divided into two parts, as illustrated in Figure 3.10. It was aimed to increase the transformation efficiency by making three cuts with the help of a scalpel on the upper part of the leaves (adaxial side). The explants prepared were left in the dark environment for O/N by transferring Pre-Induction Medium (PM) content of which is given in Table 3.5 and 3.6, with a maximum of 10-15 pieces per petri dish and the leaves touching the abaxial side environment (with a feeder (blotting paper) on it). Thanks to 2 mg/ml (in 1 liter) Zeatin (cytokinin), 0,1 mg/ml (in 1 liter) indole-3-acetic-acid (IAA) (auxin) hormones in these media, cell differentiation and plant regeneration are promoted, and adaptation of the plant to the tissue culture medium.



(a)



(b)

**Figure 3.9.** Explant preparation for PM **a)** Cotyledon leaves formed by germination; **b)** Removal of cotyledon tips

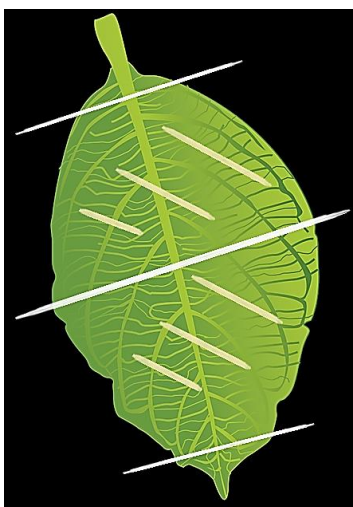


**Figure 3.10.** Representation of explant preparation method

Simultaneously, some of the EHA105 strain stocks containing pFGC-pcoCas9 vectors harboring CRISPR constructs were incubated in 50 ml liquid LB media containing 50 µg/ml Kanamycin + 25 µg/ml Rifampicin at O/N at 28°C. The next day, to determine the correctness of the cells growing in the media before plant transformation, according to the optimized conditions in Table 3.2 and 3.3, validation was performed by amplifying the gRNA regions with the CCD7-gRNA F/R and CCD8-gRNA F/R primer pairs.

In explants kept in the dark and PM environment, a slight swelling is observed after one night. This is a morphological indicator showing that the explants are ready for the infection medium. OD of O/N growing cell cultures was measured first, and falcons were centrifuged at + 4°C / 5000 rpm for 15 minutes. At the final optimum OD value of 0.6, the pellet formed after centrifugation was dissolved with MS+MES (pH:5,8) solution, and then 1:1 stock from Acetosyringone (200 µM) was added. Since the gRNA targets designed for the *SICCD7* and *SICCD8* genes are located in two different pFGC-pcoCas9 vectors (hence, located in two different EHA105 strains), the mixtures prepared in individual tubes were combined in a single sterile petri in a 1:1 ratio. The explants added to them were allowed to contact the mixture fully and were kept in this mixture for 30 minutes at room temperature (Figure 3.11). After 30 minutes, the explants were removed from excess *Agrobacterium* liquid on sterile filter paper and were transferred to Co-cultivation (CM) medium (given in Table 3.5 and Table 3.6) (approximately 15 explants per petri). Explants were kept in the dark for two nights, and the transformation process was continued in a CM environment containing Acetosyringone. *Agrobacterium*-mediated transformation was done successfully with Acetosyringone and scars on the leaf. As a control, the pFGC-pcoCas9 vector without gRNAs was transferred to 50 explants in the same way. With this method, it can be understood whether the negativities that may occur in the plant are due to the transferred gRNAs.

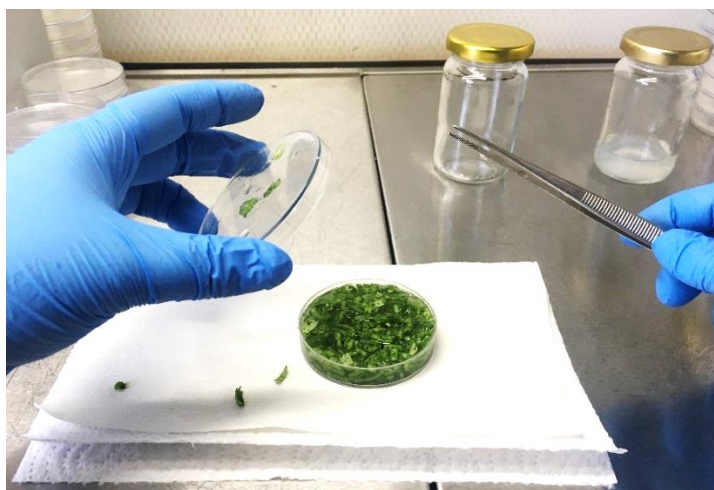


**Figure 3.11.** Explants transferred to liquid infection medium containing EHA105<sub>pFGC-pcoCas9SICCD7-gRNA1+2</sub> : EHA105<sub>pFGC-pcoCas9SICCD8-gRNA1+2</sub> in a 1: 1 ratio

As an additional preliminary study, Basta concentrations in protocol A using the Micro-Tom genotype were evaluated in terms of toxicity (“Kill curve”). In this context, the responses of the explants transferred to media containing 2, 3, and 4 mg/ml Basta

selection were observed. In this way, the concentration limit where Basta selection is most effective without damaging the plant was determined approximately. (Figure 3.12a, b, c).

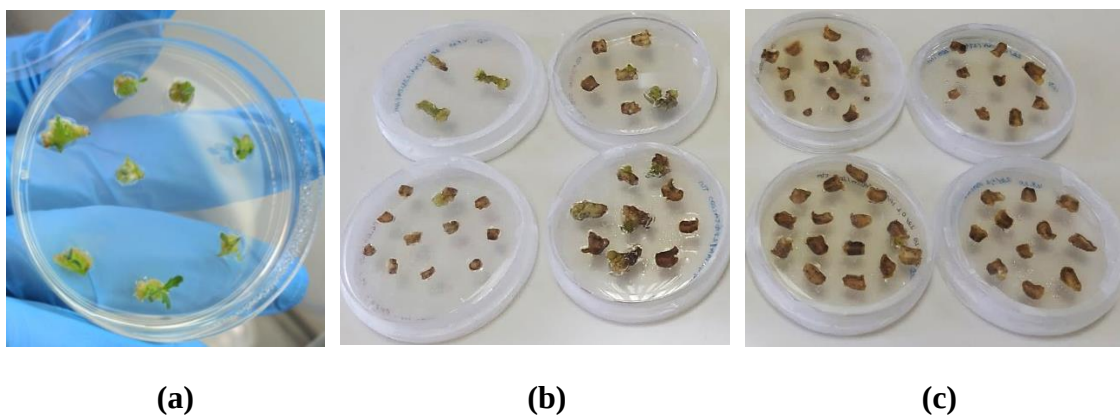


**Figure 3.12.** Creating a "kill curve" with selection media containing Basta in different concentrations **a)** Explants kept in RM containing 2 mg/ml Basta selection; **b)** Explants kept in RM containing 3 mg/ml Basta selection; **c)** Explants kept in RM containing 4 mg/ml Basta selection

3 mg/ml Basta was determined as the critical threshold for selection. Although regeneration at this concentration occurs in many explants, the selection was started with 2 mg/ml Basta to keep the number of regenerated plants high for protocol A (despite the risk of loss due to contamination, etc.). In the 2nd and 3rd regeneration media, BASTA was reduced to 1 mg to prevent plant growth difficulties. Basta was used as 0,5 mg/ml in the last regeneration medium before rooting. In the light of this information, *Agrobacterium* colonies formed around the explants waiting in CM media after two days show that the plant transformation has been completed sufficiently. The explants were transferred to Shoot Induction medium (SM) / Regeneration I with an average of 10-12 explants per petri and were kept at 26°C, 16-h photoperiod. Thanks to the final 200 µg/ml Timentin in this medium, *Agrobacterium* is eliminated without creating a toxic effect on the plant. At the same time, shoot regeneration is induced by zeatin (cytokinin) in the media. Thanks to the BASTA resistance harbored by the pFGC-pcoCas9 vector and Glufosinate-Ammonium (BASTA) selection added to the SM medium, from the cells harboring the correct constructs is ensured that the plants form a complete plant as a result of regeneration through the totipotency properties of the plants. Shoot formation usually occurs in more than 3-4 weeks as a result of differentiation of the callus structure (indirect) (or "direct shoot formation" can occur spontaneously). Callus structures were seen quite frequently at the points cut by the scalpel while making explant preparation. When shoot formation was observed (Figure 3.13 and Figure 3.14), explants were subcultured to Shoot Induction II / Regeneration II medium every ten days. When the shoot formation started, zeatin was reduced from 2 mg/ml to 1 mg/ml. Dead tissues were removed at each medium change.

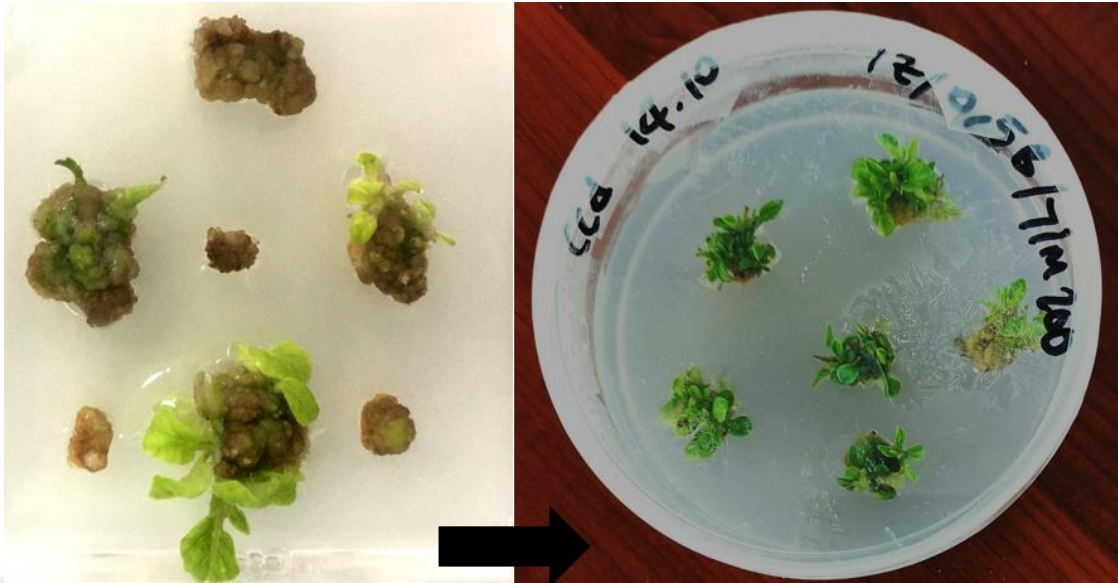


**Figure 3.13.** Callus formation and shoot regeneration with regeneration medium in Protocol A

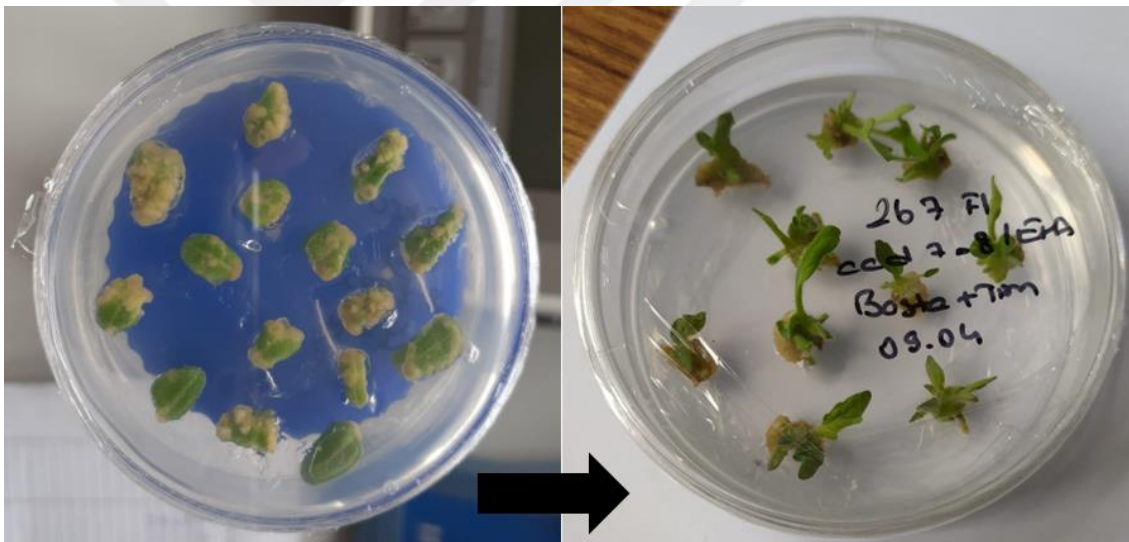


**Figure 3.14.** Callus formation and shoot regeneration with shoot induction medium in Protocol B

When the shoots reached a certain length, they transferred to the Root Induction (RM) media. Thanks to this auxin-rich environment, root formations are provided from the points where the shoots touch the media. At the same time, this environment continues to contain Timentin to prevent contamination caused by the growth of *Agrobacterium*. Rooted plantlets reaching 8 cm length in rooting medium, they were transferred to pots (Figure 3.15) containing perlite:peat in the ratio of 1:1 and kept in the growing cabin at 26°C for 16 hours of light and 8 hours of darkness, and then transferred to greenhouse conditions of  $28 \pm 2^\circ\text{C}$ .

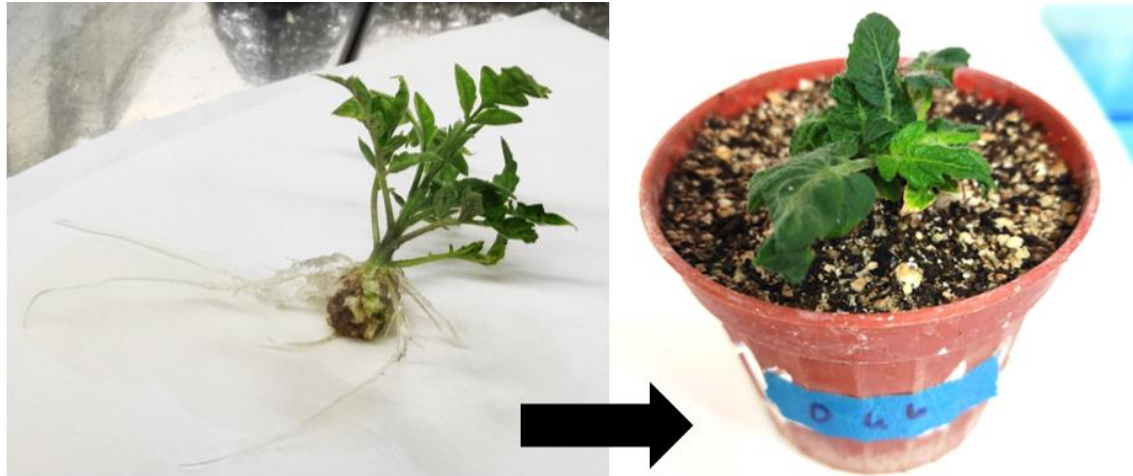


**Figure 3.15.** Rooting plants transferred to pots containing perlite and peat

### 3.6. Determination of Mutant Lines

DNA extraction was performed from plants by modifying the “CTAB Total DNA Isolation” protocol prepared by Doyle (1991) to identify mutants from plants growing seedling. An average of 0,3 g of fresh leaves from each sample was taken into micro-tubes, and 150  $\mu$ l of CTAB solution (2% CTAB, 100 mM Tris-HCl (pH: 8), 20 mM EDTA, 1.4 M NaCl, 0.2% mercaptoethanol) was added to them and grinding was done with the help of tissue grinding pestles. An additional 300  $\mu$ l of CTAB solution was added to the grinded samples and incubated for 1 hour at 65°C. After 1 hour, 450  $\mu$ l of 24:1 ratio of chloroform-isoamyl alcohol solution was added to the samples taken from the incubation, and a two-phase solution was formed in the centrifuge for 20 minutes. The upper phase was transferred to new tubes with the aid of a pipette (approximately 300  $\mu$ l). Isopropanol solution was added to the upper phase taken on this transferred phase and kept at -20 ° C for 1 hour. After one hour, samples taken from the freezer were centrifuged to form pellets at the bottom of the tubes. After centrifugation, the supernatant portion was poured, paying attention not to drop the pellet at the bottom of the tube, and it was centrifuged again by adding 500  $\mu$ l of 70% ice-cold ethanol process was repeated once more after pouring the supernatant part. After pouring the supernatant portion containing 70% ethanol, the tubes were left to dry for half an hour to remove the ethanol. Then, 100  $\mu$ l of distilled sterile water was added to the tubes, and the pellet DNA was left to dissolve at + 4°C for one night to dissolve.

Based on the literature, as a result of plant transformation with a binary vector, it was obtained that the region between the right border (RB) and left border (LB) on the vector was transferred to the plant. For this reason, plant transformation was tested with specific primers (Figure 3.16a, b): 1. BASTA primer was designed with the Primer3Plus program specifically for the *bar* gene located between LB and RB of the pFGC-pcoCas9 vector. 2. SICCD7 / F-R and SICCD8 / F-R primers (previously used for colony PCR after ligation) were used according to PCR conditions in Table 3.2 and Table 3.3 (Tm: 54°C). Extracted DNAs from T0 plants (regenerated with tissue culture protocols) were used as templates for PCR amplification. In analysis, sterile water was used as negative control and miniprep products (pFGC-pcoCas9SICCD7/8-gRNA1 + 2 vectors) as a positive control.

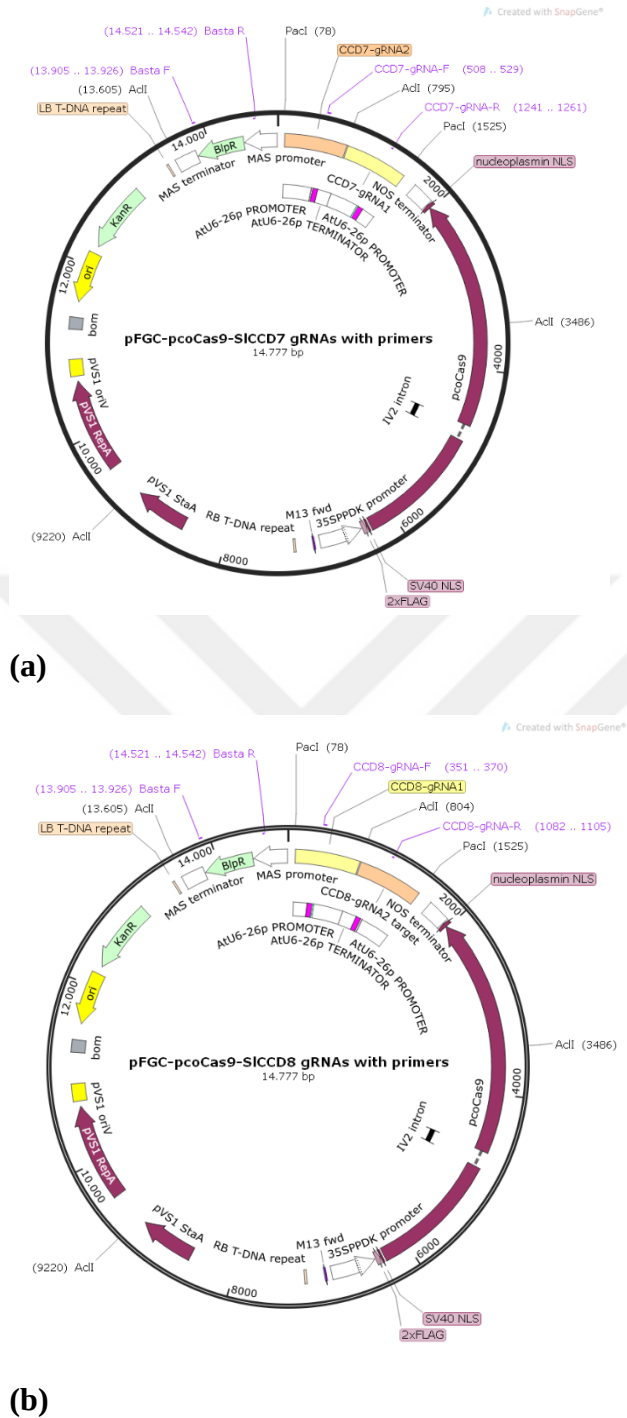


**Figure 3.16.** Representative map showing the positions of the primers used in transgenic plant identification on the pFGC-pcoCas9<sub>SICCD8/7</sub>-gRNA1+2 plasmid **a)** Positions of primers (BASTA F-R and SICCD7 / F-R) on the pFGC-pcoCas9<sub>SICCD7</sub>-gRNA1 + 2 gRNA vector used to verify plant transformation; **b)** Positions of primers (BASTA F-R and SICCD8 / F-R) on the pFGC-pcoCas9<sub>SICCD8</sub>-gRNA1+2 vector used to verify plant transformation.

After the CRISPR constructs were transferred, integrated into the plant genome, the Cas9 protein was produced. Cas9 protein is expected to make double-stranded

cleavage with the help of two cleavage domains (HNH and RuvC). After the gRNA and Cas9 protein form a complex structure, the Cas9 protein mutates in the target region by performing a double-stranded break in the target region due to the base matching of the gRNA target regions located in the gRNA gene regions between the LB and RB, according to the PAM sequence in the plant. This mutation occurs with INDELs, which are formed by repairing ds break in DNA gene repairing mechanisms in the relevant gene region according to the NHEJ method. Therefore, to determine whether the mutation occurred in the target gene, analysis was performed with primers specific to *SICCD7* / *SICCD8* gRNAs. For this purpose, PCR amplification was performed according to conditions in Table 3.2 and Table 3.3 with primer pairs (plant *SICCD7/8* gRNA targets primers) in Table 3.1. In amplification, sterile water was used as a negative control, and plant DNA (nontransformed 267-F1) was used as a positive control. PCR products were verified and then sent to the BMLabosis for Sanger sequencing.

T1 population was generated by selfing of T0 plants. All plants were grown in controlled and commensurable greenhouse conditions. Periodically, plants were phenotypically observed and compared concurrently with molecular analyzes. Extracted DNAs of 23 T1 lines were also amplified with the relevant gRNA target and Basta primers to analyze the heredity of mutation. In addition, as a second verification, testing was performed with the 52256-2 F/R primer pair designed specifically for the promoter region of the Cas9 gene located in the T-DNA region of the pFGC-pcoCas9 plasmid (Figure 3.17). According to the described conditions in Table 3.2 and Table 3.3 (Tm: 54°C), it produces a 562 bp-band when amplified with this pair of primers.

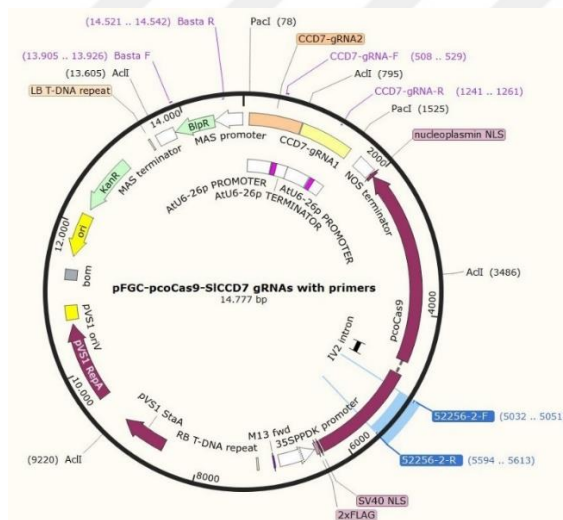


**Figure 3.17.** Representative map showing the location of the 52256-2 F/R primer on the pFGC-pcoCas9 plasmid

### 3.7. Testing of *Orobancha* spp. (*Orobancha ramosa*) Resistance in Mutant Lines with Different Mutations

Resistance testing was carried out with mutant lines obtained from 267-F1 (indeterminate type). For this purpose, T2 mutant lines were obtained by selfing of T1 lines with different mutation types (homozygous biallelic/heterozygous biallelic).

Mutation status in T2 lines was determined by analysis with PCR amplification. It was performed according to conditions in Table 3.2 and Table 3.3 with SICCD7 gRNA1 targets primers in Table 3.1. Except for the T2 mutants, two different non-mutated commercial F1 plants were used as controls. First, the sterile soil in which the testing will be carried out was prepared. For this purpose, soil: peat: sand was mixed in a ratio of 1: 1: 1. The soil was first saturated with 20 lt water. 2,5 lt fumigant (metamsodium) was applied to the homogenized mixture, and it was kept for ten days by covering it with a thick nylon greenhouse cover. The soil that became sterile after ten days was opened and aerated. 100 mg of *Phelipanche ramosa* (one of the most common species of *Orobanche* spp. in tomato cultivated areas) seeds were mixed into of per pot (Each pot contains approximately 19 liters of homogenized soil mixture). 15 of the 20 pots were planted from T2 lines having different mutations (5 plants: -186 nt homozygous biallelic deletion, five plants: -18 nt homozygous biallelic deletion, and five plants: -186 nt + -18 nt heterozygous biallelic deletion) and controls were planted in the remaining five pots (Figure 3.18). In this way, the germination and emergence rates of *Orobanche* seeds will be observed, and the relationship between mutation status and resistance will be compared. In addition, the root regions of 10 plants belonging to each mutation group were first purified from the soil with water. Every ten plants were kept in 200 ml distilled water, and root secretions of the plants were released into the water for 24 hours. At the end of 24 hours, waters with root secretion belonging to each group were filtered. It will be tested whether these secretions germinate *Orobanche ramosa* seeds in the petri dish. GR24 synthetic SL will be used as a control.

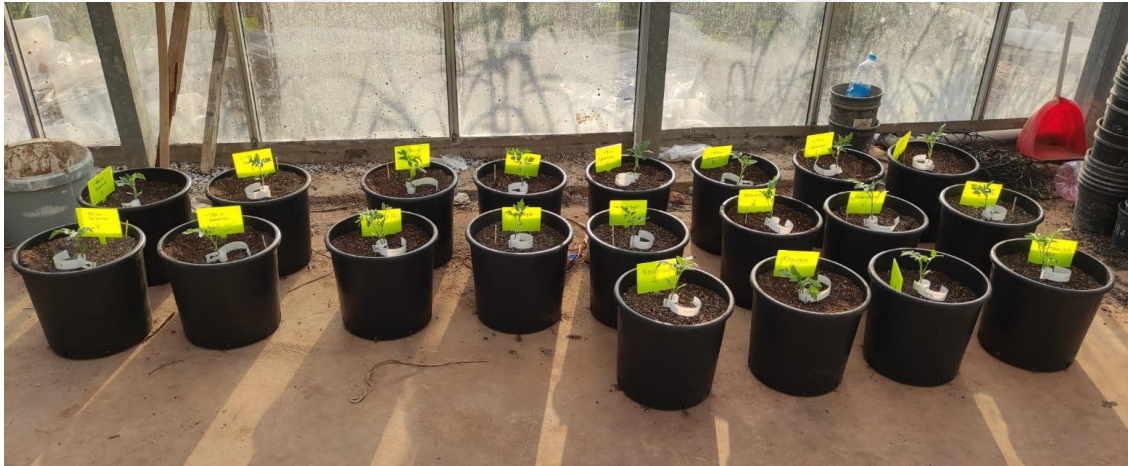


**Figure 3.18.** Mutants having different CCD7-gRNA1 mutations were potted to test *Orobanche* resistance (There are 5 plants from each of 3 different mutation groups and 1 control group consisting of 5 plants)

## 4. RESULTS AND DISCUSSION

### 4.1. The Results Obtained from Bioinformatics Studies

Sequence information for *SICCD7* and *SICCD8* genes were obtained from NCBI/Gene database employing Tomato Genome version SL3.0 (Figure 4.1 and Figure 4.2). Using this data, the detailed structure of the genes, including exons and introns, and chromosome locations, were determined. The *SICCD7* (Gene ID: 100313501) gene is located on the 1st chromosome in the tomato genome and consists of 7 exons and six introns. The *SICCD8* (Gene ID: 101254620) gene is located on the 8th chromosome and has six exons and five introns. The *SICCD7* gene is located between 84,183,259-84,186,993 nucleotides (nt) in the tomato genome version SL3.0 and it contains 3735 nucleotides. The gene has a coding sequence (CDS) of 1992 nt, which corresponds to 663 amino acids (aa) in total. On the other hand, the *SICCD8* gene consists of 2842 nucleotides located between 55,474,797-55,477,956. nt in the tomato genome version SL3.0. This gene has a CDS of 1674, which corresponds to 557 aa in total.

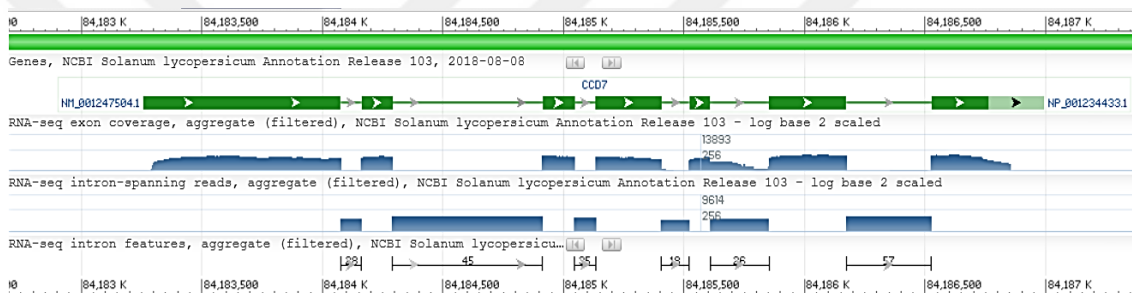


**Figure 4.1.** Features of the *SICCD7* gene in tomato genome version SL3.0

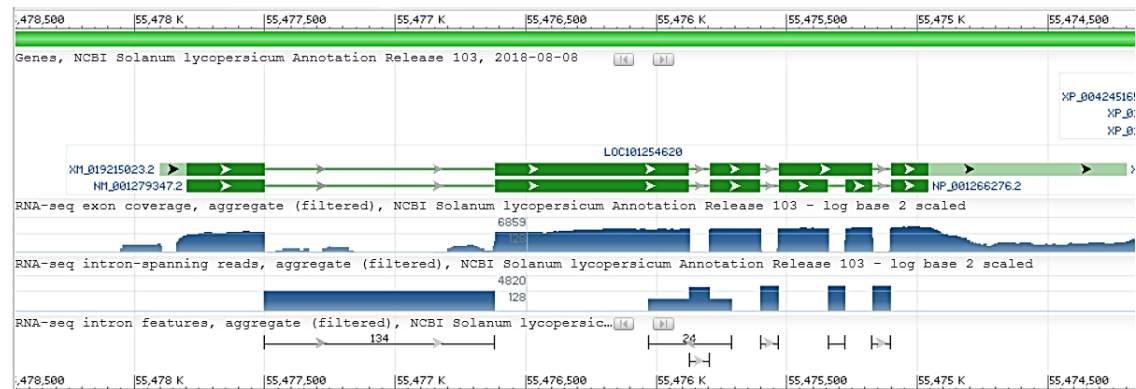


**Figure 4.2.** Features of the *SICCD8* gene in tomato genome version SL3.0

To determine the accuracy of the gene structure, the introns removed from the sequences of *SICCD7* and *SICCD8* genes and resulting mRNA translated to protein sequence using the ExPASy / Translate program. The protein sequences then used as queries in the Sol Genomics database, and matched results confirmed that the gene structure indeed belonged to CCD genes.

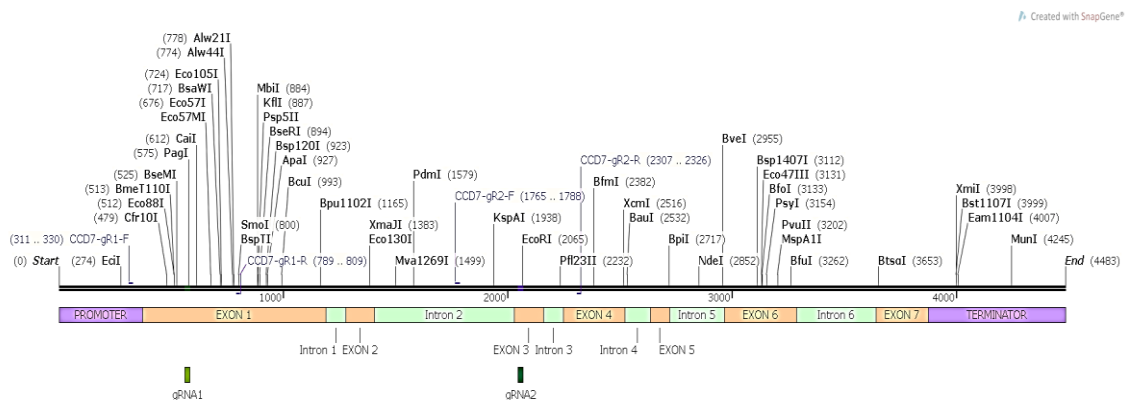
sgRNA's (gRNA), which are required for the CRISPR-Cas9 system to perform double-strand cleavage in the desired gene region, was designed using the

CRISPRDirect program. gRNAs were selected from sequences containing a proper PAM sequence (NGG) downstream, GC content higher than 40%, and no off-target when scanned against tomato genome (SL3.0). Selected gRNA's targeted the first and third exons of *SICCD7* and the first and second exons of *SICCD8*. Two gRNA's were used to increase the chance of gene editing and thus the knockout process. gRNAs with sequences (PAM sequence in bold) presented in Table 4.1 were selected for this study as they fulfilled all the criteria mentioned above.

**Table 4.1.** sgRNA target information designed rationally for *SICCD7* and *SICCD8* genes

| Gene          | Sequences<br>(5' – 3')      | Location<br>(nt)          | Size<br>(nt) | GC<br>% | gRNA<br>name | Exon<br>number |
|---------------|-----------------------------|---------------------------|--------------|---------|--------------|----------------|
| <i>SICCD7</i> | CCACGTCCCCAACTCATGA<br>AGTT | 84,183,446-<br>84,183,468 | 23           | 50      | gRNA1        | 1.             |
| <i>SICCD7</i> | CCAGCTACTACAAAGCCA<br>AGAAT | 84,184,930-<br>84,184,952 | 23           | 40      | gRNA2        | 3.             |
| <i>SICCD8</i> | CCTTCAACGACGAGTTCTC<br>CTTC | 55,477,520-<br>55,477,542 | 23           | 50      | gRNA1        | 1.             |
| <i>SICCD8</i> | ATTCTCACCACCGTATATC<br>CCGG | 55,476,113-<br>55,476,135 | 23           | 45      | gRNA2        | 2.             |

After determining the gRNAs targeting the relevant gene regions, the data were visualized in Snapgene Viewer 5.1.4.1 (GSL Biotech, 2020) (Figure 4.3a, b).



(a)

**Figure 4.3.** Positioning of gRNA targets on the tomato genome a) *SICCD7* gene and gRNA target region; b) *SICCD8* gene and gRNA target region

(Continues on next page)

Created with SnapGene®



95 AAAAAACAGGATG - AGGCCAGG - - | GCC - TAATATTAGCA - TATTCCTCCAAA - CTCTAGCCAC - GGCTATACACGCTCCACGGTGGCGCATGAGCCCG 187  
86 AAAAGCCAAATGACAGGCCAAAGCTTGCCATAATATTAAACAATATTCCTCCAAAACCTTGCCACC GGCTAAACTGCCGCTCCACGGTGGCGCATGAGCCCG 185

188 AGCCAATTAACATTGCCTA - TCACATGGCGCGTGCCATAACGATCACCACGTCGCCAACTAATGAA GTTTACACACCAGAAATCGATGACACAGTTACTG 286  
186 AGCCAATTAACATTGCCTAGCCACGTTGGCGGAGCCATAACGATCA CACAGTCCCAACTCATGAAGT TACACACCAGAAATCGATGACACAGTTACTG 285

287 CCTATTGGGATTACCAATTCTTTTCGAGTCCCAACGTTATGAATCTACCGAACCCGTTTCAC TTCGGGTCGTGAAAGGATCCATACCATCCGATTTCCC 386  
286 CCTATTGGGATTACCAATTCTTTTCGTGTCCCAACGTTCTGAAGCTACCGAACCCGTTTCAC TTCGGGTCGTGAAAGGATCCATACCATCCGATTTCCC 385

387 TTCGGGTACGTATTACCTACCGGGGCCGGGCTTATTCGCGGATGATCATGGTTCCTCGGTGACCCCTCTAGAAGACACGGCTACTTTAAAGAAAAAAA 483  
386 TTCGGGTACGTATTACCTACCGGGGCCGGGCTTATTCGCGGATGATCATGGTTCACAGGTGACCCCTCTAGAC - - - - - 458

**(a)**

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118 | CCAAGCTCTACTTTTAAAGAAATTGTATATTTTCATCAACATCACATGTAGATGTAGTAC-ACGTTAACAAGAATAATCTTTTGTGTTTTGTCTCCATAT 216
85 | TCCAACCTCTACTTTTAAAAAATTGTATATTTTCATCAACATCACATGTAGATGTAGTACAACGTTAACAAGAATAATCTTTTGTGTTTTGTCTCCATAT 184

217 | ATTCATAATAATTCAAATTCAGTTAATTTAATTCATTTTATTTTTTTTTTGTCTAAAATTTGATGCCAATTTCCAGCTACTACAAAGCCAAAAATTCAA 316
185 | ATTCATAATAATTCAAATTCAGTTAATTTAATTCATTTTATTTTTTTTTTGTCTAGAATTTGATTCCAAATTCAGCTACTACAAAGCCAAAAATTCGA 284

317 | GATCCCAAAATCATTTAATGATACTGGATTGGGCTTTTACTGATACTCACTATATATTGTTCCGGCAACCGCATCAAACCTCAATATCCCCGGTAACTAATTA 416
285 | GATCCCGAGATCATTTAATGATACATGATTGGGCTTTTACTGATACTCACTATATATTGTTCCGGCAACCGCATCAAACCTCGATATCCCCGGTAACTAATTA 384

417 | TTCGATTTCTATACTAAAAATAAATATTTCAATTTACTTATTTATTCACTAAAATTTTGCGTACCCCATCGTTGCAGGATCAATGACAGCAGTATGTGGG 516
385 | TTCGATTTCTATACTAAAAATAAATATTTCAATTTACTTATTTATTCACTAAAATTTTGCGTACGCCATCGTTGCAGGATCAATGACAGCAGTATGTGGG 484

517 | CTTTCTCCAATGATATCAGCAATTATCAGTAAATC 550
485 | CTTTCTCCAATGATATCAGCAATTATCAGTAAATC 518

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(b)

(Continues on next page)

**Continuation of Figure 4.4**

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145 TAGTTGCACCACCAGATCAAGAGGTGATTAGTAAGGAGAAAAAACTTGCTGCATGGACTAGCGTACGCCAAGAAAGATGGGAAGGAGAACTCGTCATTGA 244
111 TAGTTCCACCACCAGATCAAGAGGTGATTAGTAAGGAGAAAAAGCTTGCTGCATGGACTAGCGTACGCCAAGAAAGATGGGAAGGAGAACTCGTCATTGA 210

245 AGGCGAGTTACCAATTGTGGCTGGTATGTTATTGTTTGTGTATAAAAGTTAAATATGAACGTTTCGTGTTACATCCATTTTTTTGTTTTAAAGTGAAGTTT 344
211 AGGCGAGTTACCAATTGTGGCTGGTATGTTATTGTTTGTGTATATAAGTTAAATATGAACGTTTCGTGTTACATCCATTTTTTTGTTTTAAAGTGAAGTTT 310

345 TTCTTTTTTGGAAATCACTTATATATTATGATTTTTCTTCACCAAAAAGTATTGAGATCAAAGATGCATGTGTACCTAATACATGTTTTCTCTCAAGAAA 444
311 TTCTTTTTTGGAAATCACTTATATATTATGATTTTTCTTCACCAAAAAGTATTGAGATCAAAGATGCATGTGTACCTAATACATGTTTTCTCTCAAGAAA 410

445 ATATGAAGTATACATATTGAAACAAAAGGTTTATGAACCTTAATTATTCTGCTATTTAGTACACTACTATAGACCCAAAGATTTTCTCACTTGATAATCA 544
411 ATATGAAGTATACATATTGAAACAAAAGGTTTATGAACCTTAATTATTCTGCTATTTAGTACACTACTATAGACCCAAAGATTTTCTCACTTGATAATCA 510

545 GTCGGAAATTTTGTATTATAAAATATGATTTTCTGAGTCA 584
511 GTCGGAAATTTTGTATTATAAAATATGATTTTCTGAGTCA 550

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(c)

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6 GGAATGAT--AACTTGGGGA-GGACGCGTAGTCTGCTTAACTGAGACGATAAAAGGTTCCATTGTAATTGATCCGAACACCCCTAGATACAATTGGGAAAT 102
11 GGAGTCGTTAACTTGGGGATGGACGCGTAGTCTGCTTAACTGAGACGATAAAAGGTTCCATTGTAATTGATCCGAACACCCCTAGATACAATTGGGAAAT 110

103 TTGAATATAGTGACTCGTTAGGTGGATTGATTCATTGAGCTCATCCAGTGGTTACGGACAGTGAGTTTATAACGTTGATTCGGGATTTAATGAACCCGGG 202
111 TTGAATATAGTGACTCGTTAGGTGGATTGATTCATTGAGCTCATCCAGTGGTTACGGACAGTGAGTTTATAACGTTGATTCGGGATTTAATGAACCCGGG 210

203 ATATACGGTGGTGAGAAATGGAGGCAGGGACAAATGAGAGGAAGTATATAGGAGAGTGAGTTGTAGAGGAGGACCAGCACCAGGATGGGTTTCATTTCATT 302
211 ATATACGGTGGTGAGAAATGGAGGCAGGGACAAATGAGAGGAAGTATATAGGAGAGTGAGTTGTAGAGGAGGACCAGCACCAGGATGGGTTTCATTTCATT 310

303 CCTGTTACAGAAAATTATGTTATTGTGCCTGAGATGTCACCTAAGGTATTGTGCAAAAAAATTTGTTGAAGGCTGAGCCCAACACCACTGTATAAGTTTGAGT 402
311 CCTGTTACAGAAAATTATGTTATTGTGCCTGAGATGTCACCTAAGGTATTGTGCAAAAAAATTTGTTGAAGGCTGAGCCCAACACCACTGTATAAGTTTGAGT 410

403 GGCATCCTGATTCTAAAGCATTGTGACATGTTA 435
411 GGCATCCTGATTCTAAAGCATTGTGACATGTTA 443

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(d)

The alignments show that SlCCD8-gRNA2 had a perfect match with the target region. Other gRNA targets (SlCCD7-gRNA1, SlCCD7-gRNA2, SlCCD8-gRNA1) had one mismatch with their respective regions. Whether this single base mismatch affects the correct functioning of the CRISPR-Cas9 system will be examined later, but it is thought that a major problem is unlikely to occur because the mismatch is not in the PAM region.

A cassette was created by adding a universal sgRNA scaffold sequence, codon-optimized *A. thaliana* AtU6-26p RNA pol III promoters, AtU6-26t terminator, and appropriate restriction enzyme regions (sequences) the gRNA sequences. In this context, AclI (Psp1406I) (5'...AA/CGTT...3'), which allows gRNA1 and gRNA2 to be combined, and PacI enzyme digestion sites (5'...TTAAT/TAA...3'), which will provide ligation to the cloning vector, have been added. These sgRNA cassettes, which were synthesized and cloned to pTZ57R vector at Sentebiolab Biotechnology. However, due to the sequence length limitations of the company, the gRNA's were synthesized in two pieces (i.e., as SlCCD7-gRNA1, SlCCD7-gRNA2, SlCCD8-gRNA1, and SlCCD8-gRNA2) (Figure 4.5a, b, c, d).

AtU6-26p:gRNA:AtU6-26t cassettes, synthesized separately for each sgRNA, consist of 20 bp gRNA-target, 76 bp gRNA-scaffold, 424 bp promoter, and 192 bp terminator, in total 712 bp. Thanks to the *bla* (AmpR) gene in the plasmid, resistance to Ampicillin, Carbenicillin, and related antibiotics are provided in bacterial selection through the  $\beta$ -lactamase gene product.

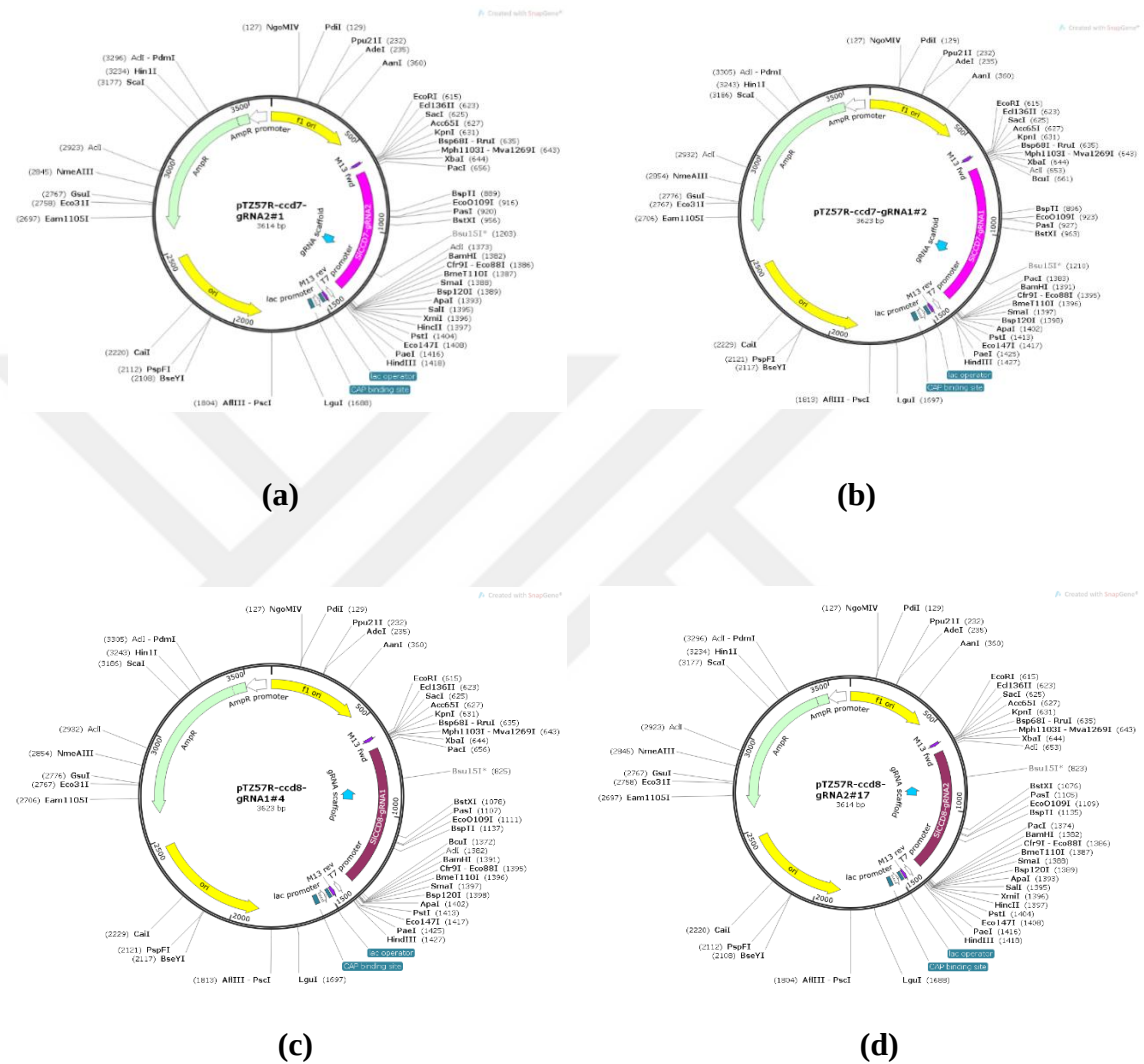


**Figure 4.5.** Representative maps of pTZ57R plasmids harboring *SICCD7* / *SICCD8* gRNAs **a)** Synthesized pTZ57R-SICCD7-gRNA1 plasmid map; **b)** Synthesized pTZ57R-SICCD7-gRNA2 plasmid map; **c)** Synthesized pTZ57R-SICCD8-gRNA1 plasmid map; **d)** Synthesized pTZ57R-SICCD8-gRNA2 plasmid map

#### 4.1.1. Determination of binary vector

The binary pFGC-pcoCas9 (ADDGENE) vector (13.330 bp) (Figure 4.6) was identified as the most suitable vector for this study. The Cas9 endonuclease from the *Streptococcus pyogenes* is located on this plasmid, which is one of the elements required for the function of the Type II CRISPR-Cas9 system. This binary plasmid containing 35SPDK:pcoCas9:NOS cassette for plant expression of Cas9 and multiple cloning sites for insertion of guide RNA. The length of the pcoCas9 gene is 4290 bp,

and it contains one 189 bp long intron region (IV2 intron of the potato ST-LS1 gene (Vancanneyt et al. 1990)). There is also a 48 bp (16 aa) nuclear localization sequence (nucleoplasmin NLS) upstream of the gene sequence. Thanks to this amino acid sequence, the protein produced are transported to the cell nucleus. This vector contains the pBR322 replication origin for maintaining it in an *E. coli* strain. The -designed-CRISPR construct was ligated between the LB (25 bp) and RB (25 bp) regions of the binary vector for integration into the plant using an *Agrobacterium* strain. Aminoglycoside phosphotransferase produced in the vector, thanks to the aphA-3 gene located outside the LB-RB region, provides Kanamycin resistance to be used for bacterial selection. Resistance to active ingredients of bialophos or phosphinothricin to be used for plant selection is provided by phosphinothricin acetyltransferase, a protein product of the 552 aa BlpR (*Streptomyces hygroscopicus* bar) gene with MAS promoter (mannopine synthase promoter (Velten et al. 1984)) and terminator in the LB-RB region. In addition, thanks to the PacI enzyme restriction site in the MCS in the vector, the ligation of the sgRNA cassettes to this region can be easily achieved.

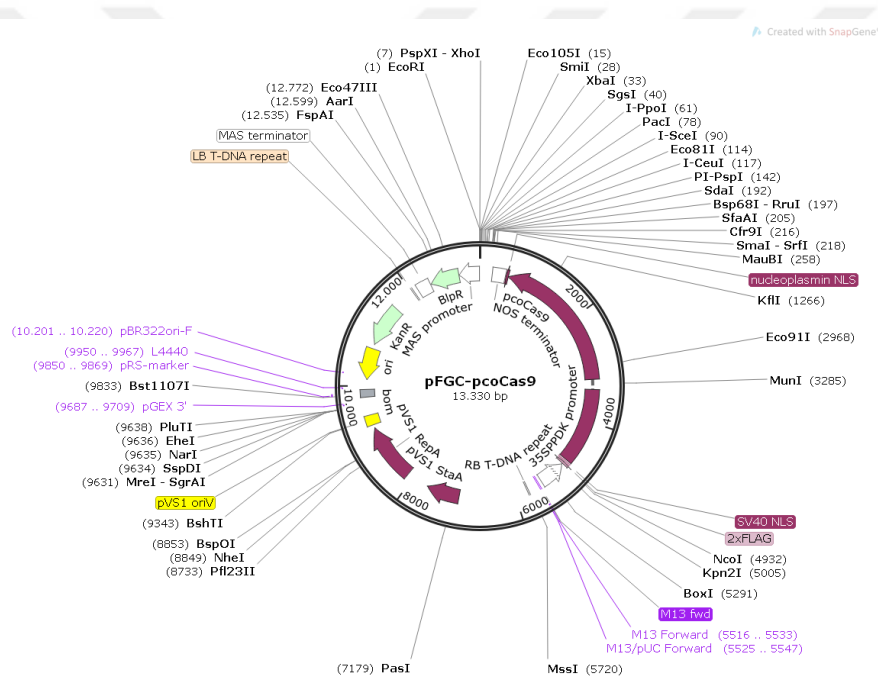


**Figure 4.6.** Representative map of the ADDGENE pFGC-pcoCas9 vector (pFGC-pcoCas9 was a gift from Jen Sheen (Addgene plasmid #52256; <http://n2t.net/addgene:52256>; RRID: Addgene\_52256)

## 4.2. The Results Obtained from Laboratory Studies

### 4.2.1. Cloning and validation of the pTZ57R-SICCD7/8<sub>gRNA1/2</sub> and the pFGC-pcoCas9 vectors

sgRNAs (synthesized by Sentebiolab Biotechnology) came in plasmids with a total volume of 35 µl (having a 2887 bp long pTZ57R backbone), and their concentrations were optimized by adding 100 µl of DNase / RNase-Free dH<sub>2</sub>O after centrifugation. After adding 100 µl of dH<sub>2</sub>O, the concentrations of plasmids were measured on the Biodrop instrument:

- SICCD7-pTZ57R-gRNA1: 44,0 ng/μl
- SICCD7-pTZ57R-gRNA2: 45,4 ng/μl
- SICCD8-pTZ57R-gRNA1: 16,0 ng/μl
- SICCD8-pTZ57R-gRNA2: 57,0 ng/μl

The prepared stock plasmids were kept at -20°C, and the heat-shock transformation method followed by plasmid purification was applied to increase the number of copies of the stocks. In this context, primarily, the media that have the necessary conditions for the life of bacteria and *E. coli* (DH5α strain) competent cells to be used in transformation were prepared according to the CaCl<sub>2</sub> treatment method. While the competent cells prepared were grown under controlled and antibiotic-free ambient conditions, it was also tested with Ampicillin containing media as a control, and whether it was able to resist the antibiotic of Ampicillin, which will be used for bacterial selection during cloning of pTZ57R-gRNA plasmids (Figure 4.7a, b). Thanks to this test, the purity of the *E. coli* DH5α strain to be used was assured.

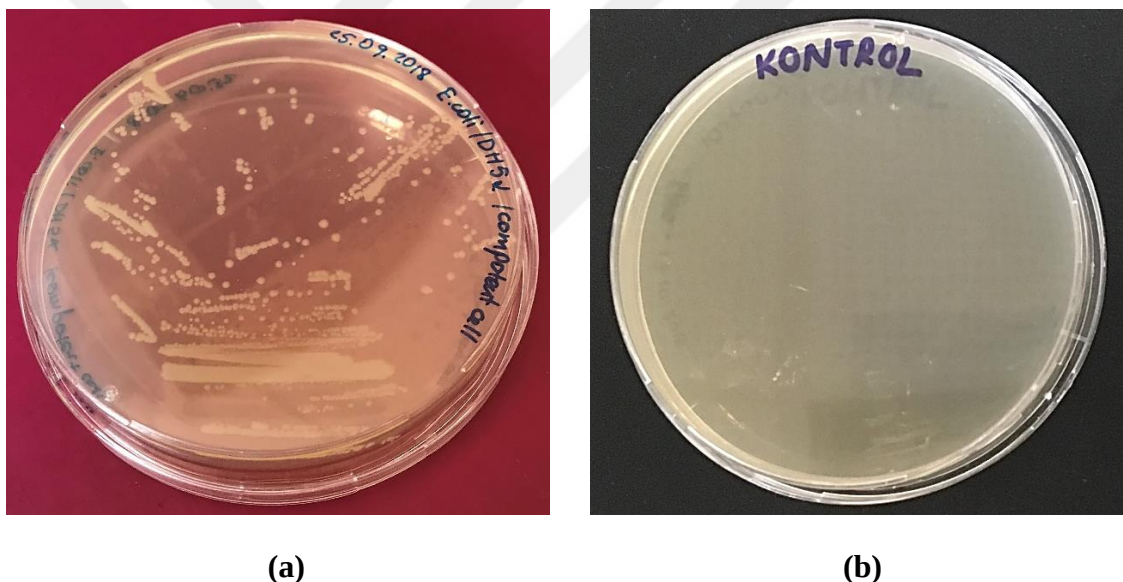


**Figure 4.7.** *E. coli* DH5α vitality test **a)** Cells of *E. coli* DH5α strain grown in an antibiotic-free medium; **b)** Cells of *E. coli* DH5α strain grown on medium containing antibiotics (Ampicillin)

The transformation of pTZ57R-SICCD7-gRNA1, pTZ57R-SICCD7-gRNA2, pTZ57R-SICCD8-gRNA1, pTZ57R-SICCD8-gRNA2 plasmids were done according to the "heat-shock" method with *E. coli* (DH5α strain) competent cells. From each petri dish containing cell colonies that were transformed and incubated in the selection medium containing Ampicillin, three colonies were selected and were grown O/N in a 37°C liquid medium containing Ampicillin. Plasmid purification was done with Plasmid Miniprep Kit (Thermo Fisher Scientific). In order to determine the correctness of the purified plasmids, the target region was amplified. For this purpose, using the Primer3Plus program, a pair of (forward and reverse) gRNA control primers from the

gRNA regions common for pTZ57R-SlCCD7-gRNA1, pTZ57R-SlCCD7-gRNA2, pTZ57R-SlCCD8-gRNA1, pTZ57R-SlCCD8-gRNA2 plasmids were designed and synthesized at Sentebiolab Biotechnology. The forward primer (gRNac/F) was designed on the AtU6-26p promoter, common to all gRNA regions, and the reverse primer (gRNac/R) on the gRNA scaffold (Figure 4.8). Therefore, it is expected that a 237 bp length product will be given for these four plasmids. As a result of the amplification obtained by the designed primers, the accuracy of the plasmid DNA of the three selected colonies was produced at 237 bp fragments (Figure 4.9).

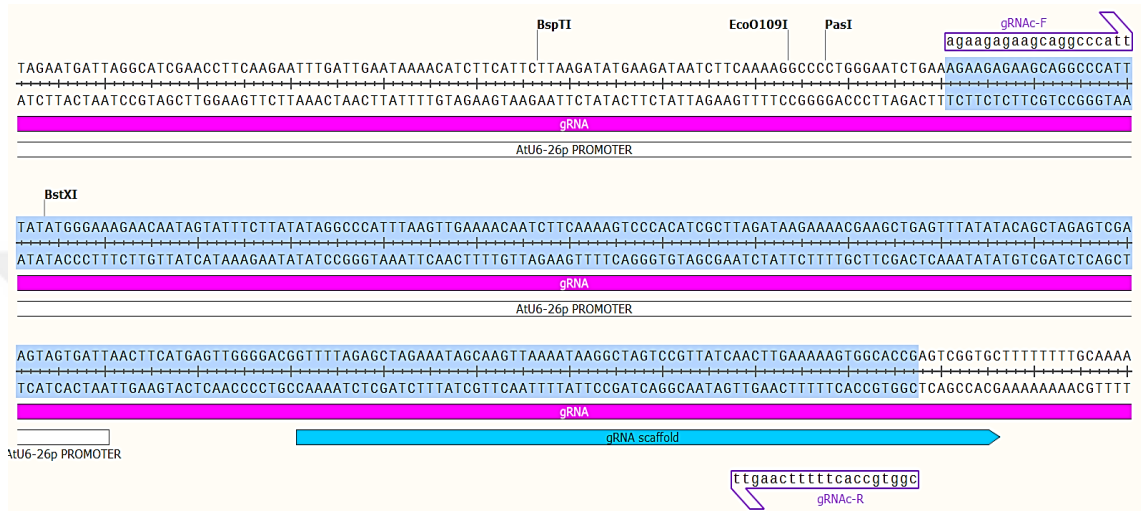


**Figure 4.8.** Primer pair designed through AtU6-26p promoter and gRNA scaffold, common to gRNA regions

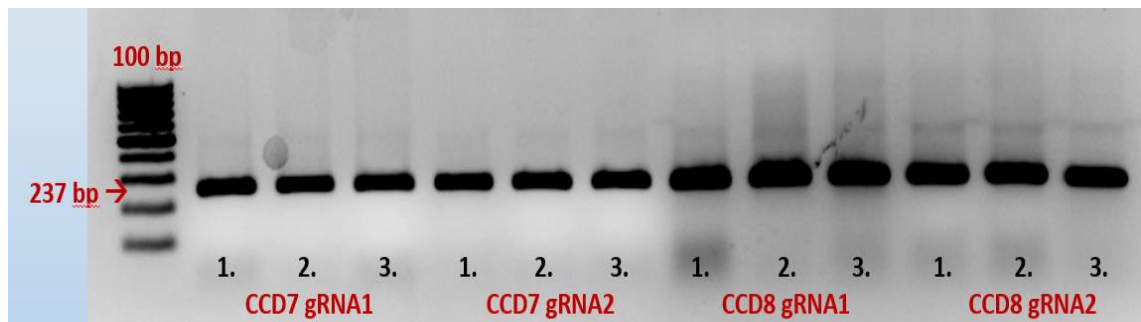


**Figure 4.9.** PCR products obtained with the designed gRNac/F-R primer pair of 3 different miniprep products of pTZ57R-SlCCD7-gRNA1, pTZ57R-SlCCD7-gRNA2, pTZ57R-SlCCD8-gRNA1, pTZ57R-SlCCD8-gRNA2, respectively (100 bp GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour)

*E. coli* DH5 $\alpha$  competent cells were tested in the medium containing Kanamycin (Figure 4.10) as a control. Two colonies selected from the pFGC-pcoCas9 colonies, which were transformed with the same method (heat-shock). They were incubated for O/N at 225 rpm at 37°C in 5 ml agar-free LB and 5  $\mu$ l Kanamycin selection (added from 50 mg/ml Kanamycin stock) and also tested with Ampicillin control medium (Figure 4.11a, b). The cells harboring the pFGC-pcoCas9 plasmid were plasmid purified

with the help of the GeneJET Plasmid Miniprep Kit and the products were stored at -20°C.

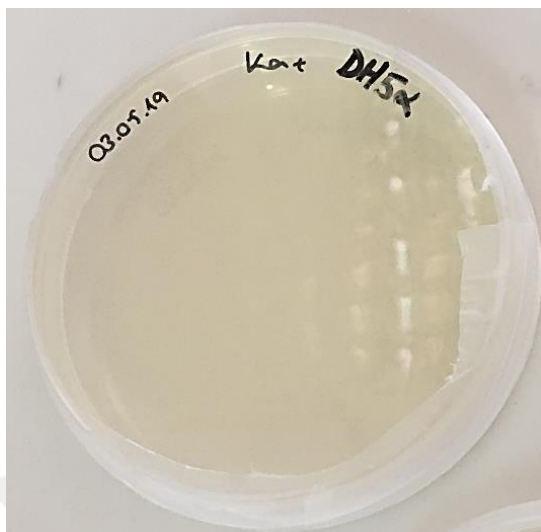
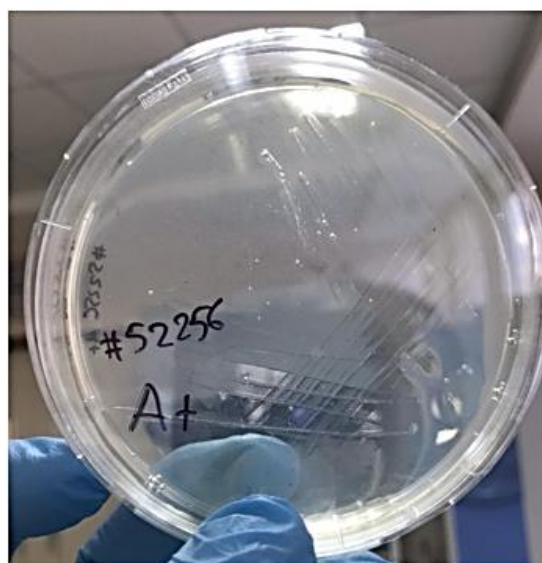


**Figure 4.10.** *E. coli* DH5 $\alpha$  competent cells tested in selection medium containing Kanamycin



(a)



(b)

**Figure 4.11.** **a)** Growing pFGC-pcoCas9 colonies on medium containing kanamycin selection; **b)** Colonies of pFGC-pcoCas9 that can't grow in a control environment containing ampicillin selection

In order to determine the correctness of the two pFGC-pcoCas9 plasmid DNAs purified, a digestion reaction was performed by selecting two enzyme restriction regions (EcoRI and NcoI) located on the pFGC-pcoCas9 plasmid itself. The accuracy of the pFGC-pcoCas9 plasmid to be used as a vector has been proven by observing two expected bands of 4931 bp and 8399 bp (Figure 4.12).

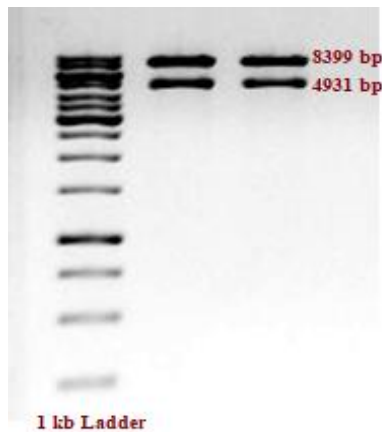


**Figure 4.12.** The result of the cut reaction of pFGC-pcoCas9 (# 52256) 1-2 plasmids with EcoRI and NcoI enzymes (1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour)

#### 4.2.2. Combining SlCCD7/8 gRNA 1 and 2 and ligation to the pFGC-pcoCas9 vector

Digestion was performed with PacI and AclI enzymes to separate relevant gRNA regions with pTZ57R-SlCCD7-gRNA1, pTZ57R-SlCCD7-gRNA2, pTZ57R-SlCCD8-gRNA1, pTZ57R-SlCCD8-gRNA2 plasmids synthesized by adding PacI and AclI enzyme restriction sites to the up and downstream of the gRNA regions. Some of the digestion products obtained were displayed on the UV transilluminator, and it was checked whether the reaction was taking place correctly and whether the bands were separated sufficiently. The remaining amount of the digestion products was displayed on the UV transilluminator after 1 hour (100 V) electrophoresis (Figure 4.13).

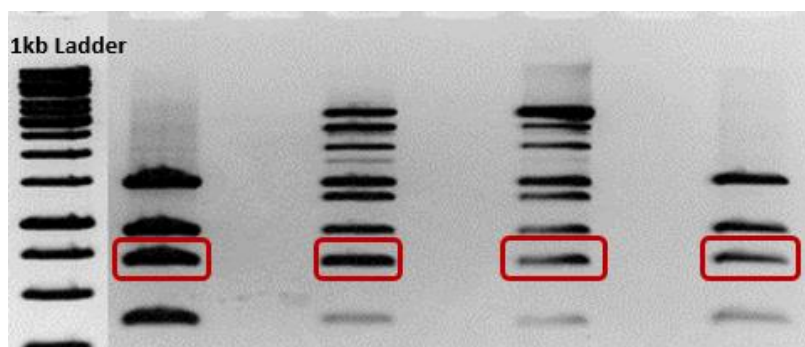


**Figure 4.13.** The digestions of pTZ57R-SlCCD7-gRNA1, pTZ57R-SlCCD7-gRNA2, pTZ57R-SlCCD8-gRNA1, pTZ57R-SlCCD8-gRNA2 with AclI and PacI enzymes, respectively (1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour)

As expected, 730 bp for SlCCD7-gRNA1, 717 bp for SlCCD7-gRNA2, 726 bp for SlCCD8-gRNA1, and 721 bp for SlCCD8-gRNA2 sized fragments were obtained in the pTZ57R plasmids (Figure 4.14 and Figure 4.15), and the bands of the correct size were cut from the gel and purified.

**pTZ57R-SICCD7-gRNA1 (AclI-PacI) Digestion = 730 bp**

**CGTT**TTACTAGTCGACTTGCCTTCCGCACAATACATCATTTCTTCTAGCTTTTTTCTTCTTCTCGTTCATACAGTTTTTTT  
 TTGTTTATCAGCTTACATTTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTAACTTTCATTTCGGAGTTTTGTATCTTGTTTC  
 ATAGTTTGTCCAGGATTAGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATAT  
 GAAGATAATCTTCAAAAGGCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCATTATATGGGAAAGAACAATAGTATTT  
 CTTATATAGGCCCATTTAAGTTGAAAACAATCTTCAAAAGTCCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATA  
 CAGCTAGAGTCGAAGTAGTGATTAACTTCATGAGTTGGGGACGGTTTTAGAGCTAGAAAAGCAAGTTAAAATAAGGCTA  
 GTCCGTTATCAACTTGAAAAAGTGGCACCAGTCGGTGCTTTTTTTGCAAAATTTTCCAGATCGATTCTTCTTCTCTGTT  
 CTTGCGCGTTCAATTTCTGGGGTTTTCTTCTGTTTTCTGTAAGTGAACCTAAAATTTGACCTAAAAAAAATCTCAAATAAT  
 ATGATTCAGTGGTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCGATGAGATAAACCAATA**TTAAT**

**pTZ57R-SICCD7-gRNA2 (AclI-PacI) Digestion = 717 bp**

**TAA**CGACTTGCCTTCCGCACAATACATCATTTCTTCTAGCTTTTTTCTTCTTCTCGTTCATACAGTTTTTTTTGTTTATCA  
 GCTTACATTTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTAACTTTCATTTCGGAGTTTTGTATCTTGTTTCATAGTTTGT  
 CCAGGATTAGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATC  
 TTCAAAAGGCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCATTATATGGGAAAGAACAATAGTATTTCTATATAGG  
 CCCATTTAAGTTGAAAACAATCTTCAAAAGTCCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAG  
 TCGAAGTAGTGATTATCTTGGCTTTGTAGTAGCGTTTTAGAGCTAGAAAAGCAAGTTAAAATAAGGCTAGTCCGTTATC  
 AACTTGAAAAAGTGGCACCAGTCGGTGCTTTTTTTGCAAAATTTTCCAGATCGATTCTTCTTCTCTGTTCTTGGCGGTT  
 CAATTTCTGGGGTTTTCTTCTGTTTTCTGTAAGTGAACCTAAAATTTGACCTAAAAAAAATCTCAAATAATATGATTCAGT  
 GGTTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCGATGAGATAAACCAATA**AA**

**Figure 4.14.** Sequence information of SICCD7-gRNA1 and gRNA2 in the pTZ57R plasmid (nucleotides in bold font are restriction regions of the AclI and PacI enzymes)

**pTZ57R-SICCD8-gRNA1 (AclI-PacI) Digestion = 726 bp**

**TAA**TATTGGTTTATCTCATCGGAAGTCAAAAACCACTAACTGAAAAGTACAAAACCACTGAATCATATTATTTGAGATT  
 TTTTTAGGTCAAATTTTAGGTTTCAGTTACAGAAAACGAAGAGAAAACCCAGAAATTGAACGCCGAAGAACAGAGGAA  
 GAAGAAATCGATCTGGAAAATTTTGCAAAAAAAGCACCAGCTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTA  
 TTTAACTTGCTATTTCTAGCTCTAAAACCAACGACGAGTTCTCCTTCAATCACTACTTCGACTCTAGCTGTATATAAACTCA  
 GCTTCGTTTTCTTATCTAAGCGATGTGGGACTTTTGAAGATTGTTTTCAACTTAAATGGGCCTATATAAGAAATACTATTGTT  
 CTTTCCCATATAAATGGGCCTGCTTCTTCTTTTTCAGATTCCCAGGGGCCCTTTTGAAGATTATCTTCATATCTTAAGAATGAA  
 GATGTTTTATTCAATCAAATCTTGAAGGTTTCGATGCCTAATCATTCTAATCCTGGGACAACTATGAAACAAGATACAAA  
 ACTCCGAATGGAAGTTAAAAAGAAGAAAACGAAAGCTACGGTTCAAGAAAATGTAAGCTGATAACAAAAAAAACCTG  
 TATGAACGAAGAAGAAGAAAAAAGCTAAGAAGAAATGATGTATTGTGCGGAAGGCAAGTCGACTAGTAA**AA**

**pTZ57R-SICCD8-gRNA2 (AclI-PacI) Digestion = 721 bp**

**CGTT**TATTGGTTTATCTCATCGGAAGTCAAAAACCACTAACTGAAAAGTACAAAACCACTGAATCATATTATTTGAGATT  
 TTTTTAGGTCAAATTTTAGGTTTCAGTTACAGAAAACGAAGAGAAAACCCAGAAATTGAACGCCGAAGAACAGAGGA  
 AGAAGAAATCGATCTGGAAAATTTTGCAAAAAAAGCACCAGCTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTT  
 ATTTAACTTGCTATTTCTAGCTCTAAAACGGATATACGGTGGTGAGAATAATCACTACTTCGACTCTAGCTGTATATAAAC  
 TCAGCTTCGTTTTCTTATCTAAGCGATGTGGGACTTTTGAAGATTGTTTTCAACTTAAATGGGCCTATATAAGAAATACTATT  
 GTTCTTTCCCATATAAATGGGCCTGCTTCTTCTTTTTCAGATTCCCAGGGGCCCTTTTGAAGATTATCTTCATATCTTAAGAAT  
 GAAGATGTTTTATTCAATCAAATCTTGAAGGTTTCGATGCCTAATCATTCTAATCCTGGGACAACTATGAAACAAGATACAA  
 AAACTCCGAATGGAAGTTAAAAAGAAGAAAACGAAAGCTACGGTTCAAGAAAATGTAAGCTGATAACAAAAAAAACCTG  
 CTGTATGAACGAAGAAGAAGAAAAAAGCTAAGAAGAAATGATGTATTGTGCGGAAGGCAAGTCG**TTAAT**

**Figure 4.15.** Sequence information of SICCD8-gRNA1 ve gRNA2 in the pTZ57R plasmid (nucleotides in bold font are restriction regions of the AclI and PacI enzymes)

For the accuracy of the purified fragments, the product of DNA amplification with the forward primer (gRNAC / F) designed over the AtU6-26p promoter, which is common to all gRNA regions, and the reverse primer (gRNAC / R) designed over the gRNA scaffold, should be 237 bp long was determined and confirmed (Figure 4.16).

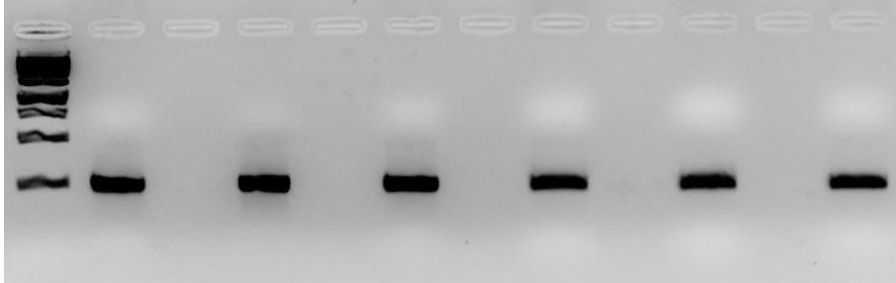


**Figure 4.16.** Amplification with gRNAC-F / R primers of SI CCD7-gRNA1, SI CCD7-gRNA2, SI CCD8-gRNA1, SI CCD8-gRNA2 which were excised from plasmid pTZ57R, respectively (1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour)

The pFGC-pcoCas9 (# 52256) plasmid was digested at the PacI restriction site in the 78th base position in the MCS and made ready for the ligation of the gRNA products. The only band produced in the correct size after the enzymatic digestion was removed from the agarose gel with a scalpel on a UV transilluminator and purified. Before the ligation of gRNAs into the vector pFGC-pcoCas9 (# 52256) -which was enzymatically digested with PacI from a single point- was treated with the rSAP enzyme. rSAP prevents self-ligation by catalyzing the phosphorylation of the 5' and 3' ends of the DNA and RNA phosphomonoesters. The products of SI CCD7 gRNA 1-2 and SI CCD8 gRNA 1-2 ligated separately to the pFGC-pcoCas9 vector using the T4 ligase enzyme (Figure 4.17 and Figure 4.18). The resulting vectors were then transformed according to the heat-shock method using *E. coli* competent cell (*DH5 $\alpha$*  strain).

SI CCD7 gRNA1+ gRNA2 = 1447 bp

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TAACGACTTGCCCTCCGCACAATACATCATTTCTCTAGCTTTTCTTCTCTCTCGTTCATACAGTTTTTTTGTATTATCA
GCTTACATTTTTCTGAACCGTAGCTTTCGTTTCTTCTTTTAACTTTCATTCCGGAGTTTTGTATCTGTTTCATAGTTTGTG
CCAGGATTAGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATG
TTCAAAAGGCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAGAACAATAGTATTTCTTATATAGG
CCCATTTAAGTTGAAAACAATCTTCAAAAGTCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAG
TCGAAGTAGTGATTATTCTTGGCTTTGTAGTAGCGTTTATAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATC
AACTTGAAAAAGTGGCACCAGTCCGGTGCTTTTTTGC AAAATTTCCAGATCGATTCTTCTCTCTCTGTTCTTCGGCGTT
CAATTTCTGGGGTTTTCTCTTCTGTTTTCTGTAACCTGAAACCTAAAATTTGACCTAAAAAAATCTCAAATAATATGATTTCAGT
GGTTTTGTACTTTTCAGTTAGTTGAGTTTGCAGTTCCGATGAGATAAACCAATAAACGTTTTTACTAGTCGACTTGCCCTT
CCGCACAATACATCATTTCTTCTAGCTTTTCTTCTCTCTCGTTCATACAGTTTTTTTGTATTATCAGCTTACATTTTCTTG
AACCGTAGCTTTCGTTTCTTCTTTTAACTTTCATTCCGGAGTTTTGTATCTGTTTCATAGTTTGTCCAGGATTAGAATG
ATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATCTTCAAAAGGCCCTT
GGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAGAACAATAGTATTTCTTATATAGGCCCATTTAAGTTGA
AAACAATCTTCAAAAGTCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTGATT
AACTTCATGAGTTGGGGACGGTTTATAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTGAAAAAGT
GGCACCAGTCCGGTGCTTTTTTGC AAAATTTCCAGATCGATTCTTCTCTCTCTGTTCTTCGGCGTTCAATTTCTGGGGT
TTTCTCTTCGTTTTCTGTAACCTGAAACCTAAAATTTGACCTAAAAAAATCTCAAATAATATGATTTCAGTGTTTTGTACTTTT
CAGTTAGTTGAGTTTTGCAGTTCCGATGAGATAAACCAATATTAAT

```

**Figure 4.17.** Sequence information of the product expected to be obtained after ligating SI CCD7 gRNA 1+2 using the T4 ligase enzyme into the pFGC-pcoCas9 vector (nucleotides in the bold font are restriction sites of the AclI and PacI enzymes)

SI CCD8 gRNA1+ gRNA2 = 1447 bp

**TAA**TATTGGTTTATCTCATCGGAACTGCAAACTCAACTAACTGAAAAGTACAAAACCACTGAATCATATTATTGAGATT  
 TTTTITAGGTCAAATTTTAGGTTTCAGTTACAGAAAACGAAGAGAAAACCCAGAAATTGAACGCCGAAGAACAGAGGAA  
 GAAGAAATCGATCTGGAAAATTTGCAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTA  
 TTTAACTTGCTATTCTAGCTCTAAACTCAACGACGAGTTCTCCTTCAATCACTACTTCGACTCTAGCTGTATATAAACTCA  
 GCTTCGTTTTCTTATCTAAGCGATGTGGGACTTTTGAAGATTGTTTTCAACTTAAATGGGCCTATATAAGAAATACTATTGTT  
 CTTCCCATATAAATGGGCCTGCTTCTTCTTTCAGATTCCCAGGGGCCTTTGAAGATTATCTTCATATCTTAAGAATGAA  
 GATGTTTTATTCAATCAAATCTTGAAGGTTTCGATGCCTAATCATTCTAATCCTGGGACAACTATGAAACAAGATACAAAA  
 ACTCGAATGGAAAGTTAAAAAGAAGAAAACGAAAGCTACGGTTCAGAAAATGTAAGCTGATAAACAAAAAAAACCTG  
 TATGAACGAAGAAGAAGAAAAAAGCTAAGAAGAAATGATGTATTGTGCGGAAGGCAAGTCGACTAGTAAAA**ACGTTT**  
**ATTGGTTTATCTCATCGGAACTGCAAACTCAACTAACTGAAAAGTACAAAACCACTGAATCATATTATTGAGATTTTTTT**  
**AGGTCAAATTTTAGGTTTCAGTTACAGAAAACGAAGAGAAAACCCAGAAATTGAACGCCGAAGAACAGAGGAAGAAGA**  
**AATCGATCTGGAAAATTTGCAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAA**  
**CTTGCTATTCTAGCTCTAAACGGATATACGGTGGTGAGAATAATCACTACTTCGACTCTAGCTGTATATAAACTCAGCTT**  
**CGTTTTCTTATCTAAGCGATGTGGGACTTTTGAAGATTGTTTTCAACTTAAATGGGCCTATATAAGAAATACTATTGTTCTTT**  
**CCCATATAAATGGGCCTGCTTCTTCTTTCAGATTCCCAGGGGCCTTTTGAAGATTATCTTCATATCTTAAGAATGAAGATG**  
**TTTTATTCAATCAAATCTTGAAGGTTTCGATGCCTAATCATTCTAATCCTGGGACAACTATGAAACAAGATACAAAAACTC**  
**CGAATGGAAAGTTAAAAAGAAGAAAACGAAAGCTACGGTTCAGAAAATGTAAGCTGATAAACAAAAAAAACCTGTATG**  
**AACGAAGAAGAAGAAAAAAGCTAAGAAGAAATGATGTATTGTGCGGAAGGCAAGTCGTTAAT**

**Figure 4.18.** Sequence information of the product expected to be obtained after ligating SI CCD8 gRNA 1+2 using the T4 ligase enzyme into the pFGC-pcoCas9 vector (nucleotides in the bold font are restriction sites of the AclI and PacI enzymes)

In order to determine the correctness of the colonies selected for plasmid isolation after transformation, five colonies selected from both media were used as template DNA, and colony PCR was performed using gRNAc-F/R primers. The PCR products were visualized after gel electrophoresis (Figure 4.19 and Figure 4.20). Plasmid maps are expected to occur after ligation was created in a representative format in the Snapgene Viewer program (Figure 4.21 and Figure 4.22).

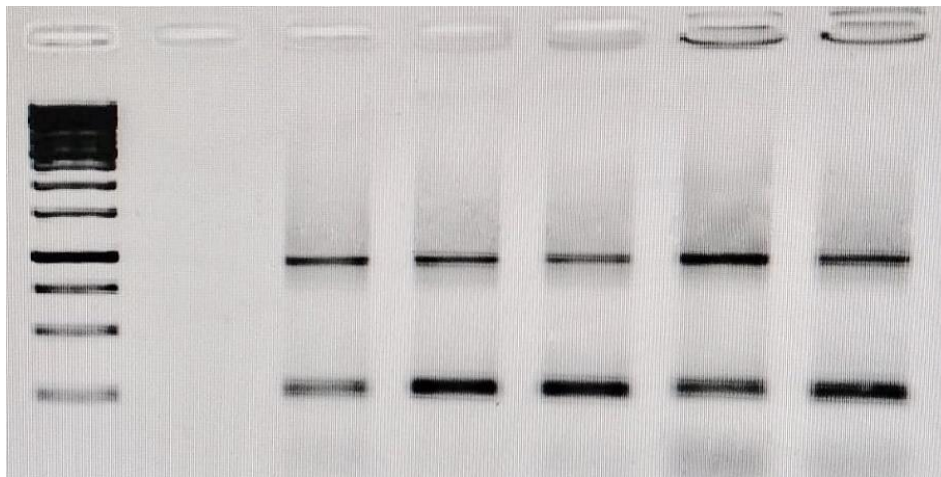


**Figure 4.19.** Colony PCR image of 5 colonies selected from the cloning performed by the ligation of SI CCD7 gRNA1+gRNA2 to the pFGC-pcoCas9 (# 52256) plasmid (1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour)

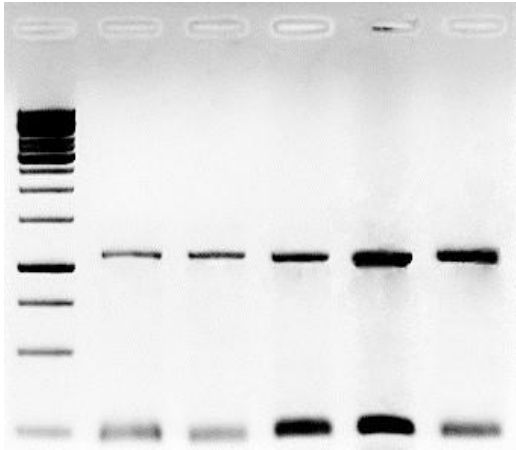


**Figure 4.20.** Colony PCR image of 5 colonies selected from the cloning performed by the ligation of SICCD8 gRNA1+gRNA2 to the pFGC-pcoCas9 (# 52256) plasmid (1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour)

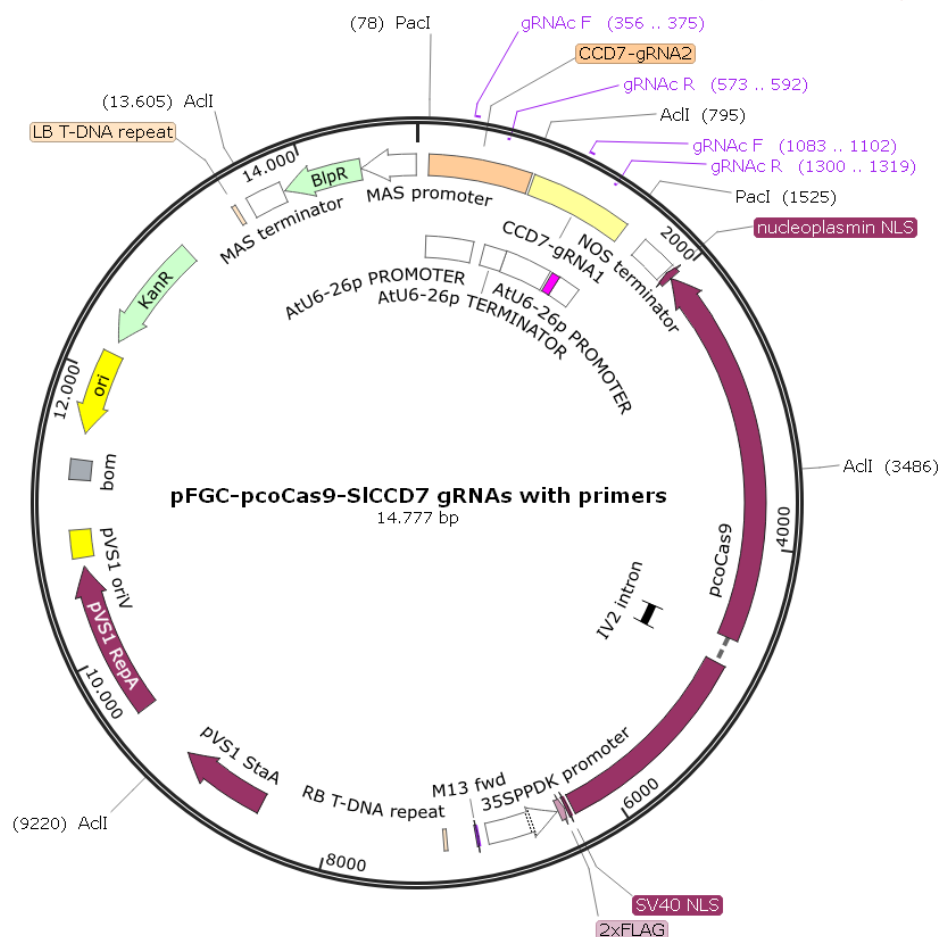


**Figure 4.21.** Representative plasmid map created after the ligation of SICCD7 gRNA1+gRNA2 to the pFGC-pcoCas9 vector and the positions of the gRNAc primers on the plasmid



Two bands were observed due to PCR performed with gRNAC F/R primers to verify gRNAs found in the pFGC-pcoCas9 vector and determined according to the *SICCD7* and *SICCD8* genes after ligation. When looking at the plasmid maps of Figure 4.17 and Figure 4.18, two bands were formed due to the binding of the gRNAC-F and R primers common in each gRNA region to more than one location. As seen in the gel, as the 1st combination, 237 bp fragment resulted from the amplification using the 1./2. gRNAC-F and 1./2. gRNAC-R primers (the lowest band that occurs as a result of UV imaging with 1kb ladder), and as the 2nd combination, 964 bp size (higher MW band resulting from UV imaging with a 1kb ladder) were obtained as a result of amplification using the first gRNAC-F and second gRNAC-R primers (Figure 4.23).

**AGAAGAGAAGCAGGCCATT**TATATGGGAAAGAACAATAGTATTTCTTATATAGGCCATT  
 TAAGTTGAAAACAATCTTCAAAAGTCCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTA  
 TATACAGCTAGAGTCGAAGTAGTGATTNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGA  
 AATAGCAAGTTAAAATAAGGCTAGTCCGTTATC**AACTTGAAAAAGTGGCACCG**AGTCGGT  
 GCTTTTTTTTGC AAAATTTTCCAGATCGATTCTTCTTCTCTGTTCTTCGGCGTTCAATTCT  
 GGGGTTTTCTCTTCGTTTTCTGTAAGTAAACCTAAAATTTGACCTAAAAAAAATCTCAAATA  
 ATATGATTCAGTGTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCGGATGAGATAAACC  
 AATAAACGTTTTTACTAGTCGACTTGCCTTCCGCACAATACATCATTTCTTCTTAGCTTTTTTT  
 CTTCTTCTTCGTTTCATACAGTTTTTTTTTTGTTTATCAGCTTACATTTTCTTGAACCGTAGCTTTC  
 GTTTTCTTCTTTTAACTTTCCATTTCGGAGTTTTTGTATCTTGTTCATAGTTTGTCCCAGGATT  
 AGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGAT  
 ATGAAGATAATCTTCAAAAGGCCCTGGGAATCTGAA**AGAAGAGAAGCAGGCCATT**TAT  
 ATGGGAAAGAACAATAGTATTTCTTATATAGGCCATTTAAGTTGAAAACAATCTTCAAAAG  
 TCCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTG  
 ATTNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTA  
 GTCCGTTATC**AACTTGAAAAAGTGGCACCG**

**Figure 4.23.** The positions of gRNAc-F (yellow) and gRNAc-R (green) primers, which are common primers for designed gRNAs, on the sequence

Some of the new plasmids created by ligating the gRNA 1 and 2 were designed to target the *SICCD7* and *SICCD8* genes into the pFGC-pcoCas9 vector. They were measured in the Biodrop device in ng/μl before sent for sequencing for validation purposes and were sent to POLGEN Biotechnology (Ankara) for their sequencing. After the ligation process, it became clear that gRNAs determined according to the *SICCD7* and *SICCD8* genes were successfully ligated to the pFGC-pcoCas9 vector.

#### 4.2.3. Transformation of pFGC-pcoCas9<sub>SICCD7/8</sub>-gRNA1+2 plasmids with *Agrobacterium tumefaciens* strain

The final product obtained as a result of the successful combination of gRNA 1 and gRNA 2, designed separately for the *SICCD7* and *SICCD8* genes after ligation, was transformed with the *Agrobacterium tumefaciens* EHA105 strain (Figure 4.24), which is known to be highly efficient in plant transformation according to the literature. After transformation, cells and empty competent EHA105 strain for control purposes were grown in the medium containing 50 μg/ml Kanamycin (bacterial selection of pFGC-pcoCas9 plasmid) + 25 μg/ml Rifampicin (to prevent possible bacterial contamination). As a result of 48 hours, pFGC-pcoCas9 plasmids harboring gRNAs were colonized in the selection medium, while the empty competent EHA105 strain cells could not survive due to the lack of Kanamycin resistance (Figure 4.25).



**Figure 4.24.** *Agrobacterium tumefaciens* EHA105 strain colonies on medium containing 40 µg/ml Rifampicin



**Figure 4.25.** Colonies formed in media containing 50 µg/ml Kanamycin + 25 µg/ml Rifampicin after transformation with EHA105 strain and control medium prepared with empty EHA105 strain, respectively

Before preparing stock cells from the colonies, colony PCR was performed with the new primers (Figure 4.26a, b) designed for the gRNAs determined according to the *SICCD7* and *SICCD8* genes and designed with the Primer3Plus program to cover the combined form of gRNA1-gRNA2 after ligation and visualized with UV in 1,5% agarose gel (Figure 4.27).

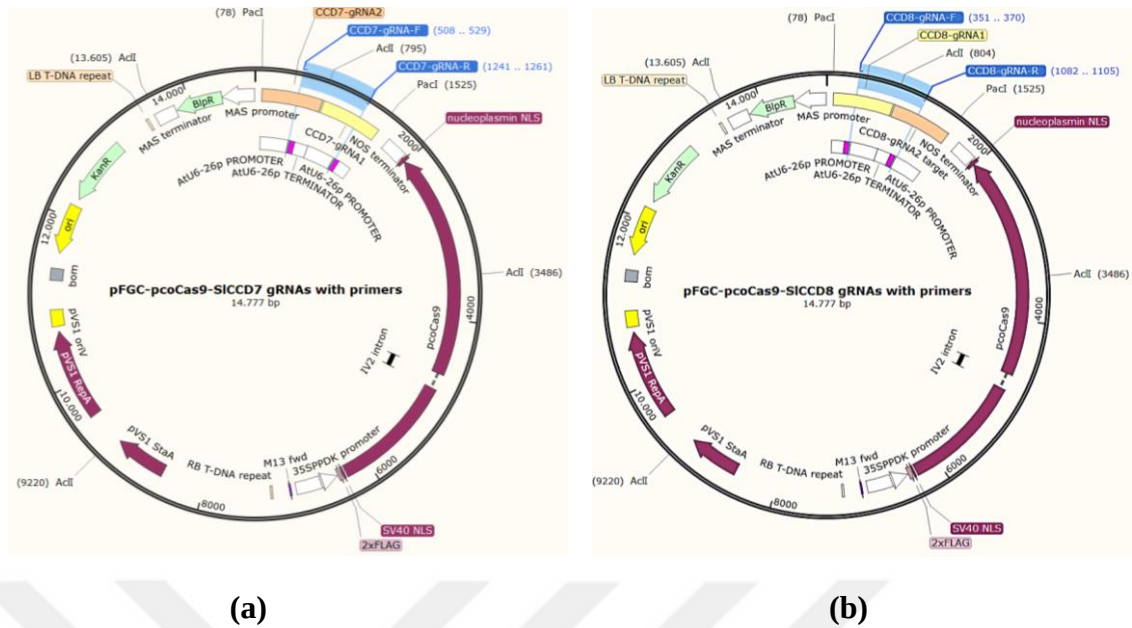


**Figure 4.26.** Representative maps of gRNA primers found on pFGC-pcoCas9 vector **a)** CCD7-gRNA F/R primer pair designed using pFGC-pcoCas9<sup>SICCD7</sup>-gRNA1+2 plasmid sequences to confirm the presence of gRNA 1 and 2 regions; **b)** CCD8-gRNA F/R primer pair designed using pFGC-pcoCas9<sup>SICCD8</sup>-gRNA1+2 plasmid sequences to confirm the presence of gRNA 1 and 2 regions



**Figure 4.27.** Colony PCR image performed with CCD7-gRNA F/R and CCD8-gRNA F/R primer pairs (water as negative control, miniprep stocks of ligation products were used as positive control) (1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour)

As a result of the amplification, the correctness of transformation with the *A. tumefaciens* EHA105 strain was checked. Stock-bacterial cultures were proved due to the amplification of the expected fragments 754 bp for pFGC-pcoCas9<sup>SICCD7</sup>-gRNA1 + 2 and 755 bp for pFGC-pcoCas9<sup>SICCD8</sup>-gRNA1 + 2.

#### 4.2.4. Plant Transformation

A preliminary study was carried out to determine how successful the *A. tumefaciens* EHA105 strain is in transforming tomato. In this context, the binary pCAMBIA1303 plasmid harboring the *GFP* (*mgfp5*) genes was transferred to tomato plants by the vacuum infiltration method. Because of transient expression, GFP presence (luminescence) in tomato leaf sections could be observed with a fluorescence microscope (488 nm excitation) for 12 days. As a result of the examinations performed on the 4th day when the expression was observed the strongest, while no GFP luminescence was observed in the non-transformed control section, intense luminescence in various tissue sections was observed successfully due to the expression of *mgfp5* in transformed plants (Figure 4.28a, b).

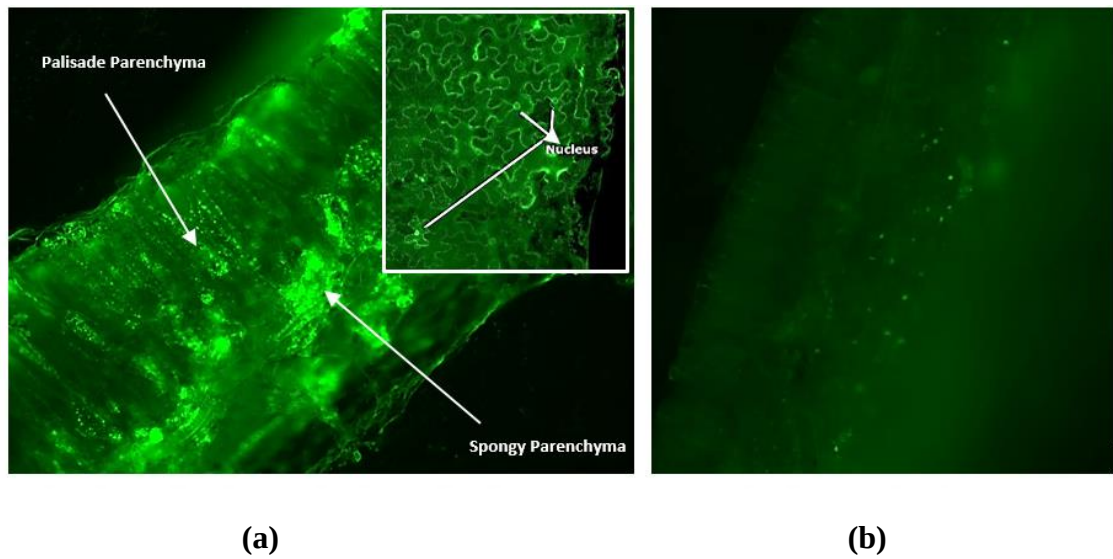


**Figure 4.28.** Analyzing the plant transformation potential of *Agrobacterium tumefaciens* EHA105 strain **a)** Observation of the *mgfp5* gene-expression (luminescence) on transformed leaf sections at 4 dpi by fluorescence microscope; **b)** Examination of non-transformed control leaf-section under fluorescence microscope (at 488 nm)

Ultimately, the success of the *A. tumefaciens* EHA105 strain in *Agrobacterium*-mediated plant transformation for tomato was confirmed in parallel with the literature. It was concluded that a successful result could be obtained in plant transformation by using the EHA105 strain in which the pFGC-pcoCas9 vectors harboring CRISPR constructs are transformed in tomato plants. 267 F1 and Micro-Tom genotypes were used in plant transformation, and two different protocols (modified version of the tomato transformation protocols created by Van Eck (2019) (Protocol A) and the optimized transformation protocol of Assoc. Prof. Dr. Hasan Pinar (Protocol B)) were applied.

To ensure plant transformation with *Agrobacterium-EHA105* cells harboring the correct plasmid, liquid bacteria culture was checked by colony PCR before co-cultivation. The gRNA regions harboring with pFGC-pcoCas vector were amplified

with CCD7-gRNA F / R and CCD8-gRNA F / R primer pairs to determine the correctness of the O/N grown cells in the medium (Figure 4.29).

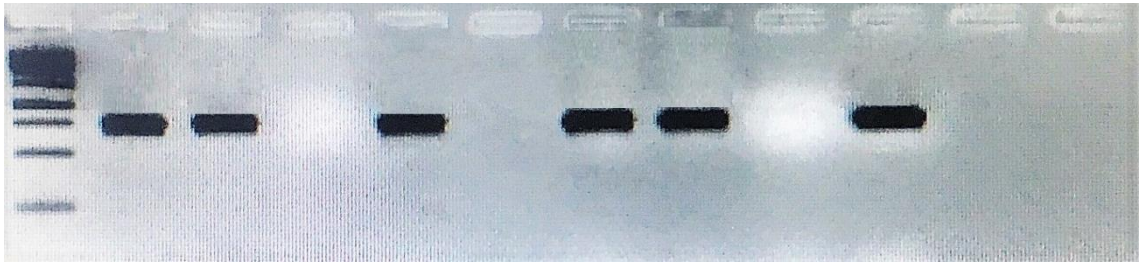


**Figure 4.29.** Amplification of 2 colonies with CCD7-gRNA F / R primer pairs, negative control ddH<sub>2</sub>O), positive control (miniprep stock of ligation products), amplification of 2 colonies with CCD8-gRNA F / R primer pairs, negative control ddH<sub>2</sub>O), positive control (miniprep stock of ligation products), respectively (1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour)

Cell culture verification was completed due to obtaining correct fragments of 754 bp for pFGC-pcoCas9<sub>SICCD7-gRNA1 + 2</sub> and 755 bp pFGC-pcoCas9<sub>SICCD8-gRNA1+2</sub>, and plant transformation was initiated. For co-transformation (to plant) of pFGC-pcoCas9 vectors harboring SICCD7 and SICCD8 gRNA targets separately, explants were kept in liquid cultures mixed at a ratio of 1: 1 (EHA105<sub>pFGC-pcoCas9SICCD7-gRNA1+2</sub> : EHA105<sub>pFGC-pcoCas9SICCD8-gRNA1+2</sub>) and infected with bacteria. Thanks to the acetosyringone and wounds created with a scalpel on the leaf, *Agrobacterium*-mediated transformation was performed, and the transformation process was maintained by taking the transformed explants into the co-cultivation media. After two weeks, in the explants which were transferred to the shoot induction environment after co-cultivation, callus hence shoot structures that are generally formed indirectly from callus are started to be observed with protocol B and modified protocol A. These formations were induced by the hormones zeatin (cytokinin) and IAA (auxin) contained in the media. Shoot regeneration has been particularly promoted, as the amount of zeatin (cytokine) (2 mg/ml) is relatively higher than that of IAA (auxin) (0.1 mg/ml). Thanks to the Timentin antibiotic in the medium (200 mg/ml), *Agrobacterium* growth was suppressed without any toxic damage to the plant. Regeneration was achieved from BASTA-resistant cells harbored by the vectors transferred to the plant by transformation (pFGC-pcoCas9).

Comparing the plants obtained by regeneration from protocol A and protocol B, the plants obtained by protocol B had a healthier green part. However, this comparison does not give a clear result because the genotypes used for both protocols were different. However, the shorter lifecycle of the Micro-Tom genotype showed a faster regeneration than the 267 F1 genotypes. The 267 F1 genotypes had lower transformation and regeneration efficiency than Micro-Tom. For this reason, Basta selection was relatively low for the 267 F1 genotypes to keep the plants alive (for protocol B). A more robust selection was achieved due to 2 mg/ml Basta in protocol A. Also, the best rooting for the 267 F1 genotypes was achieved with 0,1 mg/ml IAA, while Micro-Tom showed the best rooting at the combination of 0,1 mg/ml IAA + 0,1 mg/ml IBA. Since the expression of strigolactone was expected to decrease with the mutation to be introduced with the CRISPR-Cas9 system, the indeterminate 267 F1

genotype was more convenient for observing possible phenotypic responses plant. In contrast, the Micro-Tom genotype was more convenient in rapidly examining the success of the methods used in the study.

#### 4.2.5. Determination of transgenic plants and characterization of mutation

Leaf (tissue) samples (15 samples for 267 F1, 85 samples for Micro-Tom genotypes) were collected for DNA extraction from transgenic-candidate plants that survived under greenhouse conditions. PCR amplification, in which the obtained DNAs were used as templates, were carried out with BASTA / F-R, SICCD7 / F-R, SICCD8 / F-R primer pairs designed on pFGC-pcoCas9SICCD7/8-gRNA1 + 2 plasmids. Initially, samples were amplified with BASTA/ F-R primer (designed specifically to the *bar* gene located on the T-DNA region) to identify transformed plants without knowing which gRNA-construct transformants were. With these primers, transformant plants producing band at 638 bp were selected to determine which gRNA / gRNAs harboring construct/constructs were transferred to plants. As a result of this testing, it was determined that 8 (C36, D21, D33, D34, D39, D46, D47, D114) out of 100 plants in total were transformants (Figure 4.30, Figure 4.31).

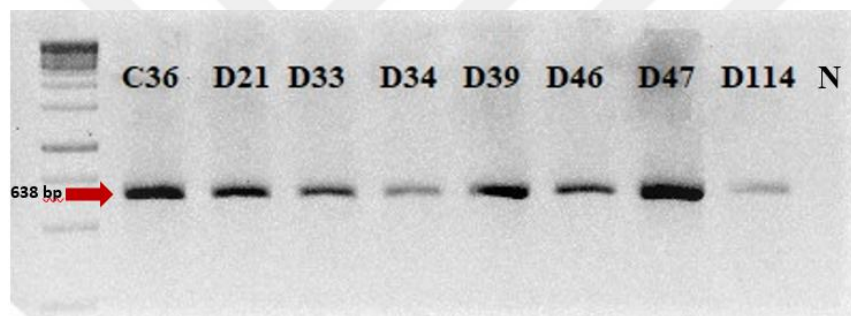


**Figure 4.30.** PCR result of 8 BASTA-positive plants with band at 638 bp (1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour / N: Negative control-water)

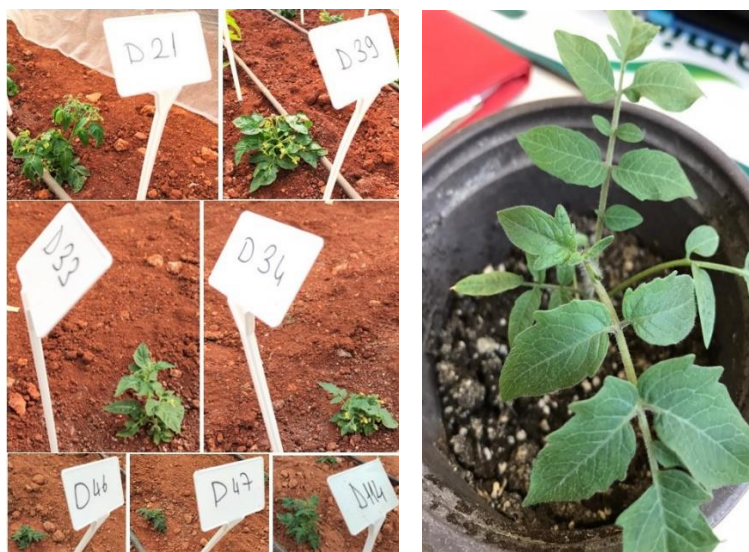


**Figure 4.31.** Plants determined to be transgenic (on the left is those from the Micro-Tom and on the right (C36) is from the 267-F1)

To find out which gRNA(s) (*SICCD7* / *8*) were transferred to 8 plants, a second PCR was performed with primers (*SICCD7* / F-R, *SICCD8* / F-R) designed specific to the gRNA constructs in the T-DNA region of pFGC-pcoCas9<sub>*SICCD7/8*</sub>-gRNA1 + 2 plasmids. For these 8 transformed samples, the expected band size was 754 bp for pFGC-pcoCas9<sub>*SICCD7*</sub>-gRNA1 + 2, and 755 bp for pFGC-pcoCas9<sub>*SICCD8*</sub>-gRNA1 + 2 (Figure 4.32).

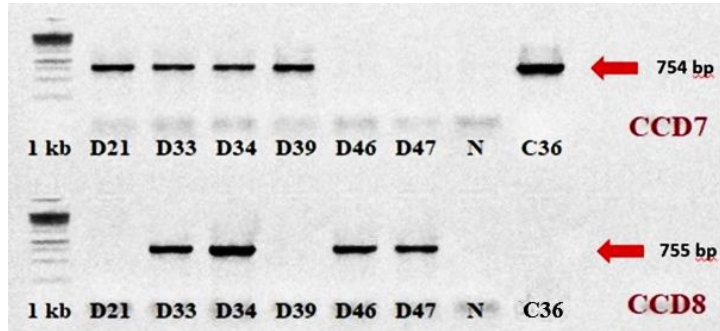


**Figure 4.32.** Plant samples transformed with pFGC-pcoCas9<sub>*SICCD7*</sub>-gRNA1+2 or/and pFGC-pcoCas9<sub>*SICCD8*</sub>-gRNA1 + 2 (1 kb Thermo GeneRuler Ladder, 100 V, after 1 hour / N: Negative control-water)

When the Figure 4.32 was examined, it was determined that 4 plants (C36, D21, D39, D114) transformed with pFGC-pcoCas9<sub>*SICCD7*</sub>-gRNA1 + 2, 2 plants (D46, D47) with pFGC-pcoCas9<sub>*SICCD8*</sub>-gRNA1 + 2 and 2 plants (D33 and D34) with both. The C36 plant was obtained from the 267 F1 genotypes, while all the others were the Micro-Tom genotype. After determining which gRNAs are carried by the transgenic plants, these regions were amplified with primers (in Table 3.1) designed specifically to the region targeted by the relevant gRNAs in the plant (Figure 4.33). In this way, mutations in the gRNA mediated targeted regions can be determined by sequencing the PCR products.

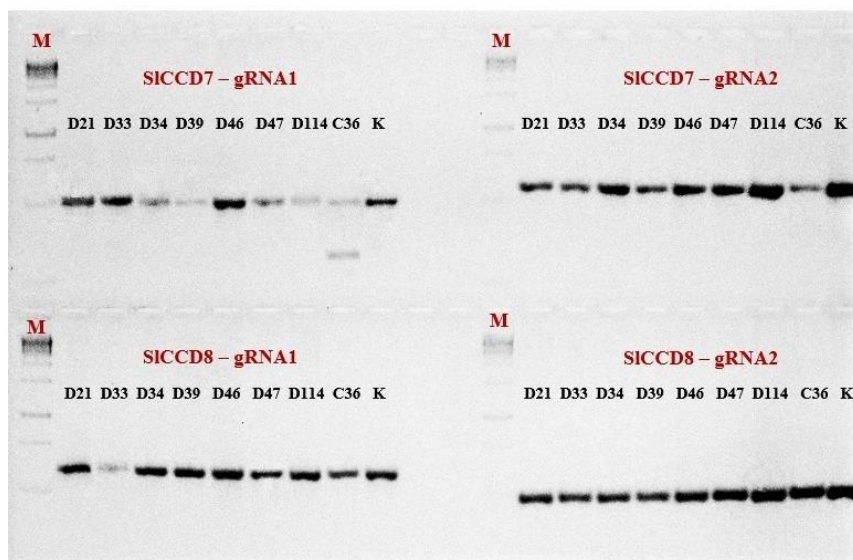


**Figure 4.33.** *SICCD7*-gRNA1/2 and *SICCD8*-gRNA1/2 plant target PCR amplifications (1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour / K: (Positive control) 267-F1)

The PCR products of 562 bp for *SICCD7*-gRNA2, 594 bp for *SICCD8*-gRNA1 and 483 bp for *SICCD8*-gRNA2 were sent to the sequencing. However, apart from the expected 448 bp products for *SICCD7*-gRNA1, extra fragments of different lengths were obtained due to the noticeable mutation in C36, even by agarose gel electrophoresis. Therefore, instead of directly sequencing the PCR products of the samples giving extra bands for the *SICCD7*-gRNA1 target, each fragment (C36-Upper / C36-Lower) was individually purified and sent to the sequencing. As a result of the important mutation seen after gel electrophoresis of *SICCD7*-gRNA1 PCR product in C36 plant, priority was given to this plant, and C36-T0 plant was selfed and C36-T1 progeny were obtained. Since this transgenic plant sample was obtained from the indeterminate type (267-F1), it was thought to be an important example in terms of detecting the morphological changes (SL-mutant phenotype) that may occur as a result of mutation.

When the chromatograms obtained by Sanger sequencing of 8 transgenic plant samples were examined, it was seen that mutation occurred in some plants (Figure 4.34) but not in others. After the PAM (NGG) sequence, distortions in the chromatograms began to occur in the mutated plants. However, no such distortions are seen in the chromatogram of the WT sample (non-mutated plant) sequenced for comparison.

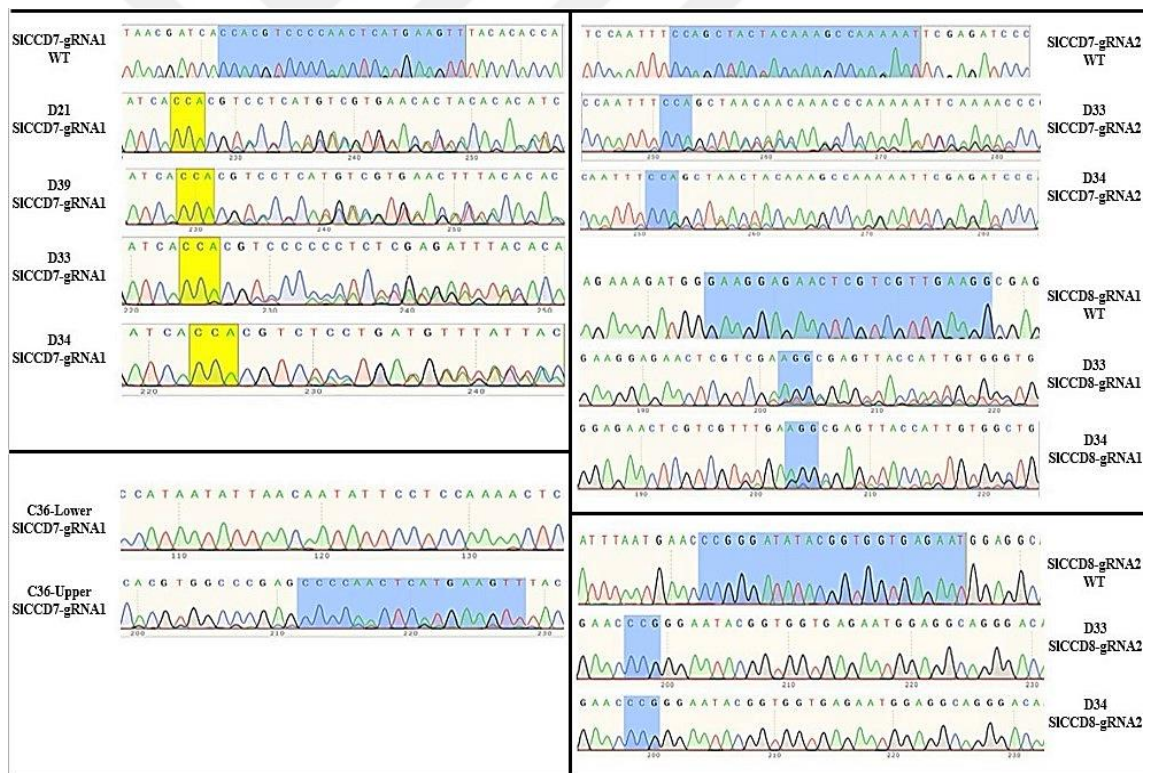


**Figure 4.34.** Chromatograms obtained by Sanger sequencing of *SICCD7* gRNA1-2 and *SICCD8* gRNA1-2 target regions of D21, D33, D34, D39, C36 plants (WT samples show chromatograms of the original sequences of *SICCD7* gRNA1-2 and *SICCD8* gRNA1-2 target regions and CCA, AGG, CCG show the PAM sequences)

When the sequence results were compared with the original *SICCD7* and *SICCD8* gene sequences, it was observed that D21, D33, D34,

D39, D39, C36 lines had mutations, but no mutations were detected for D46, D47, and D114. In order to make more qualified/accurate sequencing in plants thought to have mutations, the T1 population was generated by selfing of T0 plants, and sequencing according to the segregation was deemed suitable. However, the absence of PAM sequences at C36-lower<sup>SICCD7</sup>-gRNA1 was found, with two bands sequenced separately at the C36-T0 site giving a relatively straightforward result. This is because 186 nucleotides were deleted from the exon one region (*SICCD7* gRNA1 target) in the *SICCD7* gene during DNA repair (NHEJ). It was also observed in C36-Upper<sup>SICCD7</sup>-gRNA1 that the PAM sequence was absent, and a region of 18 nucleotides was deleted. As a result, it was determined that a heterozygous biallelic mutation occurred in the C36-T0 plant, even if only the gRNA1 (*SICCD7*) functioned. Besides, there was no distortion in the sequences with 18/186 nt deletion, so it was an “in-frame” deletion.

The segregation of the heterozygous biallelic mutation was determined by analysis of 23 C36-T1 progeny obtained from C36-T0 selfing. In this context, T1 plants were analyzed with *SICCD7* gRNA1 target (Figure 4.35) and Basta primer pairs (Figure 4.36a). Consistent results were obtained by testing with second primer pairs (52256-2 F/R) to support the results obtained with the Basta primer pair (Figure 4.36b). In the progeny obtained from C36-T0 segregation, the same sequence result as in C36-T0 was obtained by sequencing the lower and higher (upper) MW band separately.

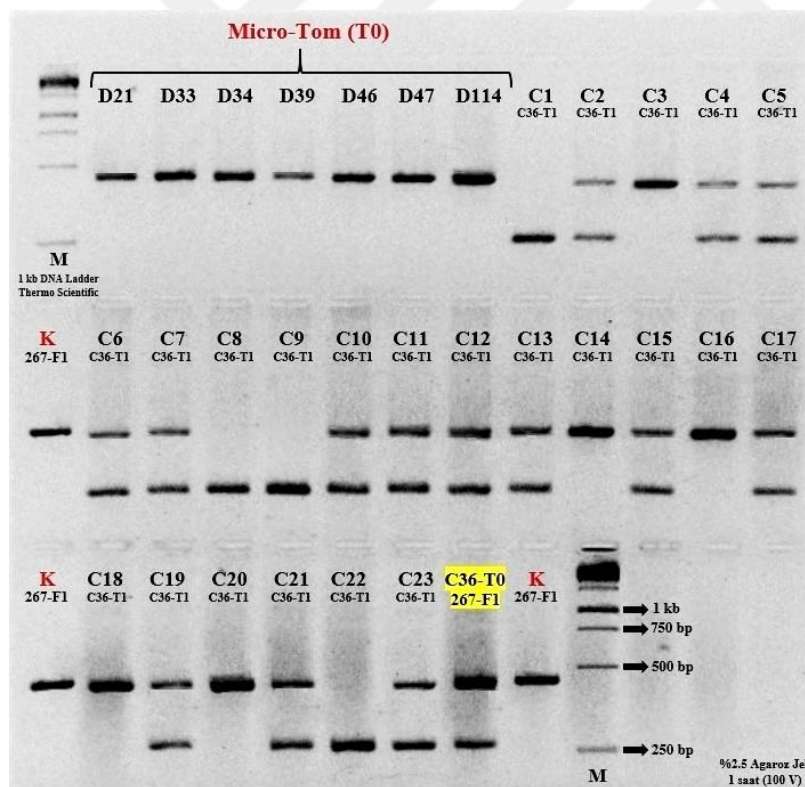


**Figure 4.35.** Image of the PCR amplification of 23 C36-T1 plants using the *SICCD7*-gRNA1 F/R primer (T0 Micro-Tom plants and 267-F1 (WT) were used as positive controls, 1 kb GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour)

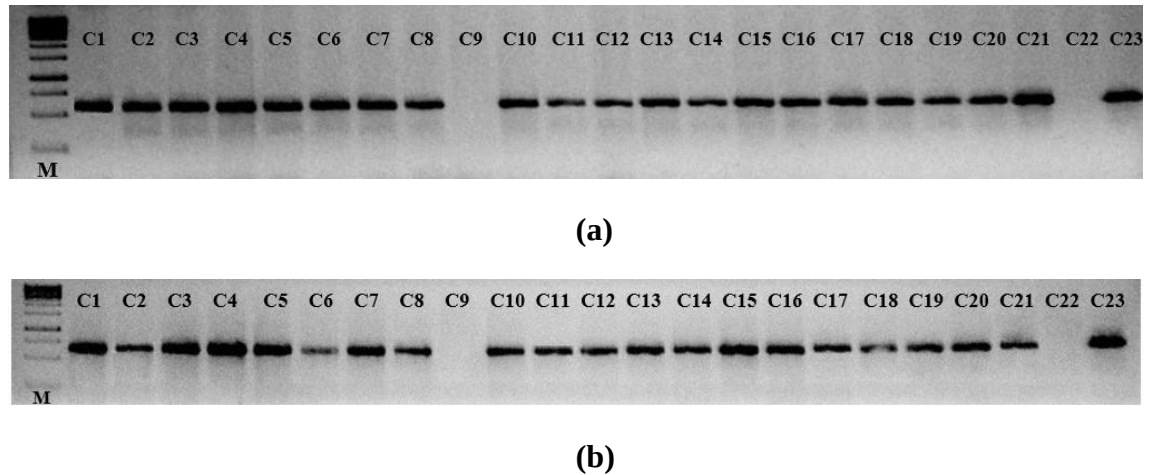


**Figure 4.36.** Image of the PCR amplification of 23 C36-T1 plants **a)** PCR amplification of 23 C36-T1 plants using the Basta F/R primer; **b)** PCR amplification of 23 C36-T1 plants using the 52256-2 F/R primer (1 kb GeneRuler Ladder (Thermo Scientific), %1,5 agarose gel, 100 V, after 1 hour)

When Figure 4.35 and 4.36a,b were analyzed, it was observed that C9 and C22 lines found to be not carrying the transgenes associated with CRISPR-Cas9, but the 186 nt homozygous deletion (mutation) was inherited. Five progeny (C3, C14, C16, C18, C20) have the homozygous mutation with 18 nt deletion. On the other hand, four (C1, C8, C9, C22) progeny have the homozygous mutation with 186 nt deletion. This 186 nt deletion occurred between Thr42-Val105 in the SlCCD7-exon 1 region of these four T1 progeny. The mutation caused the deletion of 62 amino acids (Figure 4.37) and did not cause a frameshift mutation (the base number deleted was more than three). It was evaluated as a "homozygous" in-frame "mutation. In the remaining 14 progenies, it is seen that heterozygous biallelic mutation is inherited (similar to C36-T0). By sequencing individual fragments (higher and lower MW band) obtained with the SlCCD7 gRNA1 target primer pairs in the C36-T1 progeny, the mutations in C36-T0 were identified with one-to-one similarity. No new mutations were found in the C36-T1 progeny. The result of  $p < .05$  ( $X^2 = 1,16$ ) was obtained by chi-square analysis performed in C36-T1 segregation, and no segregation distortion was observed.

|                  |                                                                |     |
|------------------|----------------------------------------------------------------|-----|
| CCD7-Exon1       | MDLQFVSLPPNSKTKAKMQAKACHNINNIPPCLLPAPKLPSTVAMSPSQLTLP SHVARAI  | 60  |
| ccd7mutant-Exon1 | MDLQFVSLPPNSKTKAKMQAKACHNINNIPPCLLPAPKLPST-----                | 42  |
|                  | *****                                                          |     |
| CCD7-Exon1       | TTITTSPTHEVMTPEIDDTVTAYWDYQFLFVSQRSEATEPVSLRWVEGSI PSDFPSGTYYL | 120 |
| ccd7mutant-Exon1 | -----VEGSI PSDFPSGTYYL                                         | 58  |
|                  | *****                                                          |     |
| CCD7-Exon1       | TGPGLFADHDGSTVHPLDGHGYLRTFEIDGSTGQVKFMARYIETEAQTEERDPVSGKWRF   | 180 |
| ccd7mutant-Exon1 | TGPGLFADHDGSTVHPLDGHGYLRTFEIDGSTGQVKFMARYIETEAQTEERDPVSGKWRF   | 118 |
|                  | *****                                                          |     |
| CCD7-Exon1       | THRGPF SVLKGGKMGNTKVMKNVANTSVLQWGGRLFCLWEGGDPYEIDSKTLNLTGKFE   | 240 |
| ccd7mutant-Exon1 | THRGPF SVLKGGKMGNTKVMKNVANTSVLQWGGRLFCLWEGGDPYEIDSKTLNLTGKFE   | 178 |
|                  | *****                                                          |     |
| CCD7-Exon1       | LIKNSDQVLEDKKISHSDFLDVAAQLLKPILY                               | 272 |
| ccd7mutant-Exon1 | LIKNSDQVLEDKKISHSDFLDVAAQLLKPILY                               | 210 |
|                  | *****                                                          |     |

**Figure 4.37.** Deletion of 186 nt between Thr42-Val105 in the SlCCD7-exon 1 region of the C1, C8, C9, C22-T1 progeny resulting in deletion of 62 amino acids (The region marked with red is the SlCCD7 gRNA1 region)

Simultaneously, periodic observations made by cultivating 23 T1 progeny under equal and appropriate greenhouse conditions, it was observed that phenotypic changes consistent with the transported mutation status occurred. Compared to plants with homozygous biallelic -18 nt deletions, it was determined that the internode distance was the shortest in those which have the homozygous biallelic -186 nt deletion. In contrast, the plants that have a heterozygous biallelic mutation (-18 nt + -186 nt) were medium length (Figure 4.38). Concerning this situation, plants with homozygous biallelic -186 nt deletion had the shortest length, while homozygous biallelic -18 nt deletions had the tallest and heterozygous ones had a medium-length (Figure 4.38).

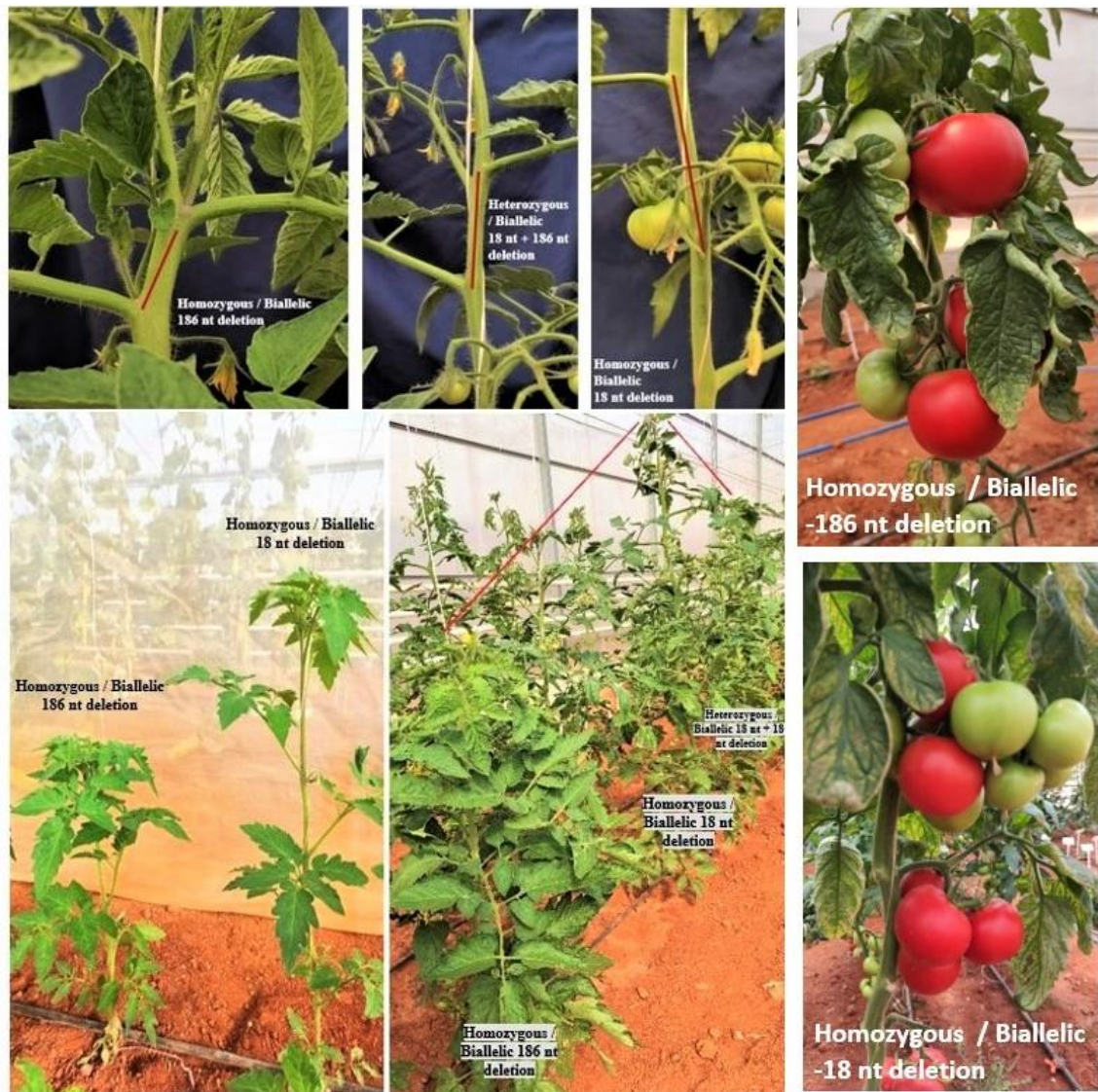


**Figure 4.38.** Comparise of C36-T1 mutant lines based on mutation types

Through the manipulations for genes involved in SL synthesis in tomato, mutant plants with similar morphological characteristics were obtained as a result of various studies in the literature:

The *Sl-ORT1* mutant was obtained by fast neutron (FN) mutagenesis in the M-82 tomato variety by Dor et al. (2010). However, this mutation provided resistance to 3 different *Orobanch* spp. species (*Phelipanche ramosa*, *Orobanch cernua*, and *Orobanch crenata*), the phenotypic changes were observed similar to the previously determined by Koltai et al. (2010). Due to the negative effect of strigolactone deficiency in the mutant plant on auxin (plant hormone) transportation, there was an increase in the number of shoots (shrub-like) and thus a change in shoot/root ratio compared to the WT plant (nonmutated).

The *SICC8* gene was silenced by the RNAi (RNA interference) method by Kohlen et al. (2012). Although a resistance success rate of 90% was achieved against *Phelipanche ramosa*, arbuscular mycorrhizal symbiosis, apical dormancy, and fruit yield were slightly affected in the mutants. In addition, an increased shoot number, adventitious root and node formation, a decrease in plant height (dwarfing), smaller flower and fruit size, less number and size of seeds per fruit were observed in mutants.

The *SICC8* gene was mutated in Micro-Tom tomato lines by Hasegawa et al. (2018) using the TILLING (targeting induced local lesions in genomes) method. The number of shoots increased due to the decrease in SL levels in the mutants. Since the mutation obtained in the study was found in Micro-Tom, which is a model cultivar and has a dwarf phenotype, the results were not examined in terms of plant breeding.

A mutation was generated in the *Slmax1* gene (responsible for SL synthesis just after *SICC7* and *SICC8* genes in the pathway) with the TILLING (targeting induced local lesions in genomes) method by Zhang et al. (2018). Compared to WT, an increase in nodes, shoot numbers, and lateral shoot length was observed in mutant lines. Total and mean lateral root lengths decreased in mutants, so there was a change in shoot/root ratio compared to nonmutant plants. In addition, due to the decrease in flower length and fruit size in mutants, problems arose in plant fertilization.

Different indels were generated in the second exon of the *SICC8* gene by using a single gRNA (5'-TTCATTCAGCTCATCCAGTGG-3') that designed by Kumar et al. (2019) within the scope of the CRISPR-Cas9 method. As a result of sequencing in T0 lines, 1 nt deletion in L1, 3 nt deletion in L2, and biallelic mutations in L5 and L11 (4 different mutations occurred: 6, 5, or 4 nt deletion, or 1 base insertion) were determined. T1 population was created by selfing of T0 lines, and it was observed that these mutations were inherited. Additional sequencing in T1 showed that L1 and L2 had a homozygous mutation (having the same mutation in both alleles). Although the resistance against *Phelipanche aegyptiaca* was obtained due to the decrease in the level of SL in the mutant lines (higher resistance was obtained in L1, L2, L5 compared to L11), morphological changes (such as an increase in carotenoid level, dwarfism, excessive shoot branching, and adventitious root formation) occurred in all mutants. However, the number of fruits per plant increased due to the increase in the number of shoots. On the other hand, a decrease in the fruit size was observed.

In another study conducted by Kumar et al. (2021), biallelic/heterozygous (WT + 9 nt deletion) and homozygous mutations (9 nt deletion) were generated in the third exon of the *Slmax1* gene with a single gRNA designed within the scope of the CRISPR-Cas9 method. As a result of the deletion of 9 nt, 3 amino acids (Lys252-Arg253-Iso254)

were deleted, and this mutation was inherited in advanced generations. Although T1 lines with *max1*-Cas9 heterozygous mutations also showed SL mutants' characteristics, these phenotypic changes were observed to be milder than homozygous plants. Although resistance was obtained against *Phelipanche aegyptiaca* due to the decrease in SL levels in mutant lines (homozygous mutants showed higher resistance compared to heterozygous mutants), morphological changes (increased carotenoid level, dwarfism, increased shoot branching, and axillary buds, excessive adventitious root formation) occurred in all mutants. In addition, the mutant plants had a significant difference in dry root weights compared to the nonmutated plant.

Unlike these SL mutants with unwanted agro-morphological changes in the literature (multi-headed, bush-like phenotype, deterioration in flower and fruit quality, etc.), C36-T1 lines did not show these SL-mutant phenotypes (except for partial dwarfism) even if there were significant "in-frame" biallelic homozygous deletion (-186 nt) in four lines. Also, as another advantage, "marker-free" plants were obtained due to the elimination of transgenes in two biallelic homozygous mutated C36-T1 lines (C9 and C22) segregation.

Pot testing was performed to determine the relationship between the occurring mutation status and *Orobanch*e resistance. It was aimed to compare the resistance between -186 nt homozygous biallelic deletion, -18 nt homozygous biallelic deletion, and -186 nt + -18 nt heterozygous biallelic deletion by testing the plants whose mutation types were determined in the generated C36-T2 population (Figure 4.39).

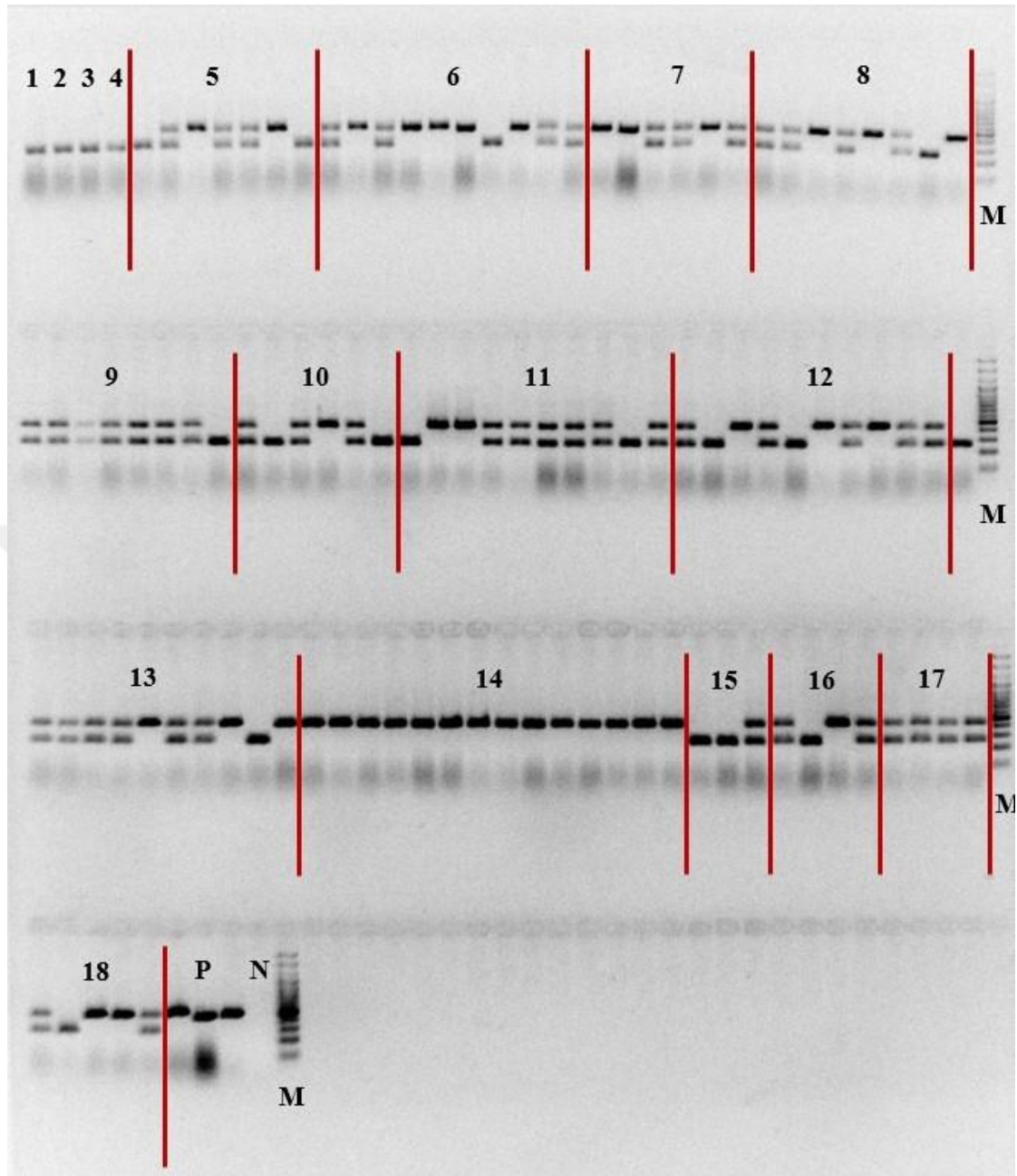


**Figure 4.39.** Image of the PCR amplification of C36-T2 plants using the *SlCCD7*-gRNA1 F/R primer (P: Commercial F1s (non-mutated “WT”) were used as positive controls, N: Negative control, 100 bp GeneRuler Ladder (Thermo Scientific), 100 V, after 1 hour). The segregations were derived from: 1-4: Bulk DNAs with homozygous biallelic -186 nt deletion (C1<sub>T1</sub>, C8<sub>T1</sub>, C9<sub>T1</sub> and C22<sub>T1</sub>, respectively), 5-13 + 15-18: mutant T1 lines with 186 nt + -18 nt heterozygous biallelic deletion, 14: mutant T1 lines with homozygous biallelic -18 nt deletion

According to Figure 4.39, mutations in the T2 generation were carried and inherited the same as the T1 generation. This thesis provided the first time in Turkey to obtain potential broomrape resistance with the CRISPR-Cas9 technique. Mutant lines with homozygous biallelic -186 nt deletion (between Thr42-Val105 in the *SlCCD7*-

exon 1 region and cause deletion of 62 amino acids) shows great promise in *Orobanche* resistance testing, which is still under investigation. There was no manipulation with CRISPR-Cas9 for the *SICCD7* gene in previous techniques, which is involved in the early stages of the strigolactone pathway. However, in genes such as *SICCD7*, *SICCD8*, and *Slmax1*, all mutants -with undesirable agro morphological properties for plant breeding- obtained by other methods (such as FN, TILLING, RNAi), also including CRISPR-Cas9. These features can be listed as multi-headedness due to shoot branching, bushiness, fertilization problems due to deterioration in flower/fruit characteristics, deterioration in shoot/root ratio, etc. For potentially *Orobanche* / *Phelipanche* spp.-resistant mutant lines obtained in this thesis, no undesirable agronomic traits were detected. Therefore, especially these "in-frame" mutants are of great importance in plant breeding and commercial terms. In addition, this mutation that provides broomrape resistance can be transferred to all desired tomato varieties with hybridization methods, and yield losses due to broomrape damage can be prevented. As a result, it is hoped that the economic, environmental, and health damages caused by the chemical control methods used against these parasitic weeds will be eliminated. The methods used within the scope of this thesis will pave the way for usage in other crops that have lost yield due to broomrape.

## 5. CONCLUSION

This study has presented a new approach in seeking a permanent solution against *Orobanche* spp. / *Phelipanche* spp., which is an important "weed" problem in tomato. The use of "CRISPR," one of the most up-to-date gene-editing technologies, as the primary method in the study has contributed to the literature in many ways. Thanks to the actual results obtained, this thesis study has the potential to be an original study. Although similar studies using CRISPR-Cas9 technology have been conducted in the usual period of this study, the results obtained show that different mutations occur in the *CCD7* and *CCD8* (*SICCD7* / *SICCD8*) gene, which play a role in the strigolactone pathway.

Although eight transgenic plants with mutations in the *SICCD7* and/or *SICCD8* genes were obtained from the studies, possible phenotypic changes caused by the mutation could be observed more quickly because the C36 coded mutant was obtained from an indeterminate type tomato explant compared to other Micro-Tom (dwarf type) based mutants. At the same time, a -relatively- significant "in-frame" mutation in the C36 mutant line made this thesis an original study. Even though the *Orobanche* (broomrapes) resistance of the C36 plant is still in the testing phase, the present results are of the nature to shed light on many studies on this subject and may offer suggestions for new hypothesis. It is hoped that the C36 will be a valuable plant material for tomato breeding.

The results obtained from this thesis are as follows:

In this thesis, it is the first time that *SICCD7* and *SICCD8* genes are mutated simultaneously. In order to increase the success of the study, two gRNAs from different exon regions of both genes were designed and simultaneously transformed into tomato via the *Agrobacterium*-mediated method (using EHA105 strain). In this way, different events in *SICCD7*, *SICCD8*, and *SICCD7* + *SICCD8* genes were mutated were obtained.

Two different protocols are used for *Agrobacterium*-mediated plant transformation in tomato. Protocol A (Van Eck 2009 protocol) was used for the Micro-Tom genotype, and Protocol B (a modified protocol) was used for the 267-F1 (indeterminate type) genotype. Protocol A requires relatively more minor chemicals, and the Micro-Tom genotype shows regeneration in a shorter time to determine the effectiveness of the CRISPR-Cas9 components explicitly designed for this thesis study in the short term and in a more straightforward way. However, the C36 mutant plant, - an important place in this study- was obtained from Protocol B.

- Effective selection was achieved by using phosphinothricin at a concentration of 2 mg/L.
- Although the different gRNAs targeting different exon regions of *SICCD7* and *SICCD8* genes were tested to mutate the target genes, the success of both gRNAs to function at the same time remained low. This situation gave rise to the thought that gRNAs may compete with different success chances within the plant genome. However, it is possible to transfer two different transformation vectors to the plant in practice.

- Inheritance of the *slccd7*- mutation was shown only in the C36 mutant. In this context, the T0 generation in which the mutation was obtained was advanced until the T2 generation. The resistance test was carried out with the plants of this generation with *Orobanch* (with "*Orobanch ramosa*," one of the most common *Orobanch* species in tomato).
- The T1 generation was obtained by selfing the C36 mutant, which was determined to have a heterozygous biallelic mutation (18 nt + 186 nt deletions) in the *SLCCD7* gene involved in the strigolactone pathway. In the T1 generation, four T1 lines (C1, C8, C9 ve C22) have a homozygous biallelic mutation (186 nt deletion) were determined due to the segregation of this mutation.
- As a result of the deletion of 186 nt with this homozygous biallelic mutation in the exon one region of the *SLCCD7* gene, a large protein fragment corresponding to 62 amino acids (the region between Thr42-Val105) was deleted. However, this deletion did not create a "frame-shift" (because it was multiple 3). Therefore it was described as an "in-frame" mutation. Due to this situation, various molecular changes could occur, including the change in protein-DNA binding sites, as the secondary and tertiary structure of the protein could be changed.
- It is aimed to obtain resistance against *Orobanch* / *Phelipanche* spp. in tomato utilizing this in-frame mutation. Moreover, apart from the partial dwarfing of the internodes in the potential *Orobanch* / *Phelipanche* spp.-resistant mutant lines, the agronomic characteristics observed in SL-mutant lines in other studies in the literature and undesirable in terms of plant breeding (shrub-like plant structure associated with the increase in the number of shoots, deterioration in flower/fruit characteristics, fertilization problems, disruption in shoot/root ratio) were not detected. Therefore, this mutant obtained may have a great commercial importance.
- Some of these "in-frame mutant lines" (C9-T0 and C22-T0) were determined to be "marker-free" plants (free of antibiotic resistance and Cas9 transgenes).
- This mutation, which provides possible broomrape resistance in C1, C8, C9, and C22 lines, can be transferred to all desired tomato varieties by the classical backcrossing method. It would be possible to eliminate the economic, environmental, and health damages caused by the chemical control methods used against these parasitic weeds as an additional advantage. In addition, the methods within the scope of this thesis study have the potential to be used in other crop plants that have lost yield due to broomrapes.

Recommendations to help similar studies that can be done in the future can be listed as follows:

- It would be sufficient to use a transformation vector to have a single gRNA during plant transformation if it does not have an essential purpose.
- Different genotypes and *Agrobacterium* strains could be included in the study to increase transformation efficiency.

- If possible, it would be essential to work with indeterminate-type tomato genotypes to make better observations of the usual morphological changes that occur due to silencing the gene / genes responsible for strigolactone synthesis.
- Care should be taken to ensure that the competitive cells are obtained from a clean source.
- The risk possibility of "regeneration of non-transformation plants" could be reduced by using 3 mg/L phosphinothricin for plant selection.



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## CURRICULUM VITAE

**CANSU BÜLBÜL**

### EDUCATION INFORMATION

|                           |                                                                                                                             |
|---------------------------|-----------------------------------------------------------------------------------------------------------------------------|
| Doctorate<br>2016-2021    | Akdeniz University<br>Graduate School of Natural and Applied Sciences,<br>Department of Biotechnology, Antalya              |
| Postgraduate<br>2014-2016 | Akdeniz University<br>Graduate School of Natural and Applied Sciences,<br>Department of Agricultural Biotechnology, Antalya |
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### PROFESSIONAL AND ADMINISTRATIVE DUTIES

|                              |                    |
|------------------------------|--------------------|
| Specialist<br>2020-Continued | Areo Seed, Antalya |
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### Publications

1- Pınar, H., Mutlu, N., Keles, D., Atilla, A. T. A., Buyukalaca, S., and Simsek, C. (2016). Inheritance of Soil Zinc Deficiency Symptoms in Pepper. *Journal of New Results in Science*, 5(11): 48-55.

2- İkten, H., Şimşek, C., Yirmibeş, B., Bak, T., Şenyurt, M., and Karadeniz, T. (2020). Analysis of Genetic Diversity Among The Kiwifruit (*Actinidia* Spp.) Genotypes Using Molecular Markers. *Bahçe*, 49(1): 51-57.

3- Kantar, F., Yemşen, S. N., Bülbül, C., YILLMAZ, N., and Mutlu, N. (2021). Phenotypic and iPBS-retrotransposon Marker Diversity in Okra (*Abelmoschus esculentus* (L.) Moench) Germplasm. *Biotech Studies*, 30(1): 7-15.

4- Bülbül, C., Sever Mutlu, S., Mutlu, N. and Gülşen, O. (2021). Evaluation of Bermudagrass [*Cynodon* (L.) Rich] Accessions with Different Ploidy Levels. *Turkish Journal of Botany*, 45(4): 315-327. DOI: 10.3906/bot-2009-31

#### **Papers presented at national scientific meetings and published in proceedings**

1- İkten, H., Karadeniz, T., Şimşek, C., Yılmaz, Y., Bak, T. and Şenyurt, M., Determination Of Genetic Diversity Among the Selected ‘Piraziz’ Apple Clones Using SSR, SRAP and RAPD Markers. 2nd International Congress on Applied Biological Sciences, International University of Sarajevo, May 27-31, 2016. Oral presentation

2- İkten, H., Karadeniz, T., Şimşek, C., Yılmaz, Y., Bak, T., Şenyurt, M., ‘Piraziz’ Elma Klonları Arasındaki Genetik Farklılığın SSR, SRAP ve RAPD Belirteçleri Kullanılarak Belirlenmesi. 5. Ulusal Moleküler Biyoloji ve Biyoteknoloji Kongresi/Konya/18-21.07.2016, ss. 32-32.

3- İkten, H., Karadeniz, T., Yılmaz, Y., Bak, T., Şimşek, C. Determination of Genetic Diversity Among The ‘Patlıcan Fig’ (*Ficus carica* L.) Clones Using SSR and SRAP Markers. 27th International Scientific Expert Congress of Agriculture and Food Industry/Bursa/26-28.09.2016, Ziraat Fakültesi Dergisi, Uludağ Üniversitesi 2016 Vol.30 No.Special Issue pp.119-119.

4- Bülbül, C., Mutlu, N., Sever Mutlu, S., Gülşen, O. Associations Between Ploidy Level And Genetic Diversity Of Bermudagrass As Assessed By IPBS Retrotransposon Marker. 5th ETS Conference 2016/June 5-8th 2016/Portugal, pp. 9.

5- Şimşek, C., Mutlu, N., Sever Mutlu, S., Irkörüçü, D. SSR Markers Determine Hybrid Half-sib Bermudagrass Progenies Developed Through Inter- and Intraspecific Hybridizations. 3. International Plant Breeding Congress/K.K.T.C./Kyrenia/15-19.10.2017, pp. 32.

6- İkten, H., Karadeniz, T., Şimşek, C., Bak, T., Şenyurt, M., Yılmaz, Y. Evaluation of Genetic Diversity of Local Growing *Prunus laurocerasus* L. Genotypes Using SRAP, ISSR and iPBS Retrotransposons Primers. 3. International Plant Breeding Congress/K.K.T.C./Kyrenia/15-19.10.2017, pp. 40.

7- Şimşek, C., Mutlu, N., Sever Mutlu, S., Gülşen, O. Associations Between Ploidy Level And Genetic Diversity Of Bermudagrass As Assessed By IPBS Retrotransposon Marker. International Agricultural Science Congress /May 9-12th 2018/Van, pp. 33, Poster Presentation.

8- Ari E., Bedir H., Şimşek C., Mutlu N., Kula N. Doubled Haploid Plant Regeneration from Microspore Cultures of Brassica napus L. Grown in Greenhouse Conditions in Antalya (International Agricultural, Biological & Life Science Conference / Edirne, 2-5 September 2018, pp.530-530.

9- Songül SEVER MUTLU, Nedim MUTLU, Alparslan KARABENİZ, Sevde Nur YEMŞEN, Bahar SANCAR, Cansu ŞİMŞEK, Ahmet GÜLGÜN Hybrid Turf Type Zoysiagrass Breeding. Uluslararası Katılımlı Türkiye 6. Tohumculuk Kongresi / Niğde, 10-13 Eylül 2018.

