

**T.C.
AKDENİZ ÜNİVERSİTESİ**



EGGPLANT GENOME EDITING USING CRISPR Cas9 TECHNOLOGY

Vese PAKASHTICA

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOTECHNOLOGY

DEPARTMENT

DOCTORAL THESIS

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This thesis was accepted by the jury on 23/02/2023 with unanimity / ~~majority~~ vote.

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ABSTRACT

EGGPLANT GENOME EDITING USING CRISPR Cas9 TECHNOLOGY

Vese PAKASHTICA

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Eggplant (*Solanum melongena* L.) is one of the most important vegetables in many countries. It has a long growing season, which makes it one of the vegetables more exposed to pests, disease attack, environmental stress and parasitic weeds.

Orobanche spp, also known as broomrape, is one of the parasitic weeds that can cause great damage to eggplant yields. It is known that in order to germinate, the seeds of these parasites must be exposed to certain chemicals called strigolactones [SLs], which are released by the roots of the host.

The CCD8 gene is involved in the synthesis of strigolactones and plays an important role in controlling growth and development. CRISPR-Cas9 was used to manipulate the *SmCCD8* gene in eggplant (*Solanum melongena* L).

The objective of the study was to create eggplants resistant to broomrape by knocking out or modifying the *SmCCD8* gene. Yamula eggplant cultivar was used for transformation.

Using *Agrobacterium*-mediated plant transformation method, gRNA and Cas9 were transferred to eggplant. Eggplant cotyledons were used as the plant material for *Agrobacterium* transformation. Successful editing of eggplant lines was confirmed by Sanger sequencing. The induced mutations were stably inherited in T1 and T2 progenies. Our results based on CRISPR-Cas9 technology open the way to the development of eggplant genotypes resistant to parasitic weeds.

KEYWORDS: *Agrobacterium tumefaciens*-mediated, CCD8, CRISPR-Cas9 technology, Eggplant, *Orobanche* spp.

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ÖZET

CRISPR Cas9 TEKNOLOJİSİNİ KULLANARAK PATLİCAN GENOMUNUN DÜZENLENMESİ

Vese PAKASHTICA

Doktora Tezi, Biyoteknoloji Anabilim Dalı

Danışman: Prof. Dr. Nedim MUTLU

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Patlıcan (*Solanum melongena* L.) birçok ülkede en önemli sebzelerden biridir. Uzun bir büyüme mevsimi vardır, bu da onu zararlılara, hastalık saldırılarına, çevresel strese ve parazit yabancı otlara daha fazla maruz kalan sebzelerden biri yapmaktadır.

Canavar otu olarak da bilinen *Orobanch* spp, patlıcan verim ve kalitesine büyük zarar verebilen parazit yabancı otlardan biridir. Bu parazitlerin tohumlarının çimlenebilmesi için konakçının köklerinden salgılanan strigolakton [SL'ler] adı verilen bazı kimyasallara maruz kalması gerektiği bilinmektedir.

CCD8 geni, strigolaktonların sentezinde yer alan genlerden biridir büyüme ve gelişmenin kontrolünde önemli bir rol oynar. CRISPR-Cas9, patlıcandaki (*Solanum melongena* L) *SmCCD8* genini manipüle etmek için kullanılmıştır.

Çalışmanın amacı canavar otuna dayanıklı patlıcan hatları geliştirmektir. Bitki materyali olarak standart Yamula çeşidi kullanılmıştır.

Agrobacterium aracılı bitki transformasyon yöntemi kullanılarak gRNA ve Cas9 patlıcana aktarıldı. Materyal olarak patlıcan kotiledonları kullanılmıştır. Patlıcan CCD8 genine ait hedef bölgenin başarılı bir şekilde düzenlendiği Sanger dizileme ile doğrulanmış. İndüklenen mutasyonlar, T1 ve T2 generasyonunda stabil bir şekilde aktarılmıştır. CRISPR-Cas9 teknolojisine genetik dayalı sonuçlarımız, parazitik yabancı otlara dayanıklı patlıcan çeşitlerinin geliştirilmesinin yolunu açmıştır.

ANAHTAR KELİMELER: *Agrobacterium tumefaciens*-aracılı gen aktarımı, CCD8, CRISPR-Cas9 yöntemi, Patlıcan, *Orobanch* spp.

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PREFACE

Any project's success, aside from me, is greatly influenced by the support and recommendations of many people. Prior to anything else, I want to thank my advisor, Prof. Dr. Nedim MUTLU, for all the support, guidance, understanding, advices that gave me throughout my graduate studies. I can't say whether thanking him is enough for his tremendous support and help. I consider myself to be extremely privileged to have been his student.

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CURRICURUM VITA	

DECLARATION

I state that this study named "EGGPLANT GENOME EDITING USING CRISPR Cas9 TECHNOLOGY", 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.

23/02/2023

Vese PAKASHTICA

İmzası



SYMBOLS AND ABBREVIATIONS

Symbols

g	: Gram
mg	: Miligram
mL	: Mililitre
mM	: Milimolar
ng	: Nanogram
μl	: Microlitre
V	: Volt

Abbreviations

bp	: Base Pair
Cas9	: CRISPR Associated Protein 9
DNA	: Deoxyribonucleic Acid
OD	: Optical Density
PCR	: Polymerase Chain Reaction
RNA	: Ribonucleic Acid
UV	: Ultraviolet

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

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1. INTRODUCTION

1.1. Background

Eggplant (*Solanum melongena* L.), which has its origin in India, is one of the main vegetables grown worldwide. Eggplant is cultivated in open fields and greenhouses in many regions of Asia, Africa, and the subtropics (including the southwest of the United States and the Mediterranean region) (Mutlu and et al., 2008). After tomato, pepper, potato, and tobacco, data from 2014 showed that the production of eggplant is close to 50 million tons annually, placing it in fifth rank as one of the most commercially significant *Solanaceous* crops (FAO, 2014). Producing 28.4 million tons, that means 57% of world's total, it brings China in first place as producer, followed by India with 13.4 millions tons, in third place comes Egypt with 1.2 million tons), then Turkey with 0.82 million tons, and Iran 0.75 million tons (Anne Frary and et al., 2007a). It was first brought to the Mediterranean, where is the highest production of eggplant.

It is an excellent source of vitamins, proteins, and minerals including iron (0.9 mg), calcium (18 mg), sulphur (44 mg), and potassium (Noda and et al., 1998; Sharma and et al., 2020). Expect being rich in minerals and vitamins, it contains also phytonutrients with an antioxidant role. The production of eggplant varieties is however, significantly decreased as a result of pest, disease, environmental stress, and parasitic weeds.

Orobancha ramosa L. and *Orobancha aegyptiaca*, more often known as broomrape is a parasitic weed that seriously damages and reduces the production of a variety of crops, including tomato (*Lycopersicon esculentum* Mill.) and eggplant (*Solanum melongena* L.). In order for the seeds of these parasites to be able to germinate, it is known that the roots of the host must secrete a chemical known as strigolactones (SLs). Strigolactones are phytohormones with various signaling functions. Strigolactones are produced by a mechanism that includes the D27 which is iron-binding protein, the carotenoid cleavage dioxygenases 7 and 8 (CCD7 and CCD8). D27 is an enzyme that changes all- β -trans-carotene into 9-cis-carotene, which is then broken down by CCD7 into a 9-cis-configured aldehyde (Alder and et al., 2012). When CCD8 links carotenoids with strigolactones and performs chemical rearrangement on 9-cis-apo-10'-carotenal, it creates carlactone, a substance having strigolactone-like biological properties. Understanding the structure of carlactone is essential for comprehending strigolactone biology and may help in the fight against parasitic weeds.

The CCD8 gene is absolutely necessary for the generation of strigolactones as well as the control of both growth and development. Using CRISPR-Cas9, CCD8 gene in eggplant (*Solanum melongena* L.) can be modified.

Trap crops, soil solarization, rotations, the development of resistant cultivars, and chemical approaches such as fumigation with methyl bromide, dazomet, and metham-sodium have all been investigated as potential control strategies. These chemical techniques directly kill the seeds in the soil (Vouzounis and Americanos, 1998). *Orobancha* spp., requires farmers to apply heavy dosages of herbicides. However, it is difficult to find "traditional" genetic sources of weed resistance. This makes it difficult to create excellent cultivars that are weed-resistant, and we believe

that using CRISPR is a viable option for developing new *Orobancha* spp. resistant eggplant varieties.

Due to the growing population, water resources depletion, climate change, and other difficult conditions, agricultural developments must produce more products with less storage space, preserve the ecosystem, and adhere to local or international laws for the production of genetically modified organisms (Kanchiswamy, 2016). Due to these challenges, using genome engineering or gene editing has emerged as a means of assisting species in their adaptation to climate change or reducing the negative effects of climate change on agriculture. Plant development with improved qualities is the primary cause of the current agricultural and other sectors' dependency on plant resources. However, these conventional breeding techniques require a lot of time and effort. The process of genome editing produces DNA alterations at specific genomic regions. It has become clear that CRISPR-associated protein 9 (CRISPR/Cas9) Clustered regularly interspaced short palindromic repeats is a new method for editing the genome that has been widely used in many organisms, including plants.

Since Danna and Nathans (1971) first disclosed the use of restriction enzymes to produce particular DNA fragments, researchers have been looking for bacterial compounds that may be used for gene editing. CRISPR technique, which was adapted from the bacterial immune system (Bolotin and et al., 2005; Mojica and et al., 2009), has revolutionized molecular biology and allowed for the precise and targeted mutation of genomes (Jinek and et al., 2012; Wiedenheft and et al., 2012). Since then, a variety of species, including bacteria, fungi, plants, and animals, have undergone site-targeted alterations thanks to the employment of the enzymes of several CRISPR systems, such as Cas9 and Cas12/Cpf1. Plants transformed with a construct containing the Cas9 gene and guide RNA expression cassette typically result in site-specific mutations.

Chromosomal DNA is cut at the target spot by cells of the transformed plants that express all CRISPR/Cas9 system components (s). After that, non-homologous end joining (NHEJ) is typically used to repair the cleaved DNA, which results in altered target-site sequences.

The effectiveness of CRISPR/Cas9 genome editing was immediately demonstrated in plants (Feng and et al., 2013). Plants often acquire site-targeted mutations through stable insertion of a Cas9 expression construct into the genome of the plant. Based on the characteristics of the constructions, the origin and type of the promoters and terminators that are utilized to express the Cas9 gene and the guide RNA, as well as the CAS9 nuclease sequence, the efficiency of introducing mutations in genes of interest can vary.

1.2. Aim and Objectives

This research was conducted with the intention of developing an effective approach for altering the genome of eggplant using CRISPR/Cas9 in order to produce a new line of eggplant that is resistant to the parasitic weed *P. aegyptiaca*.

In order to accomplish this purpose, the following objectives have been established.

- i. Employing CRISPR/Cas genome editing technology to modify the CCD8 gene in eggplant to create genetically engineered plants.
- ii. To deliver the CRISPR/Cas9 construct into the tissue of eggplant.
- iii. To figure out whether or not effective editing has taken place in the target tissue.
- iv. To confirm the resistance of eggplant against the parasitic weed *P. aegyptiaca*.



2. LITERATURE REVIEW

2.1. Origin and Distribution of Eggplant (*Solanum melongena* L.)

The family *Solanaceae*, which includes approximately 3,000 species that are evenly spread across 90 genera, includes eggplant, also known as aubergine or brinjal (Vorontsova and Knapp, 2012). It is referred to as brinjal in South Asia, Southeast Asia, and South Africa. In British English, "aubergine" is used instead of the common name "eggplant," which is used in North American and Australian English. Melongene, a garden egg, and guinea squash are some of its other common names.

Additionally, it has been suggested that eggplant is a native of Southeast Asia and is grown as the most widely in some Mediterranean nations (Concellón and et al., 2012). Due to the massive collection and wide variety revealed by Weese and Bohs (2010) India is the primary center of origin. Eggplant has been determined that *S. incanum* L., a complex of many wild species found in East Africa and the Middle East, is the closest taxon to *S. melongena* (Daunay et al. 2001a). According to (Lester and Hasan 1991) *S. incanum* L. is likely a primordial cultivar of eggplant that spontaneously returned to the wild after escaping from cultivated fields. Several DNA chloroplast studies have revealed a far too close relationship between *S. melongena* and *S. incanum*. Isozymes (Isshiki and et al., 1994; Karihaloo and Gottlieb, 1995; Isshiki, 1996) and nuclear DNA (Karihaloo and et al., 1995; Mace and et al., 1999) show that *S. melongena* *germplasm* has low polymorphism and support the close connection with *S. incanum*, which exhibits a significantly greater degree of genetic variability.

In essence, as per Lester and Hasan (1991), eggplant is an Old World crop as opposed to other *Solanaceae* family members (Sandra, 2013), and its ancestor *Solanum incanum* had been pre-dominant in the subtropical species is a native of West Asia and North Africa that is used as the source of drought resistance and variation for phenolics content in eggplant breeding programs. The *Solanaceae* is an important family of the order *Solanales* in the division *Magnoliophyta* (Bremer and et al., 2002). It includes 2450 species distributed in 90 genera with variations in morphology, ecology, and habit (Mabberley, 2008). In terms of vegetable crops and agricultural utility, *Solanaceae* family comes as third important economically taxon plant (Mueller et al. 2005).

However, *Solanum* L. remains as the important one with 1500 species (Frodin, 2004), and can be divided into 13 clades, one of them is *Leptostemonum* clade where eggplant belongs too (Sandra, 2013).

The subgenus *Leptostemonum* has 450 species (Sandra, 2013), divided in three main groups and distributed worldwide: New World, Old World, and Australia. Eggplant species that are cultivated with origin are from Old World (Figure 1).

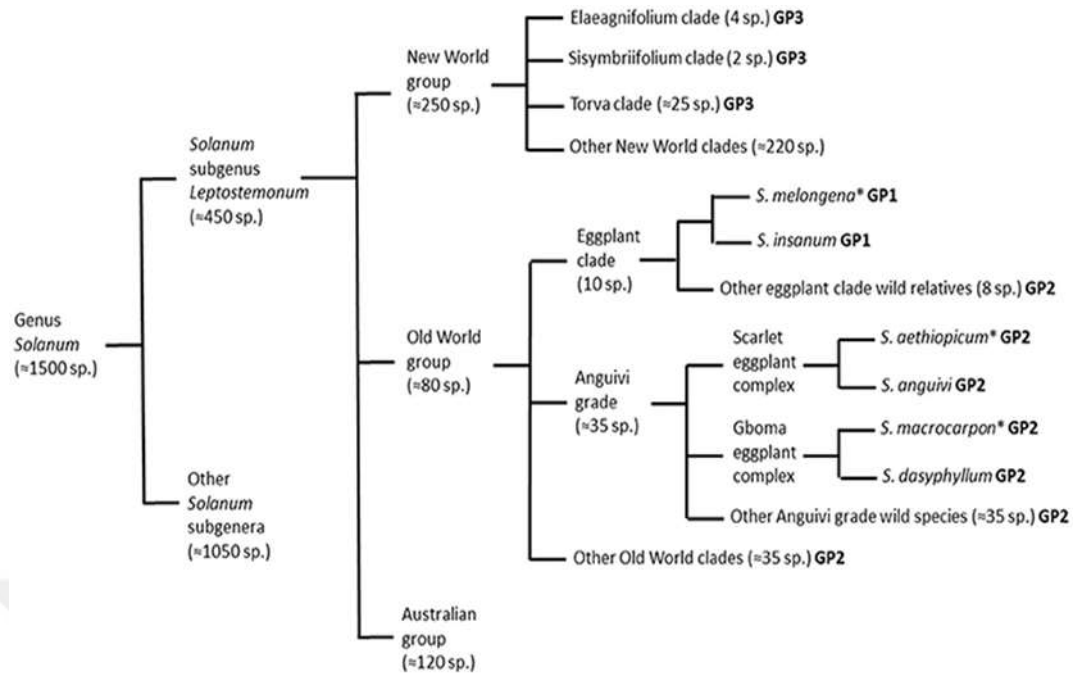


Figure 2.1. Taxonomic connections among the farmed eggplant (*Solanum melongena*), other cultivated (*S. aethiopicum* and *S. macrocarpon*), and wild species from the genus *Solanum* (Adapted from Taher and et al. (2017))

Along with the well-known and widely grown brinjal eggplant (*Solanum melongena* L.), the other two most popularly grown eggplant species are the gboma eggplant (*Solanum macrocarpon* L.) and scarlet eggplant (*Solanum aethiopicum* L.) (Taher and et al., 2017).

An annual production of 55,197,878 tonnes of eggplant occurs worldwide. China produces 35,590,700 tonnes of eggplant annually, making it the greatest producer in the world. In China alone, more than 60% of the world's eggplant is produced. With a yearly production of 12,680,000 tonnes, India ranks in second. Egypt is the third-largest producer of eggplant with an annual production of 1,180,240 tonnes. Turkey is rated 4th with 822,659 tonnes of production annually.

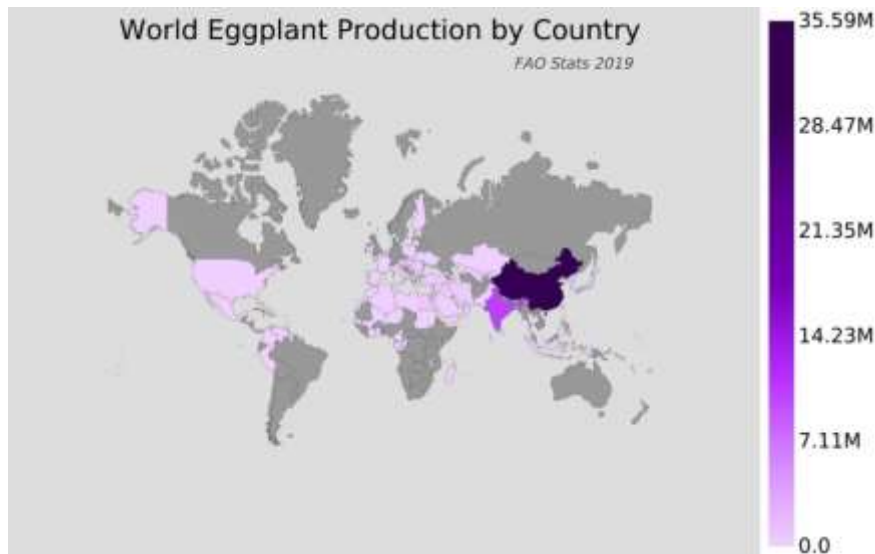


Figure 2.2. World eggplant production by country (Adapted from Anonymous1)

Botany

A dicotyledon plant, eggplant is a member of the *Solanum* genus and the *Solanaceae* family with chromosome number $2n = 24$ and a genomic size of roughly 956 Mbp (Leitch and Bennett, 2004). They are a seasonal perennial vegetable that is often grown as an annual. Eggplants, also referred to as aubergines, vary mostly in the size, shape, and color of the fruit. The huge, oblong, "Black Beauty"-type eggplant is a well-known vegetable in supermarkets and backyard gardens. However, relatives of the wild and semi-domesticated eggplant typically have small, rounded, yellow fruits, and the plants are extremely prickly (Weese and Bohs, 2010). Tropical and subtropical plants, eggplants require quite high temperatures. It can reach heights of 40 to 150 cm (16 to 59 in). Sometimes spiny, the stem.

Fruit colors range from dark purple to red, pink, yellowish, or white and might occasionally have stripes. A wide range of fruit colors are influenced by the presence or absence as well as the distribution pattern of two types of pigments, chlorophylls and anthocyanins (Daunay and et al., 2004). The leaves are described as large, ovate, and slightly lobed. The typical suspended violet blossom is solitary and is 5 cm (2 inches) across. The most common flower form is 5-merous (5 sepals, 5 petals, and 5 stamens), however 6-, 7-, and 8-merous blooms can also be found in types of fruit that are globose and circular (Anne Frary and et al., 2007a). The purple, star-shaped eggplant flowers typically appear on opposite leaves as either solitary blooms or in groups of two or more. It is self-pollinating, but bees, ants, and other insects can help with some cross-pollination. It is believed that flowering is day-neutral and not dependent on a precise number of daylight hours. In full sun,

eggplant grows at its best. It favors soils with a pH range of 5.5 to 6.8 that are fertile, well-drained, sandy, and loamy. It is propagated via seeds, which germinate in 8–12 days. The fruits can be picked in between 105 and 133 days. Between 20°C to 30 °C is the ideal growing temperature during the day. Over 35 °C, eggplant stops producing fruit and may also shed blossoms or abort immature fruit. When temperatures drop below 15°C, fruit set is also decreased.

Food Value

The fruit is advantageous to human health thanks to the presence of useful fiber as well as a variety of vitamins and minerals. Vitamins C, K, B6, thiamin, niacin, magnesium, phosphorus, copper, dietary fiber, folic acid, potassium, and manganese are all abundant in eggplants. Additionally, it contains nearly no saturated fat or cholesterol.

According to Noda and et al. (2000) and Whitaker and Stommel (2003), eggplant has the full complement of minerals, vitamins, nutritional fiber, protein, antioxidants, and phytochemicals with scavenging properties. According to Naeem and Ugur (2019) eggplants are a rich source of abundant nutrients and their contents (mentioned in Table 1).

In addition to having a richness of nutrients, brinjal has a number of medical purposes. For example, since it contains polyunsaturated fatty acids (linoleic and linolenic acid), it has a de-cholesterolizing effect that helps to treat diabetes, asthma, and liver issues (Santhosha and et al., 2017; Sharma and et al., 2020).

Table 2.1. Nutrients and their contents in eggplant (Adapted from Naeem and Ugur (2019)).

Components		Percentage of RDA			
		Minerals	mg	Vitamins	mg
Energy	(1%) 100 - 104 kJ	Ca	7.4 -9.0 mg (1%)	Vit. A	0.8 mg (1%)
Carbohydrates	(4%) 4.70- 5.88 g	Fe	0.20-0.24mg (2%)	Vit. B complex	18 - 22 mg (3 - 6 %)
Sugars	2.35-3.53 g	Mg	13.5 - 14 mg (4%)	Ascorbic Acid	1.8 - 2.2 mg (3%)
Dietary fiber	(9%) 2.80-3.40g	Mn	0.20-0.25mg(11%)	Vit. E	0.2 - 0.3 mg (2%)
Fat	0.18-0.20g	P	22.5 - 25 mg (3%)	Vitamin K	2.9 -3.5 mg (3%)
Proteins	(2%) 0.80 - 1.01 g	K	129 - 130 mg (5%)		
		Zn	0.10-0.16 mg (2%)		

Breeding Objectives

Consequently, one of the primary objectives is to modify cultivars so that they are able to thrive in a variety of environments, such as climates ranging from temperate to tropical, different growing seasons, and different production methods (open field or greenhouse), resistance to invasive species and infectious diseases is another important breeding goal (Anne Frary and et al., 2007a). Eggplants have been the subject of traditional breeding efforts, which have included the investigation of heterosis (Kakizaki, 1931) and the creation of F1 hybrids, which have since been adopted for cultivation on a worldwide scale. The range of pathogens that can be controlled by genetic resistance is limited, primarily to bacterial wilt caused by the (*Ralstonia solanacearum*), Fusarium wilt caused by the fungus (*Fusarium oxysporum f. sp. melongena*), and fruit anthracnosis by (*Colletotrichum gloeosporioides*), this is despite

the fact that there has been good progress made in the areas of quantitative and qualitative yield (A. Frary and et al., 2007b).

Interspecific hybridization has been used as a method to transfer disease resistance genes from wild relatives of eggplant into cultivated eggplant. These disease resistance genes originated in eggplant's wild relatives. For instance, *S. insanum* has been used as a source of Verticillium wilt resistance (Robinson and et al., 2001) and *S. aethiopicum* has been used as a source of bacterial wilt resistance (Ano et al. 1991). Additionally, *S. melongena* has acquired some resistance to *Verticillium dahliae* from (Acciarri and et al., 2001).

2.2. Role of Biotechnology on Eggplant Production

Latest developments and extensive molecular biology research have transformed the world of modern agriculture. The eggplant has transformed the way that plants are used in genetic engineering techniques because of its capacity to regenerate from transplanted tissues (Lynch, 1999; Saini and Kaushik, 2019). Plant genetic engineering, also known as plant genetic modification or manipulation, is the key to introducing crops with beneficial traits to produce plants that require fewer pesticides, fungicides, or fertilizers and can be more resilient to the effects of stress. Using plant genetic engineering techniques, the desired agronomic traits can be obtained by direct transfer of one or a small number of desired genes between closely or distantly related species. The use of traditional breeding to enhance eggplant is constrained by a number of obstacles, including linkage drag, pre- and post-fertilization crossover difficulties, and others (Saini and Kaushik, 2019). Transgenic technology may be employed for the genetic enhancement of eggplant to get beyond the mentioned problems of traditional breeding.

The eggplant is a plant that does well when subjected to cell, tissue, and organ culture (Kantharajah and Golegaonkar, 2004). *Agrobacterium* has been employed in every genetic transformation experiment on eggplant, with the exception of a few publications that used a biolistic system. In 1988, Guri and Sinks successfully transformed in vitro cultivated eggplant leaves with a pMON200 vector harboring the nptII gene, providing the first report on effective genetic transformation using *Agrobacterium tumefaciens* (Guri and Sink, 1988).

Numerous studies are being conducted to create a technique for *Agrobacterium* transformation on eggplant based on the selection of genetically modified explants and vectors, *Agrobacterium* cell density, duration of co-cultivation. For example, Habde's research showed that two- and three-week-old cotyledon and hypocotyl explants worked best when they were precultured for two days before being infected for one minute with an *Agrobacterium* suspension (OD₆₀₀ = 0.2-0.4) (Habde and et al., 2017).

There are only a few reports on the production of transgenic plants with tolerance to abiotic stresses, despite the fact that transgenic technology has significantly advanced in eggplant and that numerous studies have been conducted to optimize the transformation protocol for introducing foreign genes (Prabhavathi and et al., 2002). Prabhavathi conducted a study in this manner, using *Agrobacterium tumefaciens* to convert eggplant with the bacterial mannitol-1-phosphodehydrogenase (mtlD) gene. It

was observed that transgenic lines were resistant to osmotic stress brought on by salt, drought, and chilling stress (Prabhavathi and et al., 2002). In 2012, Sagare and Mohanty used the *Agrobacterium*-mediated transformation approach to convert eggplants with a binary vector pCambia2300 expressing the rd29A::DREB1A gene and produce transgenic plants that are resistant to moisture stress (Sagare and Mohanty, 2012). With the help of Kumar and et al. (2014), several experiments were done to find out how much salt the transgenic plants and the control plants could handle. The experiments used culture media in the lab and soil in greenhouses with different levels of salt, and they showed that eggplant could be made to handle more salt.

Additionally, genetic engineering was used to increase disease resistance in agricultural plants, such as eggplant. Bhat conducted a study in which he altered the hevein gene, which is responsible for fungus resistance. A binary plasmid pGPTV/hevei-carrying strain of *Agrobacterium tumefaciens* called LBA4404 into cotyledon explants revealed that the transformed brinjal plant was resistant to the fungus *F. oxysporum* (Bhat and et al., 2020). A study from Darwish found that transgenic eggplants free markers had increased resistance to *Alternaria solani* using the R/RS site-specific recombination system (Darwish and et al., 2014).

Using transgenic technology, certain beneficial agronomic features have also been introduced into eggplant. For instance, one study found that genetically modified eggplant plants could produce marketable fruits from both pollinated and unpollinated transgenic flowers under typical environmental circumstances, despite the fact that the untransformed line was completely incapable of producing fruit. This was accomplished by using the regulatory sequences of the DefH9 gene from *Antirrhinum majus* to regulate the expression of the coding area of the *iaaM* gene from *Pseudomonas syringae* pv. *savastanoi* (Rotino and et al., 1997).

Site-specific double-stranded DNA breaks may now be introduced thanks to recent advancements in the CRISPR/Cas9 system. The growth of mutant eggplant will undoubtedly be accelerated by such focused mutagenesis. In the near future, eggplant would become the primary model organism for studying genetics, physiology, development, and evolution thanks to the creation of a very effective genetic transformation system and gene expression investigations (Alam and Salimullah, 2021).

2.3. *Orobanch* spp. (Broomrapes)

2.3.1. Species, Distribution, Diversity and Biology of *Orobanch* spp.

In recent decades, parasitic plants have played a significant influence in several fields. They are regarded as research subjects and valued for their significance as agricultural pests. They used to adhere to the host plants, which served as their source of water and minerals. As a result, host crops begin to deteriorate, experience water stress, or perhaps perish completely.

In 17 plant families, parasitism exists, but the eight most significant species are those: *Scrophulariaceae*, *Orobanchaceae*, *Santalaceae*, *Balanophoraceae*, *Convolvulaceae*, *Lauraceae*, *Viscaceae*, and *Loranthaceae* are some of these.

Orobanche spp. known as Broomrapes are parasitic weeds that use to parasitic on legumes and vegetables. All are holo-parasites, they use to attach to roots (Parker and Riches, 1993).

Only five of the more than 100 species in the genus *Orobanche* are thought to be significant crop parasites (Parker and Riches, 1993). The main *Orobanche* species are: *Orobanche ramosa*, *Orobache aegyptiaca*, *Orobanche minor*, *Orobanche cernua*, and *Oroanche crenata*. *Phelipanche ramosa*, originally known as *Orobanche ramosa*, however, has an incredibly broad host range . It affects a wide range of Solanaceae plants, including eggplant, pepper, potato, tobacco and tomato, resulting in up to 75% crop production losses (Torres-Vera and et al., 2016).

Table 2.2. The main host crops of *Orobanche* species (Adapted from Parker and Riches (1993))

Family/crop	<i>O. ramosa</i>	<i>O. aegyptiaca</i>	<i>O. minor</i>	<i>O. cernua</i>	<i>O. crenata</i>
Alliaceae					
Onion	x	-	-	-	-
Cannabidaceae					
Hemp	xx	-	-	-	-
Compositae					
lettuce	xx	-	x	-	x
noog	-	-	x	-	-
safflower	-	-	x	-	x
sunflower	x	xx	-	x	x
Cruciferae					
cabbage	x	x	-	-	-
mustard	xx	xxx	-	-	-
rapeseed	xxx	x	-	-	-
Cucurbitaceae					
cucumber	x	xx	-	-	-
melon	x	xx	-	-	x
squash	x	xx	-	-	-
watermelon	x	xx	-	-	-
Leguminosae					
bird's foot trefoil	-	-	x	-	-
chickpea	x	x	-	-	xx
clovers	x	-	xx	-	-
groundnut	x	x	x	-	x
faba bean	x	x	x	-	xxx
lentil	x	x	-	-	xx
lucerne/alfalfa	-	-	xx	-	-
pea	x	x	-	-	x

(Continues on next page)

Continuation of Table 2.2

Solanaceae					
eggplant	xxx	xxx	-	xx	-
pepper	x	x	-	-	-
potato	x	x	-	-	-
tobacco	xxx	xxx	x	xxx	-
tomato	xxx	xxx	-	xx	x
Umbelliferae					
carrot	x	x	x	-	xx
celery	x	x	x	-	x
fennel	x	x	-	-	-
parsnip	x	x	-	-	-

xxx seriously attacked; xx moderately attacked; x lightly attacked; - attack doubtful.

The *Orobanch* seeds are about 0.3 x 0.2mm in size. There may be over 200,000 seeds in all on a single plant. For *O. ramosa*, the lifespan of seeds in the field has been reported to be 13 years. It is particularly difficult to regulate by rotation when there is minimal moisture and no crooking since longevity in the field might reach five years. Before seed will respond to a germinant stimulant, it needs to be condition for a period of 1-2 weeks. According to reports, the ideal temperature for germination is between 18 and 23 °C. Above 28°C, conditions are not favorable.

Germination of the main species typically takes 2-3 days at a favorable temperature. Whether *O. crenata* seeds are exposed to light during the conditioning phase or the germination phase, light inhibits germination (Hiron, 1973; Parker and Riches, 1993). In contrast to *O. ramosa*, *O. aegyptiaca* may not be inhibited by light (Ter Borg, 1986; Jain and et al., 1987; Parker and Riches, 1993) . A radicle up to 3–4 mm long emerges from the *Orobanch* seed during gemination. Although some authors have also seen cell division, the elongation of the radicle is assumed to be mostly the product of cell extension. Approaching the end of their life cycles, parasite shoot(s) only emerge above the soil to produce flowers and seeds (Fernández-Aparicio and et al., 2016; Qasem, 2021).



Figure 2.3. *P. aegyptiaca* in eggplant-growing regions in Korkuteli, Antalya

2.3.2. *Orobanche aegyptiaca*: distribution, host range, variation, and importance

This species, which is closely related to *O. ramosa*, has blooms that are virtually always longer than 20 mm, are typically more robust. It is safe to attribute *O. aegyptiaca* to plants that have both large flowers and thickly hairy anthers.

O. aegyptiaca has a smaller geographic distribution than *O. ramosa*. It primarily occurs in Southeast Europe, Northeastern Africa, and the Middle East, but it also affects parts of Afghanistan, Pakistan, India, and Nepal in the southernmost regions of the former USSR.

The *Cucurbitacea* family is *O. aegyptiaca*'s primary host group. Information on physiological variation in terms of host range is rare. The samples of *O. aegyptiaca* from winter carrots are more less successful on summer-grown *Solanaceae* than a sample from summer-grown tomato (Jacobsohn and et al., 1991).

O. aegyptiaca is a serious crop pest that is a prominent weed in Jordan, Iran, Arabia, Italy, and Afghanistan and is widespread in Turkey, Iraq, and Pakistan (Holm and et al., 1979). It is unquestionably seen as a serious issue in Israel, Lebanon, Jordan, Iran, Arabia, and all of Arabia.

Sticky papillae are created upon contact with the host root, perhaps for the purpose of adhering to the root surface. Instead of the host cells being destroyed or being penetrated intracellularly, the host root is penetrated by enzymatic action, causing the host cells to separate. The radicle stops functioning as the primary initial conducting way from the host to the parasite shoot shortly after the development of the haustorium. Instead, the haustorium gets bigger to make the tubercle, where a shoot bud finally grows, gets longer, and pops out after about a week or two to make the flowering shoot.

Since they lack chlorophyll and are holoparasites, *Orobanche* species must obtain all of their organic carbon from their hosts. This carbon is obtained from both the xylem and the phloem, primarily, it is thought, in the form of sucrose. They are also dependent on the host for water because they don't have any strong roots.

Orobanche is unable to photosynthesize, therefore it must rely on the host to increase its carbon resource. However, because it is often high in starch, it can survive and even develop after the host dies. After the host faba bean was cut down, Matthies (1989) demonstrated that the majority of *O. crenata* plants with emerging branches that were longer than 10 cm at the time were still able to grow slowly for an additional six weeks and produce some capsules.

As opposed to *Striga* species, the impacts of *Orobanche* species on their hosts are still not fully understood. Several investigations have revealed alterations in the host tissues' composition, including a decrease in K and P and an increase in Ca (Abou-Raya and et al., 1973; Parker and Riches, 1993). Stewart and Press (1990) think that the lack of carbohydrates caused by an *Orobanche* infection could make it hard for the host to get water from the soil when it is dry, even though there is no evidence yet that photosynthesis slows down because of an infection.

Through closed stomata and decreased availability of carbon dioxide, such moisture stress on the host might directly result in decreased photosynthesis.

2.3.3. *Orobanche* species control

Hand-pulling, which is the most accessible to small farmers, or supplementing other ways that have provided insufficient control, rotation out of the susceptible host crops, to sum up, is beneficial in terms of limiting fresh seed generation and allowing natural decline of the soil seed bank (Parker and Riches, 1993). It has been suggested that deep inversion plowing be used to manage *Orobanche*. This has frequently simply been based on observation, such as the fact that *O. cernua* seeds planted at a depth of 20 cm did not sprout much (Krishnamurthy and et al., 1987). Solarization is the process of raising the temperature of the sun's radiation to a point where seeds or other living things can no longer survive. It was initially noted as being effective for the control of fungal diseases in Israel (Katan and et al., 1976), and it was later demonstrated to also give excellent control of *O. aegyptiaca* in Israel (Jacobsohn and et al., 1980).

Another strategy to control *Orobanche* is chemical method. For instance, unlike typical herbicides, which can only harm plants when they have at least started to germinate, fumigants are highly volatile substances that have the ability to permeate and kill ungerminated seeds. Methyl bromide is the most effective treatment for localized *O. ramosa* infestations that has been utilized in the USA. Soil applied herbicides such as Imazethapyr have good selectivity against *O. crenata* in faba bean, lentil, chickpea, and pea, as well as against *O. cernua* in sunflower, according to the most recent data (Garcia-Torres and Lopez-Granados, 1991; Jacobsohn and Eldar, 1992). Maleic hydrazide (MH), among other post-emergence translocated herbicides, has successfully controlled *O. aegyptiaca* on tobacco, *O. ramosa* on melon, and *O. aegyptiaca* on other plants. Foy and et al. (1989) looked at these results and talked about them, but this pesticide is no longer recommended.

With more work, it might be possible to find or breed eggplant cultivars that are resistant to *Orobanche*, but it will probably take a long time to make these kinds that meet the other agronomic and quality needs of the market. It might be possible to find or breed eggplant cultivars that are resistant to *Orobanche*, but it will probably take a long time to make these kinds that meet the other agronomic and quality needs of the market. Additionally, genetic engineering may result in totally parasite-resistant crops. These should resolve the tough *Orobanche* problem in tomatoes. Until new types are available, fumigation, solarization, and flooding are the best general strategies for both crops.

2.3.4. Tolerant and Resistant Varieties

Eggplant: Seven of the 128 cultivars tested against *O. cernua* in the one investigation of eggplant varietal resistance Dalela and Mathur (1971a) were shown to be particularly resistant. These varieties include Running King, Verma's Giant, Pusa Purple Long x Manjri Gota, Pusa Purple Long x Nurki, D-12-2-66, DC-4-1-67, and E-147.

Cucurbits: The numerous research that have been written about *O. aegyptiaca* and melons, cucumbers, watermelons, pumpkins, and squash have been summarized by (Cubero, 1986, 1991; Parker and Riches, 1993). Although Kabulov conducted anatomical examinations of the roots of susceptible and resistant lines of cucumber, melon, and pumpkin and demonstrated that the differences were related to challenges in penetrating the host stele, few of these showed an evident resistance.

Brassica species: Dalela and Mathur (1971b) identified three types of rapeseed (*B. campestris*) and four varieties of mustard (*B. juncea*) among the 48 as being resistant to *O. aegyptiaca*.

Tomato: (I. Avdeev and Shcherbinin, 1976); I. Avdeev and Shcherbinin (1977); (Abdeev and Sherbinin, 1978) had more success in Astrakhan when they tested 206 lines over a five-year period and discovered that UZ-1 was almost immune to *O. aegyptiaca*. Further selection within UZ-1 led to the isolation of the homozygous line PZU-11, from which it was determined that a single dominant gene regulated resistance (Y. Avdeev, 1986), but this line's resistance hasn't been proven elsewhere.

Tomato varieties were evaluated for glyphosate resistance, but none were practical (Foy and et al., 1989). New genetically engineered varieties with glyphosate or herbicide tolerance may be able to control *Orobanche*.

Tobacco: Dalela and Mathur (1971a), who conducted their research in India using a total of 21 cultivars, discovered that just one of them, Vastakpal (Type G), had a moderate level of resistance. It is evident that there are some variations in the susceptibility of tobacco genotypes to species of *Orobanche*, but it does not appear likely that a significant amount of resistance will be discovered. The use of genetic engineering to confer resistance to herbicides is likely to become the primary focus of tobacco breeding efforts in the near future.

2.4. Strigolactone (SL), root parasitic plant species and their life cycle

Since their discovery 50 years ago as germination stimulants for root parasite plants, SLs have come to be understood as critical signaling molecules not just in the rhizosphere but also in plants.

Witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.) are two main parasitize host plant roots (Parker and Riches, 1993; D. Joel and et al., 2007; Parker, 2009). Because they retain their ability to produce chloroplasts, species of *Striga* are classified as hemiparasites. They are classified as obligatory parasites due to the fact that their photosynthesis cannot maintain their survival unless they are connected to the roots of their host (Yoneyama and et al., 2010). In addition to sorghum, maize, millet, and rice, species of *Striga* are among the most problematic weeds for essential agricultural crops.

The Chl-lacking holoparasites *Orobanche* and (*Phelipanche*) species infest economically significant dicotyledonous crops such tomato, tobacco, carrot, clover, cucumber, sunflower, and legumes (Yoneyama and et al., 2010). Up to 500,000 seeds can be produced by *Striga* or *Orobanche*, and these seeds can survive for several years in the soil. These seeds only begin to sprout after a pre-incubation period of moist conditions and appropriate temperatures (Phase I), and only germinate when they detect chemicals produced by the host plant, known as "germination stimulants," that have been released from plant roots (Phase II) (Figure 2.4.). This ensures that only seeds that are present in the host rhizosphere will sprout (D. Joel and et al., 2007; D. M. Joel and et al., 2017).

Before using up the resources in the tiny seed, parasite radicle grows in a few millimeters and reaches a host root. Phase (III) begins when the radicle makes contact with the host roots and the radicle then produces the haustorium, an organ that attaches to the root and enters its cortex and epidermis. In the end, it establishes connections with the host vascular system (Phase IV). The parasite uses this link to obtain water and nutrients from the host (Phase V). For several weeks (*Striga* spp.) to several months (*Orobanche* spp.) (Phase VI), the parasite tubercles grow underground before producing above-ground blooming shoots (Phase VII) (Yoneyama and et al., 2010).

In many plant species, stratification is necessary to break dormancy and enable seeds to germinate. However, following stratification, *Striga* spp., *Orobanche* spp., and *Phelipanche* spp. have different germination needs (Cardoso and et al., 2011). Furthermore, a germination stimulant is required for stratified root parasite seeds. Only a very low rate of spontaneous germinating (in water, without such a germination stimulant) may be identified in *Orobanche* and *Phelipanche* species in germination bioassays (1-3%), while spontaneous germination in *Striga* species is uncommon. Conditioned seeds will enter a state of secondary dormancy if they are not provided with a germination stimulant, this will cause the seeds to lose part of their sensitivity (Cardoso and et al., 2011). The survival of parasitic plant seeds in soils may last up to 14 years, and they probably go through many changes in dormancy.

As germination stimulants for root parasitic plants, three different types of chemicals have been identified: dihydroquinones, sesquiterpene lactones, and strigolactones (SLs) (Bouwmeester and et al., 2003; Yoneyama and et al., 2010).

Strigolactones are well-known as germination stimulants, host recognition signals for symbiotic arbuscular mycorrhizal fungi, and shoot branching inhibitors (Gomez-Roldan and et al., 2008; Umehara and et al., 2008; Xie and et al., 2010; Yoneyama and et al., 2010). Strigolactones, which are released into the rhizosphere by the roots of the host plants that make them, were initially discovered as germination stimulants of the parasitic *Striga*, *Orobanche*, and *Phelipanche* species.

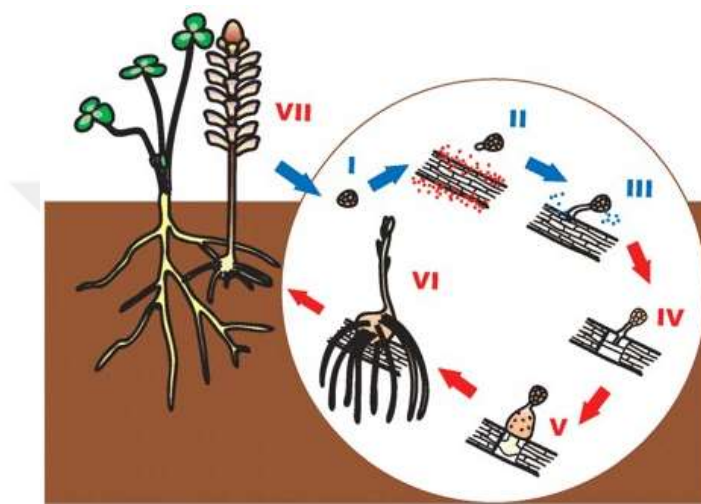


Figure 2.4. A root parasitic plant's life cycle, *Orobanche minor* (Adapted from Yoneyama and et al. (2010))

Strigol was the first SL identified from cotton root exudates as a germination stimulator for the root (Cook and et al., 1966). Later on, Siame et al. (1993) discovered that the root exudates of real hosts of *Striga*, maize, sorghum, and proso millet, contained strigol. Two strigol-related germination stimulants, sorgolactone and alectrol, were discovered from sorghum and cowpea roots. These chemicals that are related to strigol were given the term "strigolactones" by Butler (1995). It was made abundantly obvious by the isolation of orobanchol (Yokota and et al., 1998), the first *Orobanche* germination stimulant, that *Striga* and *Orobanche* spp. utilize SLs as germination signals (Yoneyama and et al., 2010).

Because effective and economically successful control strategies have not yet been established, these root parasite weeds are responsible for a significant amount of the harm that is incurred by agricultural production all over the world. These weeds are slowly but surely making their way into crop land in both developing countries.

Buildings and the tricyclic ring structure (ABC portion) of all known natural SLs is joined to a butenolide (D ring) by an enol ether bridge (Figure 2.5) (Yoneyama and et al., 2010). The C-D ring moiety is the necessary structure for demonstrating germination stimulation activity, according to extensive studies on the structure-activity

connections of SLs in the germination stimulation of parasitic plant seeds (Zwanenburg and et al., 2009).

This fundamental structure is present in all naturally occurring SLs, and it may have different substituents on the A ring or the B ring. These modifications have an impact on the stability of naturally occurring SLs in addition to the germination stimulating action.

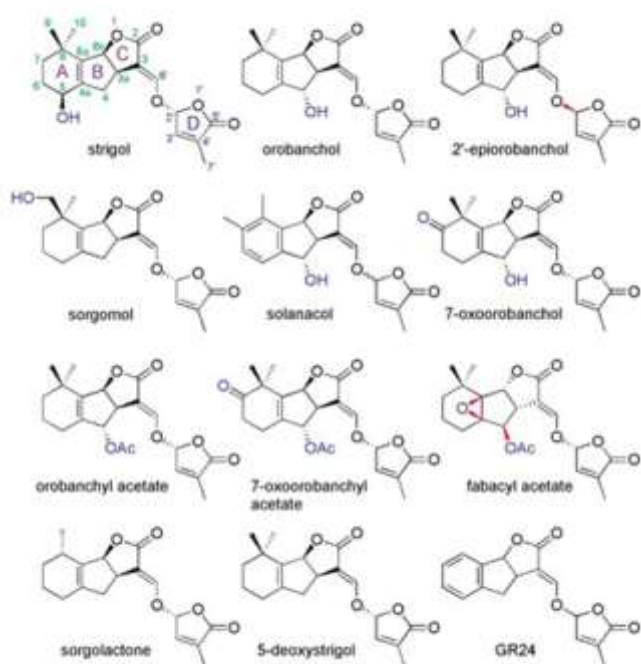


Figure 2.5. Structures of synthetically made and naturally occurring strigolactones (Adapted from Yoneyama and et al. (2010))

2.4.1. The stereochemistry and structures of naturally occurring SLs

Based on their structural characteristics, naturally occurring SLs (natural SLs) are divided into two classes: canonical (or classical) SLs are those that share structural similarities with strigol and have an enol-ether bridge connecting the ABC ring moiety, a core structure, to a butenolide (D ring) (Xie, 2016).

2.4.2. Strigolactones role

Strigolactones belong to a newly identified class of plant hormones and three distinct physiological mechanisms linked to strigolactones have been discovered: First, they encourage the growth of parasitic organisms that reside in the roots of the host plant, including *Striga lutea* and other members of the *Striga* genus. Second, strigolactones are essential for symbiotic fungi, particularly arbuscular mycorrhizal fungi, to recognize the plant because they form a mutualistic interaction with these plants and supply them with phosphate and other soil nutrients. Third, strigolactones have been discovered to operate as branching inhibitory hormones in plants. When

present, these substances suppress excessive bud growth in stem terminals, halting the branching mechanism in plants.

Recent research has increased strigolactones' importance in plant biology. As rhizosphere signaling molecules, strigolactones regulate shoot branching. These findings came from research with mutants of *Arabidopsis*, rice, and pea with poor axillary bud inhibition, giving them a bushy and sometimes dwarf appearance (Cardoso and et al., 2011).

According to the branching/tillering mutants' characterisation, carotenoids are predominantly broken down into strigolactones in the roots by the carotenoid cleavage dioxygenases CCD7 and CCD8 (MAX3 and MAX4 in *Arabidopsis*, RMS5 and RMS1 in pea, and D17/HTD1 and D10 in rice) (Figure 2.6) (Gomez-Roldan and et al., 2008; Umehara and et al., 2008; Cardoso and et al., 2011).

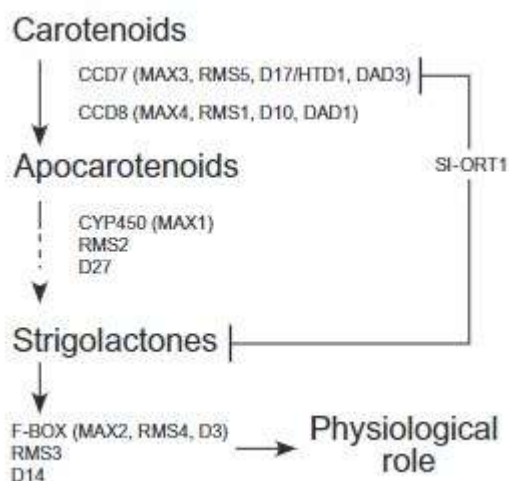


Figure 2.6. Model for the signaling and production of strigolactone. The diagram illustrates the genes (and related mutations in each species) that are now known to be involved in the strigolactone biosynthesis and signaling pathway, which ultimately results in a physiological response (Adapted from Cardoso and et al. (2011)).

Enzymes like MAX1, RMS2, and D27 that must have some kind of role in strigolactone production and perception/downstream signaling of strigolactone have been discovered (Fig. 2.3.). The genes MAX2, RMS4, D3 (encoding an F-BOX protein), RMS3, and D14 are implicated in downstream signaling.

Under phosphate deficiency, strigolactones are implicated in preventing shoot branching. According to W. Kohlen and Y. Goldwasser, strigolactones have been identified in the xylem sap and root exudates of *Arabidopsis*. When there is a shortage of phosphate, the amount of strigolactones in the xylem sap increases, which is correlated with a decrease in shoot branching. Strangely, phosphate deficiency also results in an increase in the amount of strigolactones in *Arabidopsis* root exudates (Cardoso and et al., 2011).

Recent findings indicate that strigolactones are involved in more plant physiological functions. GR24 improved tomato root elongation through cell elongation (Koltai, Dor, and et al., 2010). Higher GR24 concentrations caused asymmetric root development and root hair elongation inhibition. Additionally, MAX2 (the *Arabidopsis* max2 mutant is resistant to strigolactones and has a delayed senescence of the leaves) appears to be involved in light signaling pathways (Woo and et al., 2001; Shen and et al., 2007).

2.4.3 Strigolactone biosynthesis and signaling pathway

Until recently, it had been unclear how to fully understand the SL biosynthetic process. Before SLs were discovered as a unique family of plant hormones that control shoot branching, research utilizing carotenoid-deficient mutants and carotenoid biosynthetic inhibitors showed that SLs are biosynthesized via the carotenoid pathway. This was done before SLs were found to be responsible for the regulation of shoot branching (Matusova and et al., 2005). The study of recessive mutants with increased shoot branching, such as *Arabidopsis* showed more axillary growth (max), pea (*Pisum sativum*) ramosus (rms), petunia showed less apical dominance (dad), and rice dwarf/high-tillering dwarf (d/htd) growth, was extremely helpful in gaining a better understanding of SL biosynthesis. Mutants that are defective in CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7) and CCD8, respectively, in *Arabidopsis* max3 and max4, pea rms5 and rms1, rice d17 and d10, and other plants, were found to be SL-deficient mutants (Gomez-Roldan and et al., 2008; Umehara and et al., 2008). Using recombinant D27, CCD7, and CCD8 proteins from *Arabidopsis*, pea, and rice, Alder et al. (2012) performed an excellent biochemical analysis in vitro and demonstrated that sequential reactions by these three enzymes produce a non-canonical SL-like compound, carlactone (CL), all-trans- β -carotene via 9-cis- β -carotene and 9-cis- β -apo-10'-carotenal (Alder and et al., 2012) (Figure 2.7). Experiments revealed that CL is transformed into 4-deoxyorobanchol (4DO) and ORO, both of which are naturally occurring canonical SLs in rice, indicating that CL represents a step in the synthesis of SLs in living organisms (Seto and et al., 2014) (Figure 2.7).

A CYP711A subfamily of cytochrome P450 oxygenases transforms CL into both canonical and non-canonical SLs, and *Arabidopsis* was the first plant to be linked to the role of CYP711A in SL production. In *Arabidopsis*, CYP711A was initially connected to the production of SL. It was demonstrated that the max1 mutant of *Arabidopsis*, which lacked CYP711A1, showed the same hyper-branching phenotype as the max3 and max4 plants (Stirnberg and et al., 2002). Recombinant MAX1 protein was found to be responsible for the conversion of CL into carlactonoic acid (CLA) in *Arabidopsis* via 19-hydroxy-CL. This finding raises the possibility that MAX1 catalyzes three-step oxidations of the methyl group at the C-19 position to a carboxylic acid (Abe and et al., 2014; Mashiguchi and et al., 2021).

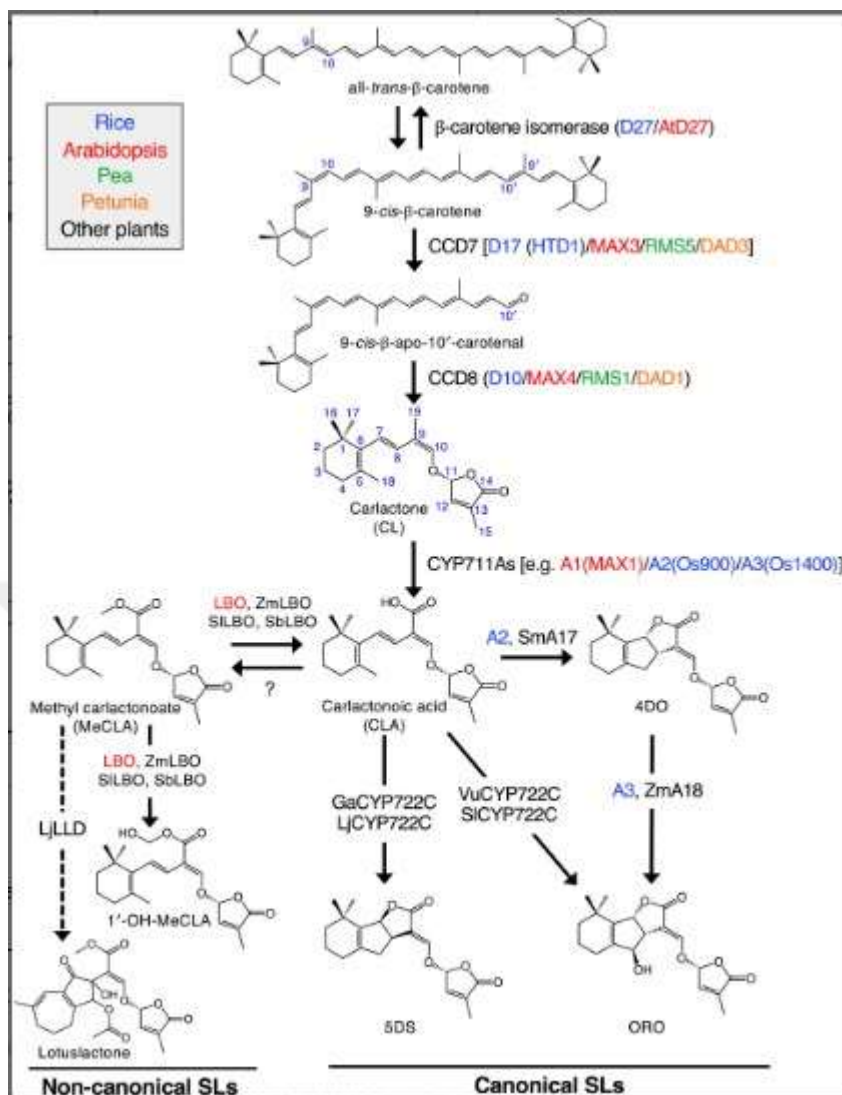


Figure 2.7. The biosynthesis process for SL (Adapted from Mashiguchi and et al. (2021))

2.5. Relationship between organism's cell and CRISPR

Cells make up every living thing. The genetic information is housed in the basic and structural unit of life, the cell. Deoxyribonucleic acid (DNA), the genetic material of the cell, is mostly found in the nucleus, and the chromosomes contain the genetic material that enables an organism to grow, operate, and reproduce (Figure 2.8). It is possible for genetic information to be transferred from one generation to the following.

Eukaryotic cells perform tasks linked to cell growth and death, organelle formation and organization, metabolite synthesis, and microenvironment sensing via a complex network of genes and genetic regulatory element (Wang and Qi, 2016). Many different types of field losses occur worldwide as a result of plant diseases and defects brought on by genetic disorders, mutations, and their connections

The most recent technology, CRISPR/Cas9, gained recognition for its capacity to alter organisms' DNA in the cell in order to modify their genetic. In the light of the present agricultural, environmental, and ecological concerns as well as the context of global climate change, the use of CRISPR to modify germplasm is very crucial (Y. Zhang and et al., 2019). CRISPR was initially used as an adaptive immune system in bacteria and archaea and subsequently developed to induce specific DNA breaks. As the result of this process DNA may be repair, and/or alter. CRISPR technology is used to precisely edit the genomes in the nucleus. CRISPR-Cas nucleases may target coding regions or regulatory areas to eliminate specific genes (Y. Zhang and et al., 2019).

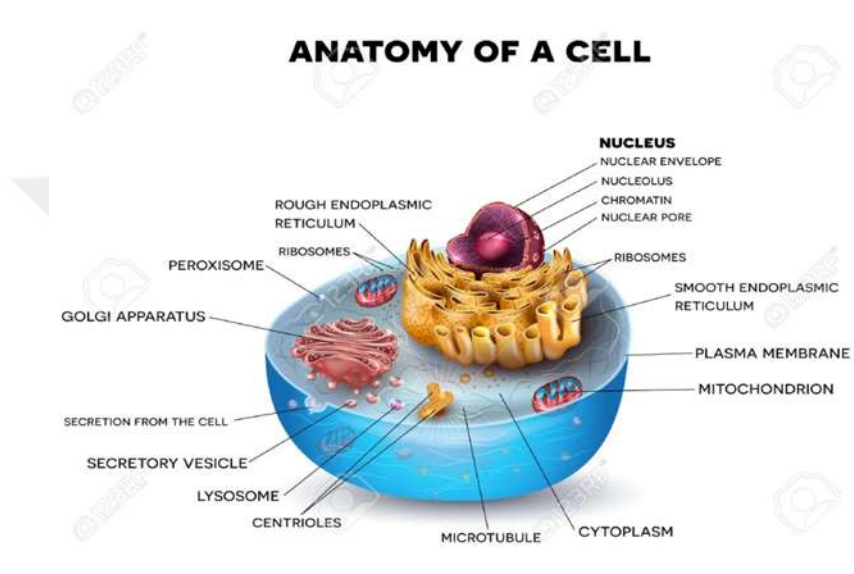


Figure 2.8. Eukaryotic Plant Cell (Adapted from Anonymous 2)

2.6. Genome Editing Methods

Exogenous DNA was demonstrated to be capable of being ingested by yeast or bacteria and randomly incorporated into the genome in the late 1970s. Genome engineering was invented during this time. Scientists can add, remove, or replace specific parts of the genome using a variety of genome engineering techniques. These modifications have the potential to permanently introduce foreign DNA without turning off one or more genes. To develop a new phenotype, different genes can be added at particular sites in the genome.

The four most common strategies for editing the genome are known as meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR (clustered regularly interspaced short palindromic. Large base pair structures known as meganucleases, sometimes known as molecular DNA scissors, are occasionally found in the genome. They were well-liked as genetic tools for DNA alteration due to their capacity to remove substantial chunks of DNA sequence (Khan, 2019).

Zinc finger nucleases were the initial step toward effective targeted nucleases (ZFNs). A 3-base pair (bp) DNA sequence fused to the endonuclease domain of the restriction enzyme FokI is recognized by each of the three to six DNA-binding zinc finger protein (ZFP) domains that make up ZFNs, which are nucleases. Since FokI needs to dimerize in order to cut DNA, ZFNs serve as heterodimers. According to Bitinaite, Wah, Aggarwal, and Schildkraut (1998). The dimerized cleavage domains produce DSBs after DNA binding that can be fixed by NHEJ or HDR. Plant genomes have been edited using this technique, but its usage is restricted by the difficulty of handling various organisms and the expense (Ramirez and et al., 2008).

2011 saw the debut of TALENs. This approach is based on the fusion of FokI endonuclease with TAL effector DNA-binding proteins, which were identified from *Xanthomonas* species. To recognize and cleave DNA at a particular place, TALENs rely on the utilization of TALE -repeat arrays. Although simple to create in the lab, TALENs have a high off-target effect and are challenging to introduce into cells for genome editing due to their size (3 kb).

The most basic gene editing technique is CRISPR/Cas9, which relies on the activity of the Cas9 nuclease and complementary binding of the single guide RNA (sgRNA) to the protospacer in the target DNA. CRISPR/Cas technology is more effective and efficient at genome editing while being less expensive than ZFNs and TALENs.

2.7. History and Adaptive Immune System of CRISPR/Cas9

The existence of Crispr/Cas9 was originally detected in gram-negative bacteria like *Escherichia coli* K12 in the late 1980s, and it was also found in the archaeal genome. In 1987, researchers at Osaka University found the palindromic sequence of the CRISPR array in the *iap* gene (isozyme of alkaline phosphatase) of *E. Coli* (Ishino and et al., 1987). In his work with archaea at the university of Alicante in Spain, Mojica, a spanish scientist, identified an equivalent palindromic sequence (Ishino and et al., 2018). Around 2006, a yogurt factory was able to confirm Mojica's findings on the characteristics of CRISPR on the adaptive immune system. Scientists had found the Cas genes. Philippe Horvath, a scientist at the yogurt factory, discovered that various bacteria cultures were reacting differently to viral attacks. Horvath discovered similar repetitions and viral genome incorporations in bacteria (Horvath and et al., 2008).

Briefly, in 2012 scientists learned how to employ this system to cut the target DNA (Gasiunas and et al., 2012; Tahir and et al., 2020). Jennifer Doudna and Emmanuelle were able to optimize the three-part structure, which consisted of a Cas9 protein, a crRNA, and a tracrRNA, to other system, which included a sgRNA and a Cas9. It was developed and widely acknowledged by other scientists that it is possible to employ Cas9 and build a target-specific RNA to make precise cuts.

In 2013, Zhang demonstrated the efficient use of CRISPR in mammalian cells and demonstrated a method to significantly favor HDR repair in eukaryotic cells, which was a crucial step towards safer gene editing in humans (Ran and et al., 2013).

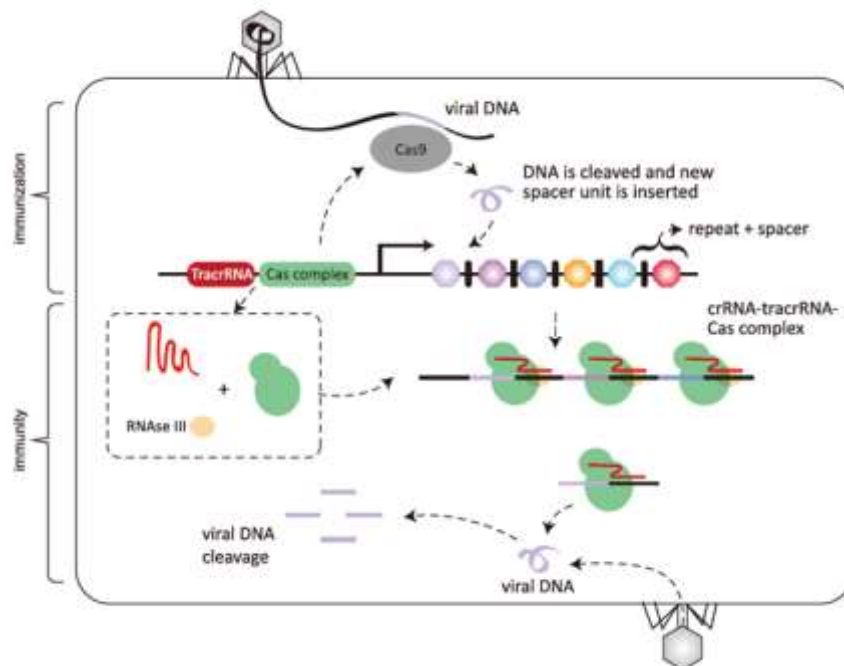


Figure 2.9. Mechanism of CRISPR-mediated immunity in bacteria

Immunization and immunity are the first two phases of the CRISPR/Cas adaptive immune system (Figure 2.9). In the initial stage, a complex of Cas proteins cleaves foreign viral DNA that has been incorporated as repeat-spacer units into the bacterial CRISPR locus.

These repeat-spacer units are then translated to become CRISPR RNA (crRNA) precursors during the immunization stage (Brouns and et al., 2008). The Cas9 endonuclease connects with crRNA precursors under the guidance of trans-activating crRNAs (Deltcheva and et al., 2011). The cleavage of RNase III produces the complex of crRNA, Cas, and tracrRNA. As a short guide RNA, mature crRNA functions to interfere with viral DNA by triggering Cas cleavage.

The protospacer adjacent motif which is called PAM, is a short sequence of three to five base pairs, that follows the DNA region targeted for cleavage by the CRISPR (Mojica and et al., 2009).

2.7.1 Components of CRISPR/Cas9

Two main components of CRISPR-Cas9 technology are a single-guide RNA (gRNA) and a CRISPR-associated endonuclease (Cas). The two of them are joined to create a ribonucleoprotein complex.

In prokaryotes, the crRNA and tracrRNA created two RNA structures that guided Cas9 to a particular location in the invading DNA. When created as a single RNA chimera, they were discovered to drive Cas9 and produce equivalent dsDNA cleavage in eukaryotes in 2012 (Jinek and et al., 2012). Originally described as a crRNA-tracrRNA chimera, this structure is now referred to as a single-guide RNA (sgRNA) (Figure 2.10).

An sgRNA has a 20 nucleotide recognition sequence that it uses to access the complementary DNA strand (Jinek and et al., 2012). The sequence is made up of the nucleotides that are situated at positions 5 to 12 along a U-shaped structure that connects the 3' end of the crRNA to the 5' end of the tracrRNA. Transcription depends on the first nucleotide in the target sequence, which is the nucleotide 20 places next to the U-shaped structure. The first nucleotide in the gRNA sequence needs to be guanine (G), as the U6 RNA polymerase III likes a G as the first base in its promoter (Ran and et al., 2013).

The "molecular scissors" nuclease activity of the enzyme Cas9 has the capacity to cut DNA strands at a specific spot in the genome. To carry out the cleavage, Cas9 should be placed close to the 5'-NGG- 3' PAM sequence.

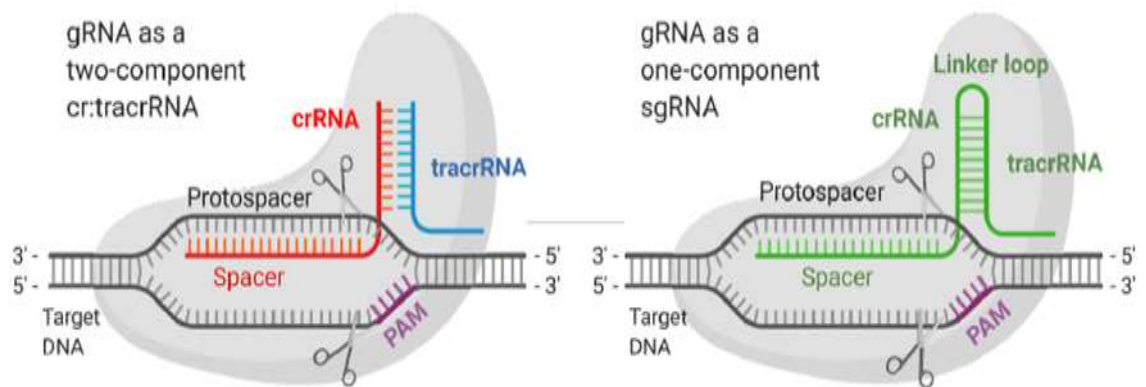


Figure 2.10. Components of CRISPR/Cas9 with two different gRNA configurations

2.7.2 The DNA repair mechanisms

A double-strand break is caused by sgRNA-driven Cas9 protein (DSB). Rather, the damaged DNA strand's repair mechanism is what causes the mutation (Rouet and et al., 1994; Maresca and et al., 2013). There are two methods for repairing DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR) (Rouet and et al., 1994). NHEJ is the name of the first significant repair pathway (Ferreira and Cooper, 2004). The cell uses NHEJ to try to join the blunt ends of the DNA after CRISPR has sliced it. The original strands may be restored during the NHEJ repair phase, or the indel mutations may be assimilated before the strands are reconnected (Figure 2.11). NHEJ is referred to as error-prone, which means that there is a high likelihood of nucleotide insertions or deletions at the site of the DSB. As a result, a DNA sequence that differs from the original may occur, which may have a variety of implications on the gene's expression (Addgene, 2018).

Using the gene's HDR technique, a specific point mutation is induced. Another application of HDR where the gene loses function is the knockout method. The cell uses a homologous sequence as a template for the repair process, which is intended to contain a mutation that causes an early stop codon in the gene and renders the gene

inactive. These repair templates can be used to cause a targeted point mutation, a targeted genomic deletion, or targeted insertions of genetic elements linked to genomic deletions. They can take the form of double-stranded DNA or a single-stranded DNA oligonucleotide (Chen and et al., 2011). Additionally, rather of double-strand breaks, it may result in site-specific single-strand DNA breaks.

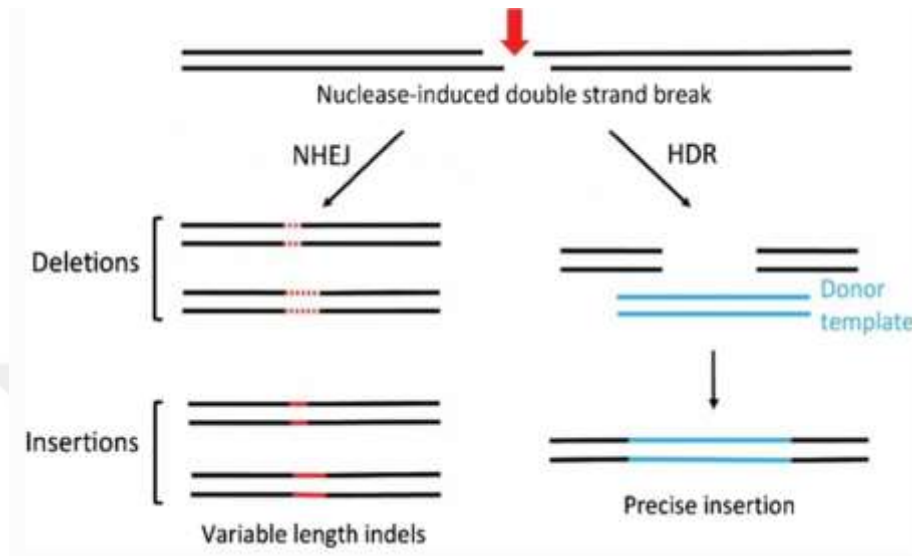


Figure 2.11. HDR and NHEJ, the two alternative repair pathways

2.7.3 CRISPR-Cas Classes and Types

Using the Cas protein classification, CRISPR/Cas systems have been divided into two classes and six types. The Cas9 protein SpyCas9, which was obtained from the bacteria *Streptococcus pyogenes*, is the most well-known CRISPR-Cas system. Two classes are identified based on the Cas genes.

Class I CRISPR/Cas systems include types III and IV, while types II, V, and VI CRISPR/Cas systems are classified as belonging to class 2. *Streptococcus pyogenes*' well-researched CRISPR/Cas9 system is a type II CRISPR system and falls within the class 2 classification.

Class 2 type II: The CRISPR-Cas9 system is one of the most widely utilized and well-known systems. Because the protein can cut and target without enlisting other molecules, it differs from other varieties. Pre-crRNA and tracrRNA must both mature in order for RNaseIII to function. This type includes the RuvC and HNH domains, which can cleave dsDNA and produce blunt ends.

Class 2 Type V: Produces the distinctive Cas12 protein, which uses the RuvC domain to target dsDNA and carry out staggered editing on the target genome. Type V CRISPR-Cas systems use a single crRNA to localize the target DNA instead of tracrRNA, which is necessary for Cas guidance (Tang and Fu, 2018). Numerous organisms have exploited the Cas12 mechanism for genome editing. In the beginning, its effectiveness in *E. coli* was examined, and the findings revealed a high degree of specificity and a minimal off-target effect (X. Zhang and et al., 2017).

Class 2 type VI: This type is well recognized for targeting RNA molecules. It operates on the basis of the Cas13 protein, which can target RNA without the aid of PAM or guide RNA. Cas13 is made up of two different ribonucleases: an RNase (REC) that breaks down pre-crRNA to create mature crRNA, and a ribonuclease that stays in the area where Cas13 joins with it after cleavage. The target RNA cleavage site is formed by two HEPN domains (Higher eukaryote-prokaryote nucleotide binding domain) and one additional RNase activity (NUC) during the viral interference step (Ahmadzadeh and et al., 2019). Collateral cleavage is the term used to describe the action of Cas13 molecules when they are coupled to a complementary target RNA. The HEPN region can cleave any exposed RNA molecule, including free, non-complementary RNA, in addition to the target RNA, leading to the degradation of the target as well as the cleavage of additional RNA sequences (Gootenberg and et al., 2018). Class I Type I: It includes the Cascade complex, which is made up of a single crRNA and five Cas proteins (Cse11, Cse22, Cas5e1, Cas76, and Cas6e1). When Cascade binds to the target DNA, Cas3 is recruited, and it cleaves and digests the target DNA (Van Der Oost and et al., 2014).

Category I, type III: RNA and DNA can be broken down by the class I type III Cas10 protein.

Class 1 type IV: The characteristics of type IV include the Cas proteins Cas7, Cas8, and Cas5. The research on this category is lacking. Recent research has demonstrated the wide diversity of the many subtypes.

2.8 Transformation in *Solanum melongena* L.

2.8.1. *Agrobacterium* mediated transformation

DNA from one organism is inserted into another using transformation techniques. *Solanum melongena* is the intended organism in this instance. Plant transformation can be divided into two categories: stable transformation and transient transformation. Stable transformation aims to completely integrate the gene into the host genome so that it can continue to express itself in the target plant and in succeeding generations. Because the goal of transient transformation is to temporarily express the inserted genes, it is easier to accomplish. It is essential to employ stable transformation techniques in order to obtain consistent and dependable eggplant varieties. There are two types of delivery systems: mechanical and biological. While biolistic bombardment is a direct mechanical delivery method, *Agrobacterium*-mediated transformation is an example of biological delivery. The most common way of delivering genes to plant cells is an *agrobacterium*, which has a clean insertion, a low copy number of inserted genes, is simple to handle, has a greater efficiency, and has a more predictable pattern of integrating foreign DNA (Sah and et al., 2014).

The soil-dwelling bacteria *Agrobacterium tumefaciens* is used in *agrobacterium*-mediated transformation to transfer important genes to plants. Dicot plants are naturally infected by the bacteria, which also causes tumors. The integration of bacterial transfer DNA (T-DNA) into its plant host is what causes that process. The activation of vir genes on the tumor-inducing plasmid marks the beginning of the infection process, which starts when bacteria recognize phenolic compounds released by wounded plants.

These chemicals signal the bacteria to begin the process of infection (Ti plasmid). To properly integrate the T-DNA into the plant's genome, these genes encode proteins that have the transfer, excision, and integration activities.



3. MATERIAL AND METHOD

3.1. An Overview

Following the identification of the CCD8 gene homologs in eggplant using sequence alignment and examination of tissue-specific expression patterns, the vector construct was introduced into the plant tissues using *Agrobacterium*-mediated transformation. To assess for the presence of transgenes in the plant genome using PCR, the DNA from regenerated shoots was collected.

Mutant plants were first planted in the tissue culture chamber before being moved to the greenhouse. We examined these plants in a hydroponic root screening system for a month with two replicates to see whether they were resistant to *P. aegyptiaca*, using wild type as a control. Following that, they will undergo a 90-day test using *P. ramosa* seedlings in pots in a greenhouse. We compared the characteristics of edited plant morphology to those of unedited plant morphology. The experimental flow is presented in the (Figure 3.1).

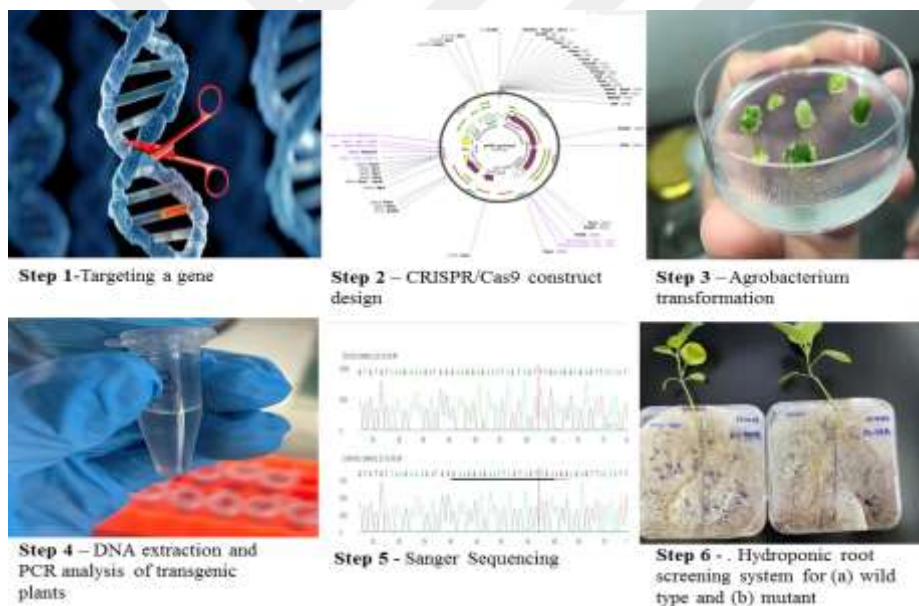


Figure 3.1. A summary of the experimental strategy, from target gene selection through the hydroponic root screening method.

3.2. Identification of CCD8 gene

An important step in the process of genome editing is construct design for CRISPR. Selecting the proper Cas proteins, creating and choosing guide RNAs (gRNAs), and choosing the regulatory components that allow gRNAs and Cas proteins to be produced are all crucial aspects in the process (Hassan and et al., 2021). We employed a construct developed by Dr. Cansu SIMSEK that she prepared for her CRISPR/Cas9 study on tomato, to enable CRISPR-based genome engineering in eggplant and give a design resource for CRISPR targeting protein-coding genes.

The sequence details for the SICCD8 gene regions was retrieved from the National Center for Biotechnology Information's (NCBI) "Gene" database.

3.3. Design of Cloning Vector and gRNAs

Using specialized synthetic oligonucleotides (Oligos) with a Spacer sequence (20 nucleotides) corresponding to the genomic region or gene of interest targeted for alteration is the basis of the CRISPR-Cas9 gene editing technique. These Oligos may be designed using a different bioinformatic software and are known as guide RNAs (gRNAs).

To determine which sgRNAs were required for the CRISPR-Cas9 system to perform double-strand cleavage in the desired gene location and result in a mutation, the CRISPRDirect program (Natio et al., 2015) was used for this study. The first and second exon regions of SICCD8 gene were targeted. The ExPASy/Translate tool (Gasteiger and et al., 2003) was used to translate the nucleotide sequence data of the genes into mRNA sequences, and remove the intron part of the gene. The potential "off-target" possibilities of gRNA sequences have also been investigated. The target gene area was the only sequence that the gRNAs matched. These sets of gRNAs were digested and ligated into the pFGC-pcoCas9 (Addgene, plasmid #52256) plasmid to create a transformation vector.

For the best expression, a codon-optimized *A. thaliana* AtU6-26p RNA Pol III promoter (424 bp) and AtU6-26t terminator (192 bp) were also added, along with a universal sgRNA scaffold sequence (76 bp) to the chosen gRNAs (20 bp without PAM sequences). In order to combine gRNAs 1 and 2, which were developed separately for SICCD8, a cassette was made by adding the proper cut sections (sequences) to a vector to be used in cloning (Figure 3.2) and sending it to Sentebiolab Biotechnology (Ankara) for their synthesis.

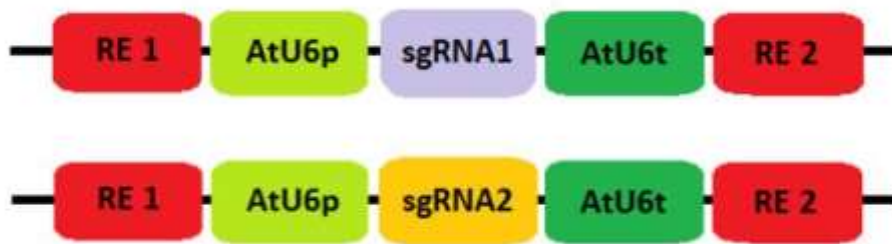
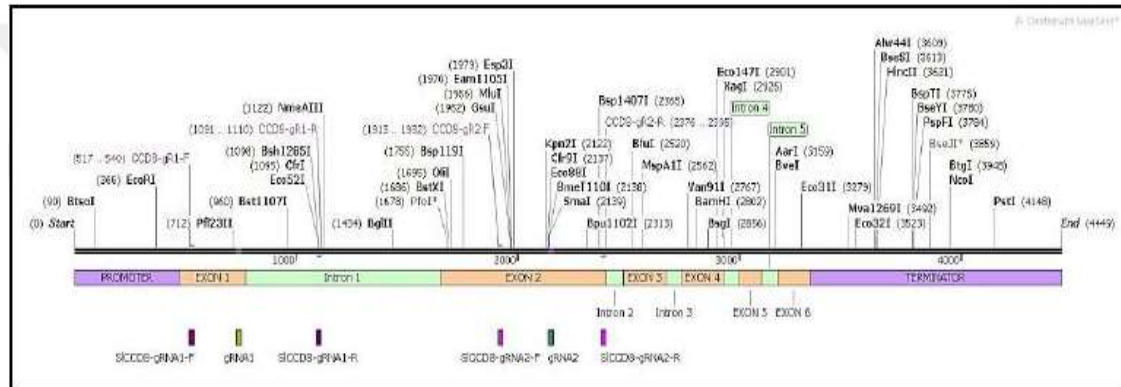


Figure 3.2. The produced sgRNA 1 and sgRNA 2 cassettes (RE 1: PacI (5'...TTAAT/TAA...3'), RE 2: AclI (5'...AA/CGTT...3')) are shown in this illustration. (Adapted from C. Simsek, 2021).

A PCR was built up using tomato DNA to determine whether the planned gRNAs are in the correct area in the tomato plant. Using the Primer3Plus database, primer pairs were created to amplify the SICCD8 gene's specific exon regions.

Table 3.1. SICCD8 gRNA1-2 primer pairs (Adapted from C. SIMSEK, 2021).

Primer Name	Sequence (5'→3')	Length (bp)	Tm (°C)
SICCD8-gRNA1-F	CAAGATCCTTCCTGACATGTTTGA	24	56
SICCD8-gRNA1-R	TAAGCTAACTCGGCCGAAAA	20	56
SICCD8-gRNA2-F	GGTGCATCCCTAACCGATAA	20	56
SICCD8-gRNA2-R	GTTGCCACTGGCTTTACACA	20	59

**Figure 3.3.** Primer design for the tomato SICCD8 gene's first and second exons (Adapted from C. Simsek, 2021).

The Bio-Rad T100TM thermocycler was used for all PCR amplifications under optimal conditions. Single bands of 594 bp for SICCD8 gRNA1 and 483 bp for SICCD8 gRNA2 are expected as a result of electroporation. These single bands, one for each gRNA region, were cut on a low-beam UV device using a scalpel (Transilluminator, UVP). The agarose gel's sliced pieces were cleaned using Thermo Fisher Scientific's GeneJet Gel Extraction Kit. The eluted products from the agarose gel were forwarded to the BMLabosis (Ankara) laboratory for Sanger sequencing.

The binary pFGC-pcoCas9 (Addgene, plasmid #52256) vector was chosen as the best cloning vector for this investigation. It has a Cas9 construct (pcoCas9) and MCS, which makes it simple to integrate gRNA cassettes. The vector is binary and contains the *Pseudomonas* plasmid pVS1 replication origin for *Agrobacterium* and the pBR322 replication origin for *Escherichia coli* (*E. coli*). The choice of the vector containing the aphA3 gene by the bacterium is influenced by kanamycin resistance, according to this fact, *E. coli* (such as the DH5 strain) can be served as the replication line of the vector when Kanamycin is present, the antibiotic that is suited for bacterial selection and prior to that, the binary vector contains an additional element called the bar gene, BASTA (Glufosinate ammonium), which is used for plant selection.

3.4. Cloning experiment

3.4.1. The Cloning of Plasmids that contain gRNAs along with the pFGC-pcoCas9 Vector and Preperation of Media and Competent Cells of *E. coli* - DH5 α strain

The gRNAs were delivered in plasmids that had a total volume of 35 μ l and contained a pTZ57R backbone that was 2887 bp in length. Following centrifugation at 4 °C, 6000 x g for 1 minute, 100 l of DNase/RNase-Free dH₂O was added, and their concentrations were adjusted accordingly. In order to do additional research, it was necessary to first transform these plasmids into *E. coli* so that there would be a greater number of copies available.

It was necessary to make three different mediums of LB Broth Miller (Sigma-Aldrich) in order to provide bacterial cultures with a suitable living environment. One contains 100 g/ml of ampicillin antibiotic, which has a stock concentration of 100 mg/ml and is intended for use in the bacterial selection of the plasmids that were manufactured by Sentebiolab Biotechnology and contain gRNA cassettes. In preparation for the transformation of the Addgene pFGC-pcoCas9 (#52256) vector, 50 g/ml of the kanamycin antibiotic, which has a stock concentration of 50 mg/ml, was added to the second medium. This antibiotic has a stock concentration of 50 mg/ml. The control sample was administered in the third medium, which did not contain any antibiotics. As soon as the medium in the petri dish began to solidify, the dishes were wrapped in parafilm, placed in the refrigerator, and maintained at 4 °C until use.

The bacteria utilized must first be competent in order to produce a greater number of identical clones by using the division of a bacterial cell of plasmid DNA. The bacteria utilized must first be competent in order to increase the number of clones by using the division of a bacterial cell of plasmid DNA. For this aim, bacteria cells (such as the *E. coli* DH5 strain) are treated with positively charged ions like CaCl₂ (CaCl₂ treatment), which causes openings in the cell membranes to open up in preparation for the application of the heat-shock transformation procedure. There are multiple stages to this process:

First the stock *E. coli* DH5 strain culture from -80°C was streaked on the antibiotic-free plates, and was placed in the incubator overnight (O/N) to make sure that *E. coli* grows at 37°C.

After 1 night, 3 bacterial colonies were chosen and transferred to 15 ml falcon tubes containing 10 ml of medium (agar-free LB Broth Miller). It was shaken at 225 rpm and 37°C for 18 hours to reproduce.

The next day, a spectrophotometer measured the cells' Optical Density (OD). When the O.D value reached 0,509, 100 ml of cell culture was placed in two 50 ml x 50 ml pre-cooled falcon tubes and incubated on ice for 10 minutes.

After incubation, liquid cell cultures were centrifuged at 4°C at 5000 rpm for 10 minutes, and the pellet was dissolved in 0.1M cold CaCl₂ (Merck). Each falcon

received 5 mL of the solutions. Cell culture was incubated on ice for 30 minutes with pellets that contained dissolved CaCl_2 .

And as the last step, the supernatant portion was removed after 10 minutes of incubation and centrifugation at 4°C and 5000 rpm, and the pellet was gradually dissolved in 0.1M CaCl_2 + 15% Glycerol (Isolab) solution. Each falcon was given 2.5 mL of the solution. A total of 5 mL of competent cells were obtained; the cells were then divided into 100 µl and streaked over the ready, competent cell's agar-free media before being transferred to -80°C. By streaking them on agar medium containing ampicillin (NeoFroxx), a control Petri was made. This assay allowed for the verification of the *E. coli* DH5 competent cells' purity.

3.4.2. Heat-shock transformation and purification of pTZ57R plasmid

Molecular biology involves heat-shocking plasmid DNA into *E. coli*. Foreign plasmids or ligation products are inserted into bacteria (Froger and Hall, 2007). The plasmid DNA is transferred into these cells by creating pores in the cell membrane during the hot-cold treatment that will be applied to the competent cell and plasmid DNA mixture. This electrostatic repulsion is caused by the presence of CaCl_2 in the medium between the competent cell and the plasmid DNA.

For transformation, 100 µl of competent cells from -80°C were melted on ice along with 3 µl of plasmids. In a 1.5 ml microtube, the competent cell and plasmid DNA (separately for each DNA) were combined and incubated on ice for 30 minutes and after that, the mixture was heated for 2 minutes at 42 °C.

After the heat shock phase, the tubes were held on ice for an additional two minutes. After that, 1 ml of LB broth was added, and suspensions that had been serially diluted were made and incubated for 1 hour 225 rpm in a shaker at 37 °C. In this manner, cell division is permitted in the cells.

After an hour, 100 µl of pTZ57R-SICCD7/8-gRNA1/2 plasmid DNA-containing products were streaked in petri dishes containing the antibiotic ampicillin for bacterial selection. The petri dishes were inverted and placed in an incubator set to 37°C and O/N.

The presence of colonies in petri dishes was examined, and three colonies were chosen, transferred, and given 5 ml of liquid LB and 5 µl of antibiotics in a 15 ml falcon and left the O/N at 37°C while leaving a hole for an oxygen inflow and exit via the coverings.

After overnight incubation at 37 °C and 225 rpm, the cell culture was centrifuged at 4 °C, 5000 rpm, and 15 minutes to precipitate. After removing the supernatant phase, the plasmid was isolated following the manufacturer's instructions using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). A 1% agarose gel containing 5 l of the plasmids was loaded for DNA control. After moving the rest of the components to -20 °C.

3.4.3. Testing the precision of the purified plasmid DNAs and cloning the pFGC-pcoCas9 vector

To create a colony, the pFGC-pcoCas9 vector, which was received as a cell culture in the medium, was streaked over agar media containing selection (Kanamycin). 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). From these liquid samples plasmids were purified using Thermo Fisher Scientific's GeneJET Plasmid Miniprep Kit. For the transformation, the heat-shock procedure was used. A 100 µl of competent cells (*E. coli* DH5) and 3 µl of a particular Miniprep product were used. To create a colony, the transformed cell culture was cultured at 37°C, O/N, in an agar medium containing kanamycin and, using the GeneJET Plasmid Miniprep Kit, plasmid purification was carried out and stored at -20°C. By choosing two enzyme cut areas, a digestion process was carried out to verify the accuracy of the two pFGC-pcoCas9 plasmid DNAs that had been purified. With 1X EcoRI and 1X NcoI enzymes from Thermo Fisher Scientific, 2X Tango Buffer from Thermo Fisher Scientific, and 1 µl of plasmid DNA, a total of 20 µl of reaction mixture was made. It was then incubated at 37 °C for 2.5 hours. Based on the map information of the pFGC-pcoCas9 plasmid, two bands of sizes 4931 bp and 8399 bp are expected to come from the digesting procedure.

3.5. Digestion and Ligation

Due to their lengthy sequences, two different gRNAs (pTZ57R-SlCCD8-gRNA1 and pTZ57R-SlCCD8-gRNA2) were cut from pTZ57R plasmid. To drop the desired gRNA fragments from the backbone, enzyme sites were introduced to the ends of gRNAs (Figure 3.4).

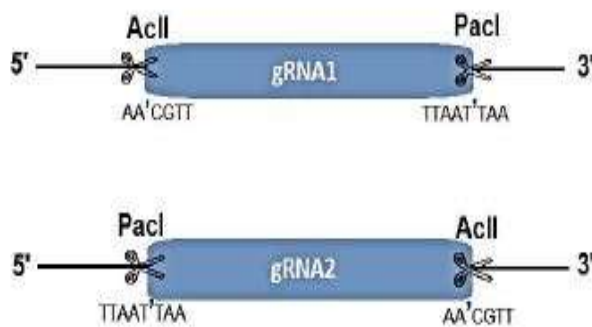


Figure 3.4. Sites of restriction enzymes at the beginning and end of gRNAs (Adapted from C. Simsek, 2021).

With 1X AclI (NEB), 1X PacI (NEB), and 1X CutSmart Buffer (NEB) enzymes, 5 µl of pTZ57R plasmids containing gRNAs were digested for two hours at 37°C. After digestion, 726 bp for SlCCD8-gRNA1 and 721 bp for SlCCD8-gRNA2 are expected. The digested products were run for 4 hours at 75 V and 0.8% agarose gel. On a UV transilluminator, the appropriate-sized bands were cut and purified. Next, using gRNACCD8-F / R primers, the precision of band fragments was examined.

In other side pFGC-pcoCas9 (#52256) plasmid, where gRNAs will be ligated to, was first linearized by PacI enzyme digestion (Figure 3.5). A 20 µl digestion reaction containing 2 µl (2X) of pFGC-pcoCas9 plasmid DNA as a template, 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 was set for enzyme inactivation. The linear plasmids size 13,330 bp, was obtained as a result, and it was cut on a UV transilluminator and purified.

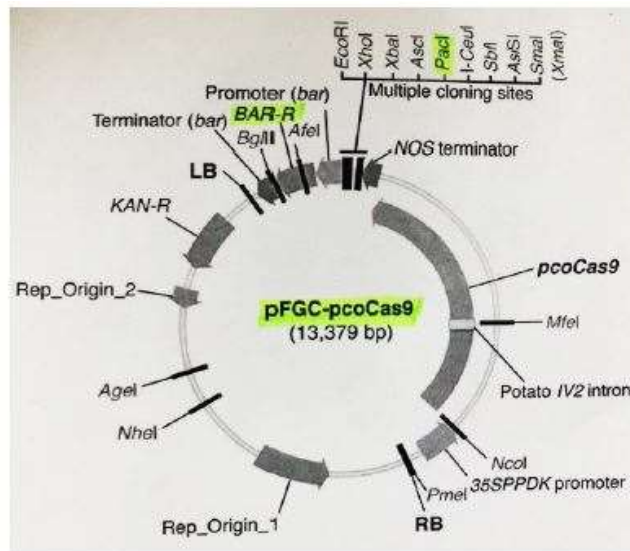


Figure 3.5. Map of the pFGC-pcoCas9 vector's plasmids (Adapted from C. Simsek, 2021).

The rSAP (Shrimp Alkaline Phosphatase (rSAP), NEB) was used to prevent self-ligation of the pFGC-pcoCas9 plasmid, which was digested from a single point (base position 78). The rSAP prevents self-ligation by catalyzing the phosphorylation of the 5' and 3' ends of the DNA and RNA phosphomonoesters. The reaction was set up using 1X Cut Smart Buffer (NEB), 1X rSAP enzyme (NEB), and 8X pFGC-pcoCas9 gel purification product. It was then incubated for 2.5 hours at 37°C before being inactivated for 5 minutes at 65°C.

For the ligation of gRNA1 and gRNA2 to pFGC-pcoCas9 vectors was used T4 Ligase enzyme. A total of 10 µl of the 1X pFGC-pcoCas9 (#52256) rSAP product, 1X T4 Ligase Enzyme, 1X T4 Ligase Buffer, 3X gRNA1, and 3X gRNA2 reaction were used. Approximately 18 hours were spent incubating the combination at 16°C for O/N, and the enzyme inactivation step took place for 10 minutes at 65°C. Following that, transformation utilizing heat shock was carried out using 100 µl of *E. coli* competent cell (DH5 strain) and 8 µl of ligated product. The finished product was placed into media containing Kanamycin-LB plates at a concentration of 50 g/ml, and it was then incubated at 37°C for O/N. The following day, five colonies were placed into liquid LB medium containing 50 g/ml kanamycin, and colony PCRs using gRNAC-F/R primers were conducted concurrently for verification. For Sanger sequencing, the appropriate plasmids were isolated and delivered to Sentegen.

A new primer was created for the post-ligated pair of gRNAs (gRNA1-gRNA2) for the *SlCCD8* gene using the Primer3Plus tool (Table 3.2). DNA amplification produced a 755 bp fragment for *SlCCD8* to confirm the gRNA region.

Table 3.2. Designed primers for post-ligation *SlCCD8*- F/R.

Primer Name	Sequence (5'→3')	Length (bp)	% GC	Hairpin Tm (°C)	Tm (°C)
SlCCD8/F	CAACGACGAGTTCTCCTTCA	20	52,4	-	58,3
SlCCD8/R	GTAGTGATTATTCTCACCACCGTA	24	39,1	39,5	58,9

3.6. Electroporation of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is used for plant genetic engineering because it can mediate inter-kingdom DNA transfer (Nonaka and et al., 2017). Binary vector transformation assay was used to produce mutant plants. This technique depends on the *Agrobacterium tumefaciens* EHA105 strain having a helpful vector or virulence plasmid (Vir-plasmid), which enables the transfection of a binary plasmid into the T-DNA area, which is bordered on the right and left sides. According to previous research, we picked EHA105 as the one of the most effective strains for eggplant transformation.

Firstly, competent *Agrobacterium tumefaciens* EHA105 cells were prepared using EHA105 strain (provided by Associate Professor Amit MITRA, University of Nebraska-Lincoln). *Agrobacterium tumefaciens* was transformed using the electroporation technique, which employs an electrical field to increase the permeability of the bacterial cell membrane and improve DNA absorption.

For the *Agrobacterium* transformation, plasmids carrying gRNAs were optimized to include 80 ng/μl, 1 μl of each was collected, and 100 μl of EHA105 electro-competent cell was gently combined by pipetting on ice. Then the mixture was transferred to electroporation cuvettes.

2,5 kV current was used to transfer the plasmids into the competent cells after the cuvette was positioned in the area of the electroporation machine where the electric current would be generated. The cells were maintained alive by adding 1 ml of liquid LB medium to the cuvettes right after the transfer. Without the use of antibiotics, the cell culture from the cuvette was placed in a microcentrifuge tube and allowed to develop for 2 hours at 28°C.

100 μl of high-density cells were cultured in YEP medium with 50 μg/ml Kanamycin (pFGC-pcoCas9 plasmid selection) and Rifampicin (to prevent possible bacterial contamination). Two days were spent at 28°C. As a control, the empty competent EHA105 strain was grown on 50 μg/ml Kanamycin + 25μg /ml Rifampicin

YEP agar. Kanamycin selection media shouldn't develop competent cells without constructs.

Five colonies were chosen after 2 days. They were left O/N and shaken at 28°C with 5 ml of liquid LB containing 50µg /ml Kanamycin + 25µg /ml Rifampicin. Colony PCR was carried out using chosen colonies and generated primers according to Table 3.1. Miniprep stocks of the ligation products were utilized as a positive control and water as a negative control. Under UV, results were seen on 1.5% agarose gel. Cell cultures with verified colonies were combined with 80% sterile glycerol and stored at -80°C.

3.7. Confirmation of Homology between Tomato CCD8 Gene and eggplant *Solanum melongena* L. CCD8 homolog

Due to the fact that tomato and eggplant are both members of the Solanaceae family, we believed that the construct that had been developed for the CCD8 gene in tomato by Dr. Cansu Simsek, should also work for eggplant. We first verified for homology before applying this construct to the eggplant genome, to see if the construct sequences and gRNAs designed for CCD8 tomato (*Solanum lycopersicum* L.) align with the genome of the eggplant (*Solanum melongena* L.). This verification was carried out with the help of SOL Genomics Network.

First, information about the tomato CCD8 gene's locus and chromosome was found (Figure 3.6). Next, the coding sequence was found and aligned with the genome's coding sequences of the eggplant (Figure 3.7 and Figure 3.8). Coding sequences contain the exons from which gRNAs are created.



Figure 3.6. CCD8 informations in tomato genome

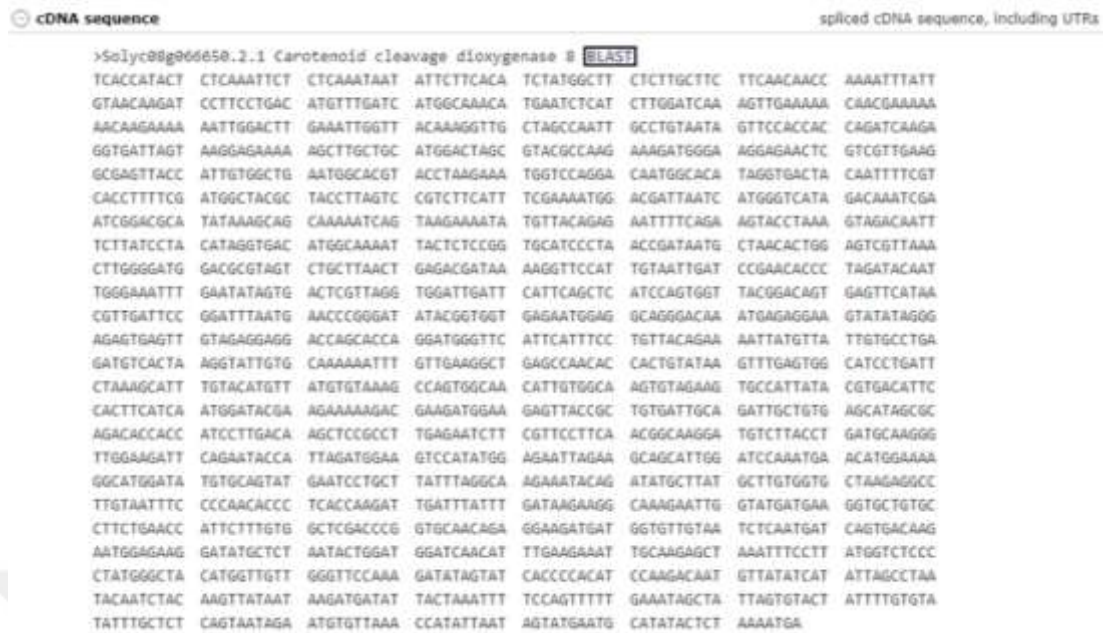


Figure 3.7. Coding sequences for CCD8



Figure 3.8. Alignment between coding sequences of CCD8 tomato and coding sequences of eggplant

3.8. Plant Transformation

Success of the plant-Agrobacterium relationship determines how well Agrobacterium mediates plant transformation. The optimized transformation protocol of Assoc. Prof. Dr. Hasan Pınar was applied in two genotypes of eggplant Yamula and Aydin Siyahi for plant transformation. Yamula is standart eggplant variety, which is cultivated in the region of Kayseri. It is distinguished by its distinctive striped structure and firm fruit flesh, and is used in a variety of ways, such as dried food, fresh consumption, and brine (Uysal and et al., 2019).

In dicotyledonous plants, the integration of CRISPR/Cas9 components by Agrobacterium-mediated transformation is the technique that has gained the most

popularity. The EHA105 strain, which contains pFGC-pcoCas9 vectors containing CRISPR constructs and whose efficacy in plant transformation was confirmed by a preliminary research, was employed for Agrobacterium-mediated plant transformation. Two types of culture media were used for the Agrobacterium strain: YMB (Yeast extract Mannitol Broth) and LB (Luria Broth). The former was used to maintain the Agrobacterium strain and the latter was used to infect the explants. Five milliliters of the liquid LB, containing 5 mg/l kanamycin and 5 mg/ml rifampicin, were inoculated from a new bacterial plate using Agrobacterium, and grown for 19 hours and a temperature of 28 degrees Celsius on a rotary shaker operating at 200 rpm.

3.8.1. Tissue Culture

The procedure began with the preparation of all of the mediums, the primary components, including MS (which contains macro- and micronutrients that are essential for the plant's growth), sucrose (a source of carbon), and MES (a buffer containing MES and $MgCl_2$, with a pH of 5.8-6.0), were dissolved in water to the extent that the volume was reduced by half, and the pH was adjusted to a range of 5.7-6.0. After adding plant agar and sterile water to the final volume, it was autoclaved at 121°C for 20 minutes. After bringing the medium down to 55 °C, the materials that were going to be added after the autoclave, and medium was poured to petri dishes.

Firstly, sterilization of the 100 seeds of Yamula and Aydin Siyahi was carried out, so that we could get the explants ready and prevent any contamination from happening. First, during the process of sterilization, we treated the seed with 70% ethyl alcohol for one minute. Next, we exposed the seed to a 10% solution of calcium hypochlorite. Finally, we rinsed the seed with distilled water five times. After that, the seeds were placed in a glass container so they could germinate in the dark for 5-6 days at 25°C. Eggplant seedlings sprouted and reached full size in less than 12 days in an MS medium with sucrose (Figure 3.9).



Figure 3.9. On the left are shown Yamula seeds transferred on glass jar for germination and on the right it's shown seed germination after 5 days.

Pre-induction

The cotyledon leaves were collected from the tips of plants that were 10–12 days old and split into two parts. To improve the effectiveness of the transformation, we made three cuts with a scalpel on the upper half of the leaves (adaxial side). By transferring the Pre-Induction Medium (PM) content, which is listed in the (Table 3.3), with a maximum of 5 pieces per petri dish, the explants were prepared and left in the dark for O/N (Figure 3.10).



Figure 3.10. Explants in Pre-Induction Medium

MS=4,3 gr
MES=0,56 gr
SUCROSE=30 gr
pH=5,6
AGAR=7 gr

Table 3.3. Hormones used after autoclave in pre-induction medium

Hormones	Volume	Stock solution
Thiamine	1ml	3 mg/ml
Pcpa	8 μ L	1 mg/ml
Zeatin	2 ml	1 mg/ml
Kinetin	100 μ L	1 mg/ml

The binary plasmid pFGC-pcoCa9 from the *Agrobacterium tumefaciens* strain EHA-105 was utilized for transformation in the study. *Agrobacterium* was inoculated

into five milliliters of liquid YMB that contained 5 mg/l of kanamycin, and the bacteria were then cultured for 19 hours at 28 °C at 200 rpm on a rotary shaker. After that, the culture was centrifuged for ten minutes at 20 °C at 6000 rpm. To achieve an acceptable bacterial density of 0.5 for infection, the supernatant was removed and the bacterial precipitate was resuspended in an adequate volume of liquid MS. Cut into two parts, the cotyledonary leaves were then submerged in the bacterial mixture for 20 minutes of incubation. The bacteria were then removed from the explants by blotting them dry using filter paper. The explants were co-cultured in petri plates for two days in the dark after infection and incubation.

Callus induction and Shoot regeneration

The co-cultivated explante were transferrered to selction medium for callus induction and root regeneration (Figure 3.11).

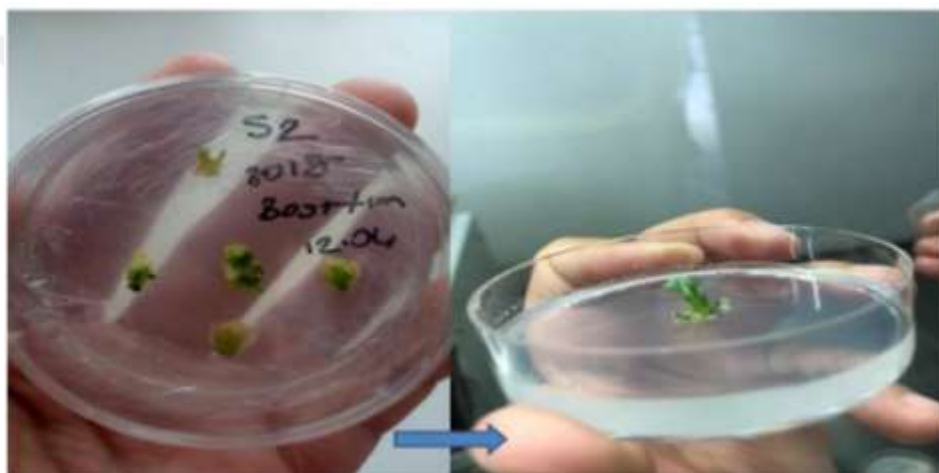


Figure 3.11. Callus induction and shoot regeneration

Selection I (1Liter)

MS= 4,3 gr
MES=0,56 gr
SUCROSE=30 gr
PH=5,7
AGAR=7 gr

Table 3.4. Hormones used after autoclave for Selection I medium

Hormones and antibiotics	Volume	Stock solution
Basta	2 mL	1 mg/mL
Timetin	1 mL	200 mg/mL
Zeatin	1 mL	1 mg/mL
IAA	1 mL	1 mg/mL

Rooting

After being placed in the selection medium, well-developed shoots were then moved to the rooting medium (Figure 3.12), where they remained for about three weeks until they produced enough roots to move on to the acclimatization stage (Figure 3.13).



Figure 3.12. Developed shoots transferred to rooting medium

Rooting medium (1 Liter)

MS= 4,3 gr
MES=0,56 gr
SUCROSE=30 gr
pH= 5,7
AGAR= 7 gr

Table 3.5. Hormones used after autoclave for Rooting medium

Hormones and Antibiotics	Volume	Stock Solution
Basta	1 mL	1 mg/mL
Timentin	1 mL	200 mg/mL
IBA	2 mL	1 mg/mL

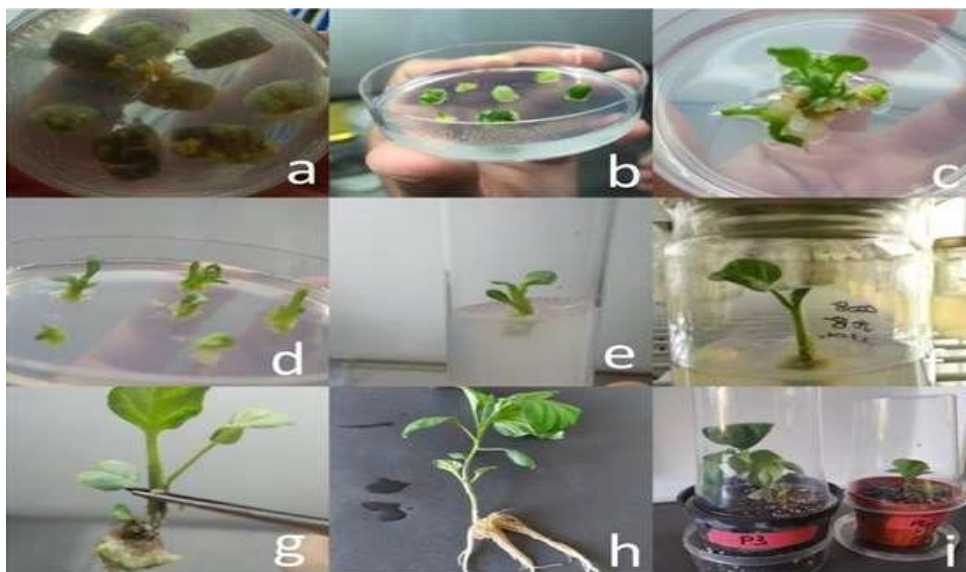


Figure 3.13. Different phases of transgenic eggplant growth in vitro: Initiation of callus at the cut ends of cotyledon explants, followed by greenish compact callus and shoot proliferation (a, b, and c); strong shoot and shoot elongation after one month (d, e, f, g); 4-5 leaf stage shoots and their rooting (h); acclimation of rooted shoots (i).

Acclimatization of Plants

Different from field-grown plants are plants grown in a laboratory. Tissue culture plants must gradually acclimate to the field circumstances since they are cultivated in extremely high humidity and under carefully regulated conditions on a medium rich in sucrose. Because they contain non-functional stomata, a weak root system, and a poorly formed cuticle, it is necessary to transplant individual plantlets to potting soil and let them to acclimate under certain humidity, temperature, and light conditions. After a few weeks, well-rooted plants were gently removed from the jars and their roots thoroughly rinsed with running water. Following that, they were put in little pots with a perlite mixture. For humidity maintenance, plastic cups were placed over these pots. The greenhouse where they were kept (Figure 3.14).



Figure 3.14. Acclimatization of plants

3.9. Molecular characterization

3.9.1. Analysis of CCD8 mutants

The T0 plants screened to identify those that could have undergone mutation. Through the use of gene-specific primers and PCR amplification of SlCCD8 gRNA, the presence of a trans-gene in transformed shoots was verified. From the leaves of transformed shoots, genomic DNA was extracted. Yamula plant DNA that had not been mutated was used as a positive control during amplification, whereas sterile water was used as a negative control. Following verification, PCR results were forwarded to Sanger sequencing.

Plants with the mutation chosen from T0 were chosen for selection, and then those plants were selfed to produce the T1 and T2 generations. That's because, in the case that a gene mutation occurs, this alteration may only be seen in one of the two alleles (in the T0 generation). To choose homozygous mutants or to achieve this variation in the subsequent generation, the T0 plants are developed, and their seeds, T1 or first generation, are taken and evaluated (T2). In the T2 generation of plants, a second round of sequencing was performed to verify for the inheritance and homozygosity of mutations.

DNA extraction

In order to obtain DNA from the plants that may have been transformed, young leaves were harvested and placed in 1.5 mL tubes with the assistance of sterile scissors and tweezers. These implements were then washed with 70% ethanol before being used to harvest tissue from a different plant in order to prevent contamination. In order to extract DNA, the CTAB protocol was used. The NanoDrop spectrophotometry was used to evaluate the DNA's purity and concentration, and the samples were kept at a temperature of 4 °C.

DNA amplification using PCR

The parts of the plant's genome that were supposed to be edited by the CRISPR-Cas9 technology were amplified using a set of targeted primers that focused on the various areas of interest of the CCD8 gene. Sterile water was employed as a negative control throughout the process of amplification, while wild type genomic DNA was also amplified and used as a positive control. Following verification, PCR results were sent to the BMLabosis for Sanger sequencing. Thermo Fisher's 6x concentrated DNA Gel Loading Dye was added to the samples before loading them onto the gel wells since the PCR results were free of loading dye. Later, 20 µl of the PCR results were run on a 1% agarose gel, at 120V for 40min. In order to determine the size of the amplified DNA, bands were observed under the UV light.

DNA sequencing findings' multiple alignment

A multiple sequence alignment was done between the Sanger sequencing findings of the in theory modified plants' CCD8 gene and the genomic sequence of the *Solanum melongena* in order to compare the DNA from the transformed plants with the

CCD8 gene of WT plants. The sequences were entered into the NCBI's BLASTn website tool to ensure that the results returned matched the CCD8.

The SnapGene (GSL Biotech) chromatogram viewer program was used to examine the sequencing chromatograms in order to assess the quality of the Sanger sequencing. The ICE tool from Synthego allows for further characterization of mutated events.

3.10. An examination of the phenotypes of mutant plants

Plant growth in the presence of *P. aegyptiaca* was tested, and its effects on the newly produced mutant plants' growth, root development, and behavior were compared to those of WT plants under the same circumstances. Both mutant and wild type plants were tested with *P. aegyptiaca* seeds in hydroponic systems as well as in pots for the purpose of this experiment. Additionally, changes in the morphology of plants were explored too.

3.10.1. Testing Resistance of Mutant Lines with *P. aegyptiaca* seeds through Hydroponic Root Screening System

We conduct an infection test using *P. aegyptiaca* to determine the effect of reduced SL on the interaction of Yamula mutants with root parasitic plants. The seeds of *P. aegyptiaca* were surface sterilized with 1% (v/v) sodium hypochlorite (NaOCl), combined with 0.01% Tween 20, for five minutes, followed by a thorough rinse for six minutes times with water that has been sterilized. Approximately 10 mg of surface-disinfected seeds of *P. aegyptiaca* were placed on 9 mm diameter glass fibre filter paper disks, each one moistened with 3 ml of sterile water. The petri dishes were sealed with parafilm, wrapped in aluminum foil and placed in incubator at $21\pm1^{\circ}\text{C}$ for 7 days for seed conditioning.

Then two compartments that were bonded together but separated by a glass plate made up the hydroponic system. The eggplant seedlings with healthy root growth were first picked, and it was then re-rooted in the glass plate. Filter paper sheets were used to cover the glass plate dividing these two compartments, which had first been filled with 175 ml of vermiculite and then uniformly scattered with preconditioning with *P. aegyptiaca* seeds. The eggplant was then picked out and put on the glass plate with healthy roots (Fig. 3.15). The two treatments that follow were created: (a) wild type is the control; (b) mutant line is the treatment. Each treatment included five replicates and repeated two times. Each plant was placed in a tissue culture environment with a photoperiod of 16 hours of light and 8 hours of darkness at 24°C . Hoagland nutrient solution was used to moisten them (Hoagland and Arnon, 1950). The fertilizer solution applied to plants every two days. The plants were picked during the 30- day incubation period in order to analyze the *P. aegyptiaca* growth differences between wild type and mutant at roots of eggplant. The rates of germination tubercles were photographed (Leica, LAS EZ) and numbered using a binocular microscope. When the radicle appeared, the *P. aegyptiaca* seeds were considered to have germinated.



Figure 3.15. Hydroponic root screening system for (a) wild type and (b) mutant.

3.10.2. Data Collection and Statistical Analysis

Statistical significance difference between mutants and wild type were subjected to ANOVA using SAS statistical package (SAS institute, Cary, NC, USA) based on biomass of tubercles of *P. aegyptiaca* collected from roots of mutants and wildtype (Figure 3.16). The roots of plants that were hydroponically tested using *P. aegyptiaca* seeds were utilized for a biomass test after one month. Five replicates of each root were collected from each plant together with *P. aegyptiaca* tubercles. Tubercles were picked from the roots, and were weighed after being dried at 65°C for 24 hours, and the fine tubercles biomass was calculated in grams.



Figure 3.16. Tubercles biomass of *P. aegyptiaca* on eggplant

3.10.3. Field Screening

Also, throughout the seasons of 2022–2023, outdoor experiments were conducted with the intention of confirming the hydroponic experiment's findings. The eggplant seedlings were put into pots that already had the mixture in them. Then, 100 mg of *P. aegyptiaca* seeds were put into each pot. Plants will be grown in a greenhouse and exposed to natural light (Figure 3.17). After 90 days of being inoculated with *P. aegyptiaca* seeds, the roots of the host plants that will be used to create the control and mutant plants will be gathered. We are going to count and take pictures of the number of *P. aegyptiaca* tubercles attached to the eggplant roots that are larger than 2 millimeters in diameter.



Figure 3.17. Mutants with different CCD8-gRNA1 mutations were put in pots and taken to a greenhouse to see if they were resistant to *P. aegyptiaca*.

3.11. qPCR Analysis

RNA Isolation

Consequently, measuring the levels of the corresponding messenger ribonucleic acid (mRNA) is one of the most important ways to determine a gene's function. In this procedure, complete cellular RNA is isolated, and the relevant mRNA is then analyzed (Guan and Yang, 2008). RNA isolation is a crucial step that determines whether a qRT-PCR experiment is successful. RNA of high enough quality for qRT-PCR can be obtained using RNA isolation techniques including acid phenol extractions, purification with glass-fiber filters or magnetic beads, and lysis-based reagents.

Only freshly emerged, properly grown leaves were gathered, and they were grinded using liquid nitrogen. RNA was isolated following the instructions on the Monarch Total RNA Miniprep Kit. The steps below are used to extract RNA from eggplant leaves: homogenization, separation, RNA precipitation, washing and solubilization. The NanoDrop spectrophotometer was used to validate the quantity and

quality of the extracted RNA. After isolation RNA samples were stored at -80 °C. Quantity and purity measurements of the isolated RNA samples were made.

DNase digestion

DNase was used to process RNA samples so that they wouldn't be contaminated with genomic DNA. After removing 1000 ng of RNA from the sample, adding 2 µL of DNase I Reaction Buffer and 1 µL of DNase I enzyme, and then bringing the total volume to 15 µL with ddH₂O, the volume was considered complete. It was incubated for 1:30 hours at 37 °C. Following the incubation step, 1 µL of EDTA at a concentration of 50 mM was added to the sample. It was kept in an incubator at 65°C for 10 minutes.

As a control, we carried out PCR using Actin primers in accordance with the parameters outlined in the (Table 3.4 and Table 3.5), to be sure if samples aren't contaminated with genomic DNA.

Table 3.6. PCR reaction content

Component	Quantity
RNA Template	1 µl
ddH ₂ O	13.75 µl
10X Taq Buffer ((NH ₄) ₂ SO ₄ -MgCl ₂)	2 µl
MgCl ₂ (25 mM)	1.6 µl
Taq DNA Polymerase (5 U/µl)	0.25 µl
dNTP (10 mM)	0.4 µl
Primer-Forward (10 µM)	0.5 µl
Primer-Reverse (10 µM)	0.5 µl
Total	20 µl

Table 3.7. PCR conditions

95°C	5 minutes	1 cycle	Denaturation (I)
95°C	30 seconds	44 cycles	Denaturation (II)
62°C	30 seconds		Annealing
72°C	1 minute		Extension (I)
72°C	10 minutes	1 cycle	Extension (II)

qRT-PCR quantification

Quantitative PCR was used to measure the expression level of the targeted CCD8 gene in eggplant. Using the gene-specific primers, the qRT-PCR of each sample was repeated two technical and three biological replicates. iTaqTM Universal SYBR® Green Supermix mix, 0.8 µL forward and 0.8 µL reverse primer (Table 3.7), 1 µL of DNA (around 200 ng), and 6.9 µL of ddH₂O make up the qPCR mix. PCR cycling included pre-denaturation at 95 °C for 6 min followed by initial denaturation at 95°C for 60 s, 2 step amplification at 95°C for 10 s and 59°C for 30 s, and melting as last step 95°C for 10 s, 65°C for 60 s and 97°C for 1 s. The $2^{-\Delta\Delta C_t}$ was used to quantify the target genes' transcripts.

Table 3.8. Primers designed for gene expression CCD8 primers and Actin primers

Primer Name	Sequence (5'→3')	Length (bp)	T _m (°C)
SlCCD8/F	ATGGCTTGCTTTGCTTCTTC	20	55°C
SlCCD8/R	CGTCACAAGTGGATGAGCTG	21	55°C
Actin/F	GGGATGGAGAAGTTTGGTGGTGG	24	62°C
Actin/R	CTTCGACCAAGGGATGGTGTAGC	24	62°C

Table 3.9. RT-PCR reaction content

Component	Quantity (1x)
RNA Template	0.5 µl
ddH ₂ O	6.9 µl
RT enzyme (20x)	1 µl
Primer-Forward (10 µM)	0.8 µl
Primer-Reverse (10 µM)	0.8 µl
Reaction mix (2x)	10 µl
Total	20 µl

Statistical Analysis

In order to conduct statistical analysis and make evaluations of the data that we acquired for our research, we used the GraphPad Prism 7.0 tool. When doing the statistical analysis of the data, the student's t-test was the chosen method. The significance of the findings was determined using the $p < 0.05$ level for the confidence interval, which was 95%. The mean of the data is represented here by the symbol $\pm$ SEM.

3.12. The Use of High-Performance Liquid Chromatography for the Determination of the Amount of Absciscic Acid

The biosynthesis of both strigolactones (SLs) and abscisic acid (ABA) begins with carotenoids. The interaction between these two hormones at the biosynthetic and/or regulatory level may be expected given their shared origin (Haider and et al., 2018). All higher and lower plants need abscisic acid to regulate a variety of physiological functions. Lopez Raez et al. (2010) that ABA is one of the regulators of strigolactone production in his research, which was the first to show a correlation between the concentrations of the phytohormone ABA and the family of phytohormones strigolactones (López-Ráez and et al., 2010). According to Torres-Vera (2014), the tomato mutant *slccd8* lacking SL had decreased ABA levels (Torres-Vera and et al., 2014).

High performance liquid chromatography was used to analyze abscisic acid from the leaf. Eggplant freshly harvested plant material of P4-2, P3-28, P3-47, P38-26, and wild type, was immediately put on ice, then brought to the lab and stored at a temperature of -20 degrees.

- The next day after (24h) , the leaves were weighed, and a quantity of freeze-dried leaf tissue weighing 2.5 grams were homogenized by adding 50 ml of methanol with the help of a pestle.
- The leaf samples, which became homogenized in the mortar, was taken into a 100 ml beaker and made ready for the next ultrasonication process.
- Leaf samples were placed in an ultrasonic bath and exposed to sound waves at 37 kHz at 30°C for 15 minutes. The reason for this is that the sound waves break the cell wall and allow the cytoplasm to mix better with the solvent.
- After the ultrasonic bath, the pH level of the solution was adjusted to 3.5 by using formic acid to release abscisic acid bound to the cell wall or various molecules.
- After pH adjustment, 3 ml of this mixture was taken and passed through C18-E (strata- Phenomenex) and analyzed in filtrate HPLC-DAD. Absciscic acid was not detected at this stage.
- Because the C18-E column held the abscisic acid, preventing it from passing. Afterwards, abscisic acid analyzes were performed by passing 3ml 80%MeoH 20% UPW(ultrapure water) through the column.
- After the column was washed, 0.22µm syringe tip was filtered, 500 µl of this mixture was taken, 500µl 80%MeoH 20% UPW was put on it, and the analyzes were carried out by diluting it by half.

Absciscic acid standard was analyzed at 7 different wavelengths as 210, 265, 270, 280, 290, 380 and 400 nanometers (nm) by using DAD detector in the HPLC system, and since the smoothest and symmetrical peaks were obtained, all analyzes were prepared at 290 nanometers.

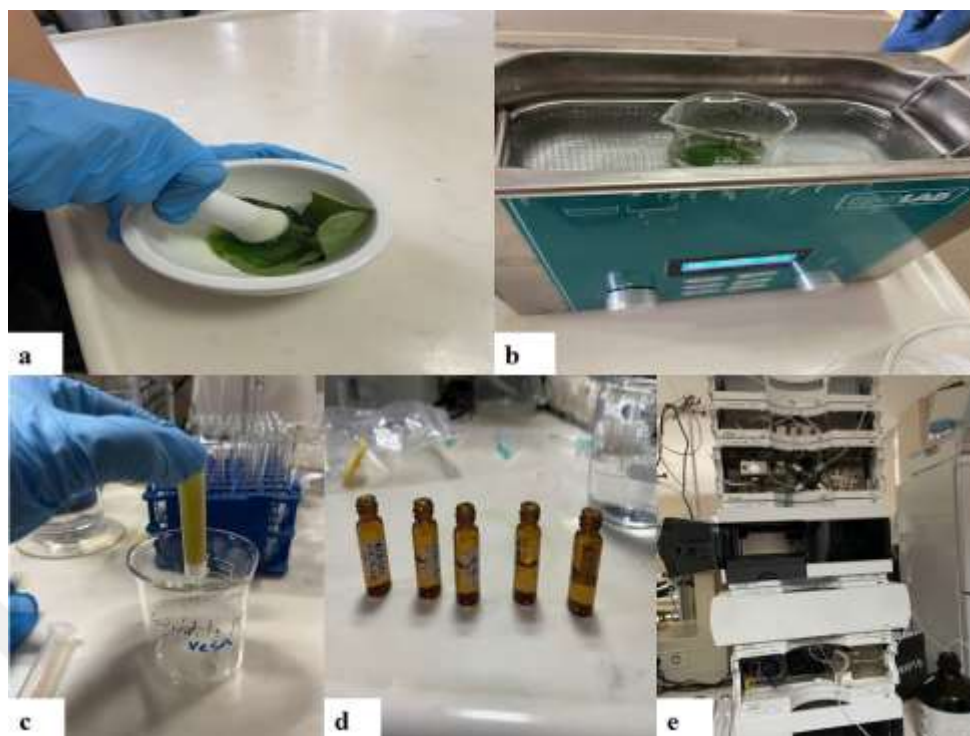


Figure 3.18. Absciscic acid extraction: a) grinded leaves in 50 ml of methanol; b) samples incubated in an ultrasonic bath for 15 minutes; c) filtration with C18-E (strata-Phenomenex) ; d) samples mixed; and e) absciscic acid content measured with HPLC

4. RESULTS AND DISCUSSION

4.1. Identification of CCD8 gene on tomato

The goal of this experiment was to build a vector that targets a part of the CCD8 gene. Using the NCBI/Gene database, specific information on the *SlCCD8* gene were obtained. In tomato, the CCD8 gene is found on the eighth chromosome, and it has six exons and five introns, as was estimated from the information presented here. The gene consists of 2842 nucleotides that may be found in the tomato genome version SL3.0 between the 55,474,797 and 55,477,956 nt. This gene has 557 amino acids.

To assess gene structure, introns were deleted from *SlCCD8* gene sequences and was converted to protein sequence using ExPASy/Translate. Results proved the gene structure was CCD8.

4.2. gRNAs design

CRISPRDirect was used to create sgRNA's (gRNA) necessary for CRISPR-Cas9 to accomplish double-strand cleavage in the target gene region. Based on PAM the gRNAs were selected where, GC content was more than 40%, and there was no off targets (Table 4.1). These gRNAs specifically targeted *SlCCD8*'s first and second exons.

Table 4.1. Two gRNAs designed for *SlCCD8* gene (Adapted from C. Simsek, 2021).

Gene	Sequences (5' – 3')	Location (nt)	Size (nt)	GC %	gRNA name	Exon number
SlCCD8	CCTTCAACGAC GAGTTCTCCTTC	55,477,520- 55,477,542	23	50	gRNA1	1.
SlCCD8	ATTCTCACCACC GTATATCCCGG	55,476,113- 55,476,135	23	45	gRNA2	2.

Using Primer3Plus, gRNA-specific primers were created. By sequencing the PCR-amplified area (Table 3.1), it was revealed that *SlCCD8* gRNAs targeted the right locations. The sequences were aligned using Clustal Omega, and the coverage of the designed *SlCCD8* gRNAs (shown in blue) in (Figure 4.1 a, b).

```

145 TAGTTGCACCACCAGATCAAGAGGTGATTAGTAAGGAGAAAAAAGTTGCTGCATGGACTAGCGTACGCCAAGAAAGATGGGAAGGAGAACTCGTCATTGA 244
111 TAGTTCCACCACCAGATCAAGAGGTGATTAGTAAGGAGAAAAAGCTTGCTGCATGGACTAGCGTACGCCAAGAAAGATGGGAAGGAGAACTCGTCATTGA 210

245 AGGCGAGTTACCAATTGTGGCTGGTATGTTATTGTTTGTGTATAAAAGTTAAATATGAACGTTTCGTGTTACATCCATTTTTTTGTTTTAAAGTGAAGTTT 344
211 AGGCGAGTTACCAATTGTGGCTGGTATGTTATTGTTTGTGTATAAAGTTAAATATGAACGTTTCGTGTTACATCCATTTTTTTGTTTTAAATGTGAAGTTT 310

345 TTCTTTTTTGGGAATCACTTATATATTATGATTTTTCTTCACCAAAAAGTATTGAGATCAAAGATGCATGTGTACCTAATACATGTTTTCTCTCAAGAAA 444
311 TTCTTTTTTGGGAATCACTTATATATTATGATTTTTCTTCACCAAAAAGTATTGAGATCAAAGATGCATGTGTACCTAATACATGTTTTCTCTCAAGAAA 410

445 ATATGAAGTATACATATTGAAAACAAAGGTTTATGAACCTTAATTATCTGCTATTAGTACACTACTATAGACCCAAAGATTTTCTCACTTGATAATCA 544
411 ATATGAAGTATACATATTGAAAACAAAGGTTTATGAACCTTAATTATCTGCTATTAGTACACTACTATAGACCCAAAGATTTTCTCACTTGATAATCA 510

545 GTCGGAAATTTTGTATTATAAAATATGATTTTCTGAGTCA 584
511 GTCGGAAATTTTGTATTATAAAATATGATTTTCTGAGTCA 550

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(a)

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6  GGAATGAT--AACTTGGGGA-GGACGCGTAGTCTGCTTAACTGAGACGATAAAAGGTTCCATTGTAATTGATCCGAACACCCTAGATACAATTGGGAAAT 102
11 GGAGTCGTTAAACTTGGGATGGACGCGTAGTCTGCTTAACTGAGACGATAAAAGGTTCCATTGTAATTGATCCGAACACCCTAGATACAATTGGGAAAT 110

103 TTGAATATAGTGACTCGTTAGGTGGATTGATTCATTCAAGTCATCCAGTGGTTACGGACAGTGAAGTTCATAACGTTGATTCGGGATTAAATGAACCCGGG 202
111 TTGAATATAGTGACTCGTTAGGTGGATTGATTCATTCAAGTCATCCAGTGGTTACGGACAGTGAAGTTCATAACGTTGATTCGGGATTAAATGAACCCGGG 210

203 ATATACGGTGGTGAGAAATGGAGGCAGGGACAAATGAGAGGAAGTATATAGGGAGAGTGAAGTTGTAGAGGAGGACCAGCACCAGGATGGGTTCAATCATTT 302
211 ATATACGGTGGTGAGAAATGGAGGCAGGGACAAATGAGAGGAAGTATATAGGGAGAGTGAAGTTGTAGAGGAGGACCAGCACCAGGATGGGTTCAATCATTT 310

303 CCTGTTACAGAAAATTATGTTATTGTGCTGAGATGTCCTAAGGTATTGTGCAAAAAATTTGTTGAAGGCTGAGCCAAACACCCTGTATAAGTTTGAGT 402
311 CCTGTTACAGAAAATTATGTTATTGTGCTGAGATGTCCTAAGGTATTGTGCAAAAAATTTGTTGAAGGCTGAGCCAAACACCCTGTATAAGTTTGAGT 410

403 GGCATCCTGATTCTAAAGCATTTGTACATGTTA 435
411 GGCATCCTGATTCTAAAGCATTTGTACATGTTA 443

```

(b)

Figure 4.1. gRNA sequences of SICCD8 **a)** target region of SICCD8-gRNA1 with their respective areas had one mismatch.; **b)** target region SICCD8-gRNA2 had a perfect match.

4.3. Vector Generation

A. thaliana AtU6-26p RNA pol III codon-optimized promoters, AtU6-26t terminator, and suitable restriction enzyme regions (sequences) were combined to form a cassette. A number of enzymes have also been included, including AclI (Psp1406I) (5'...AA/CGTT...3'), which joins gRNA1 and gRNA2, and PacI enzyme digesting sites (5'...TTAAT/TAA...3'), which ligates the cloning vector. These sgRNA cassettes were created and then cloned into the pTZ57R vector (Figure 4.2). Resistance to Ampicillin, Carbenicillin, and similar antibiotics is supplied through bacterial selection by the (AmpR) gene in the plasmid via the β -lactamase gene product.

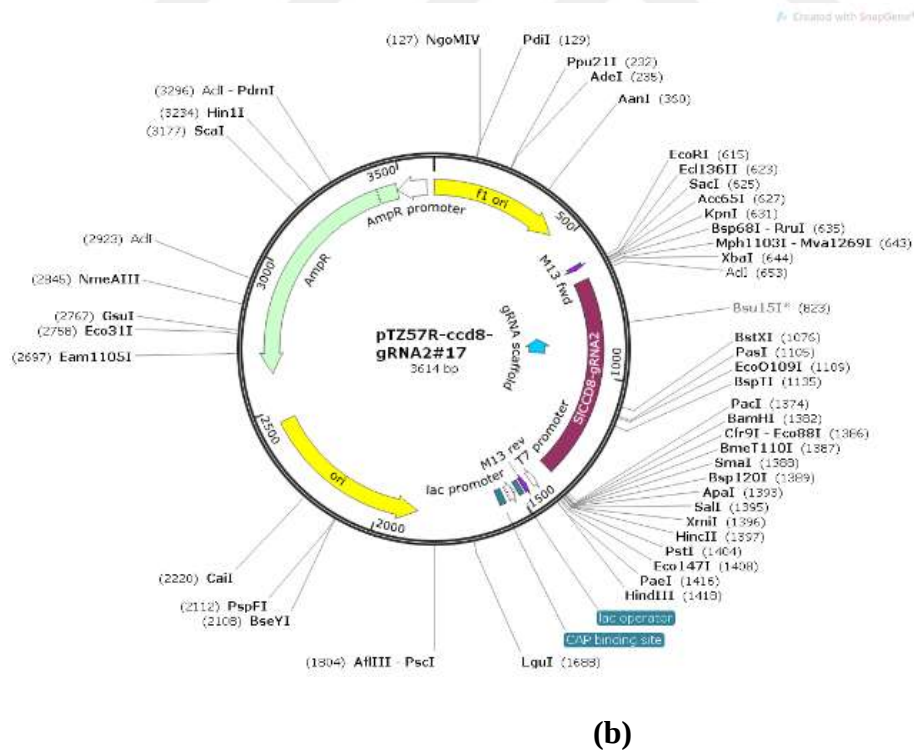
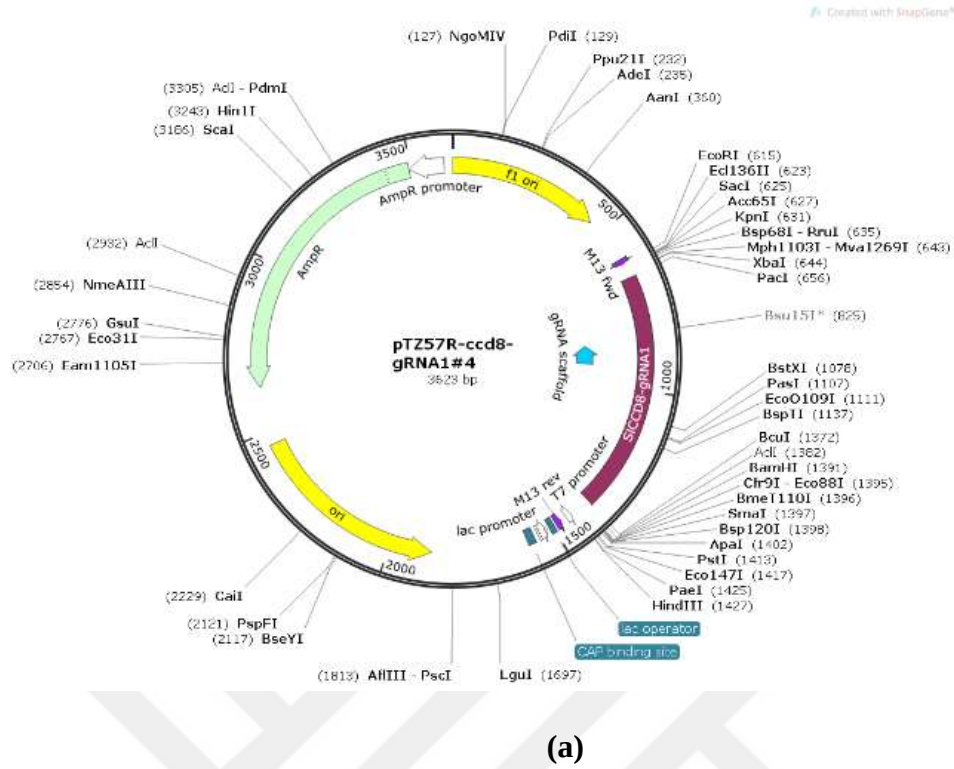


Figure 4.2. Maps of pTZ57R plasmids containing SICCD8 gRNAs **a)** pTZ57R-SICCD8-gRNA1 plasmid **b)** pTZ57R-SICCD8-gRNA2 plasmid (Adapted from C. Simsek, 2021)

CRISPR/Cas9 was utilized to modify the genome using Vector pFGC-pcoCas9 (Addgene) (Figure 4.3). Its 13.330 bp length makes it acceptable for using *Agrobacterium tumefaciens* to produce plants that have undergone stable transformation. This plasmid comprises the *Streptococcus pyogenes* Cas9 endonuclease, a 35SPPDK: pcoCas9: NOS cassette for Cas9 plant expression, as well as several cloning sites for guide RNA insertion. The nuclear localization sequence (nucleoplamin NLS), 48 bp (16 aa), is located upstream of the gene sequence. This amino acid sequence enables the transfer of the generated proteins to the cell nucleus. For sustaining pBR322 in an *E. coli* strain, this vector includes the replication origin. For integration into the plant, the -designed- CRISPR construct was ligated between the LB (25 bp) and RB (25 bp) sections of the binary vector using an *Agrobacterium* strain. Additionally, this vector includes aminoglycoside phosphotransferase, which is generated by the aphA-3 gene outside of the LB-RB region and confers kanamycin resistance, allowing it to be utilized for bacterial selection. It also has a PacI enzyme restriction site in the vector's MCS, making it simple to ligate sgRNA cassettes to this area.

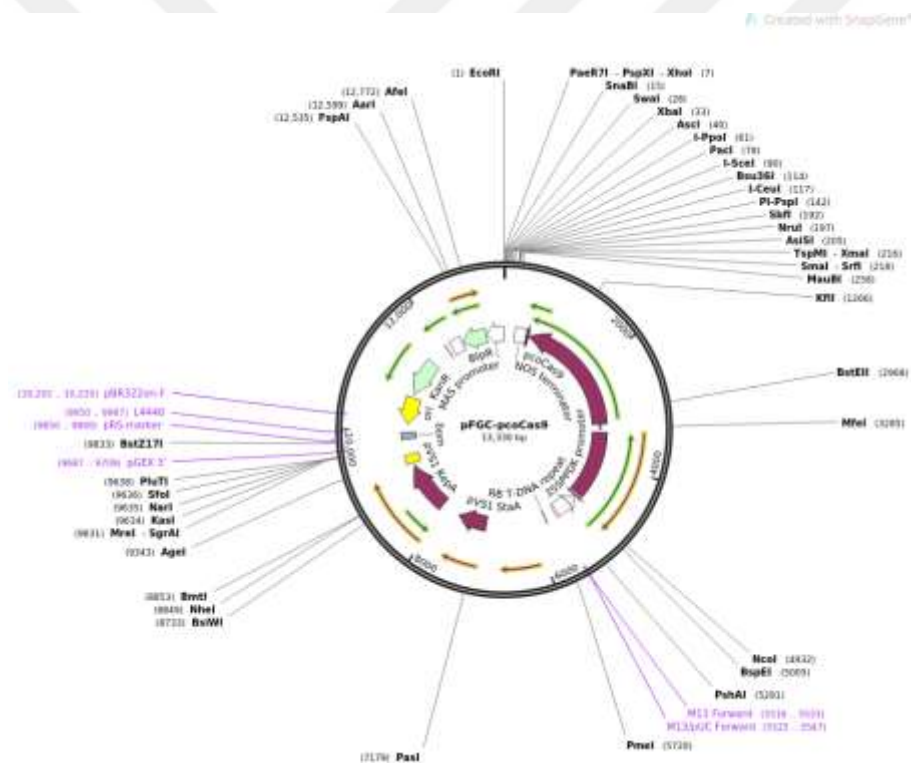


Figure 4.3. Vector pFGC-pcoCas9 shown schematically (Addgene plasmid #52256; <http://n2t.net/addgene:52256>; RRID: Addgene_52256)

4.4. Cloning experiment

Plasmids containing sgRNAs (produced by Sentebiolab Biotechnology Ankara, Turkey) were available. To multiply the stocks, the heat-shock transformation approach was used first, followed by plasmid purification. Competent *E. coli* (DH5 α strain) cells were used to perform heat shock transformation. To see whether these cells could withstand the antibiotic of Ampicillin, which would be employed for bacterial selection during the cloning of pZT57R-gRNA plasmids, these cells were first examined with

antibiotic-free medium and Ampicillin-containing media as a control. Through this test, it was confirmed that the *E. coli* DH5 α strain can be used (Figure 4.4a, b). To test the purified plasmids, the target area was amplified using gRNA primers and the expected bands were obtained.

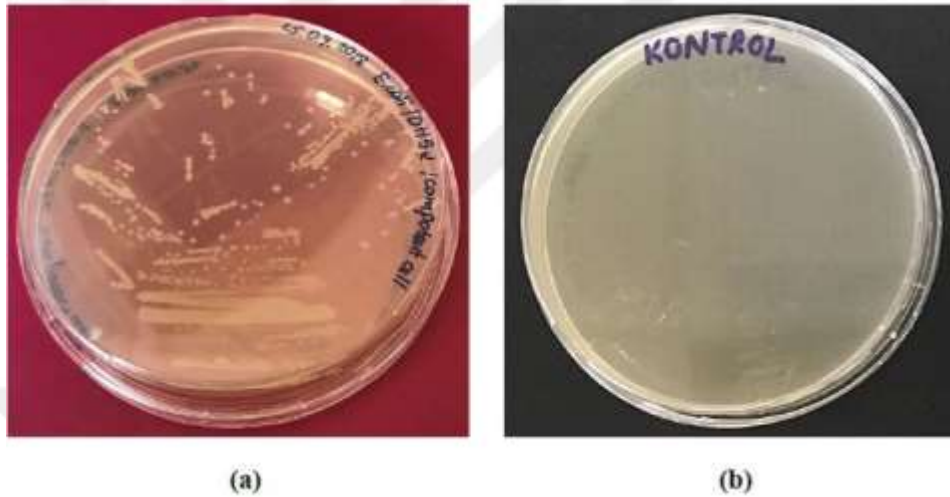


Figure 4.4. *E. coli* DH5 α viability test a) Cells grown in antibiotic-free media; b) Cells cultured in antibiotic-containing medium (Ampicillin) (Adapted from C. Simsek, 2021)

On the other hand, *E. coli* DH5 competent cells were examined in Kanamycin-containing media as a control (Figure 4.10). From the pFGC-pcoCas9 colonies that were transformed using the same technique, two colonies were chosen. They were evaluated with an ampicillin control medium as well as incubated for O/N at 225 rpm at 37°C in 5 ml agar-free LB and 5 l Kanamycin selection (added from 50 mg/ml Kanamycin stock) (Figure 4.5 a, b).

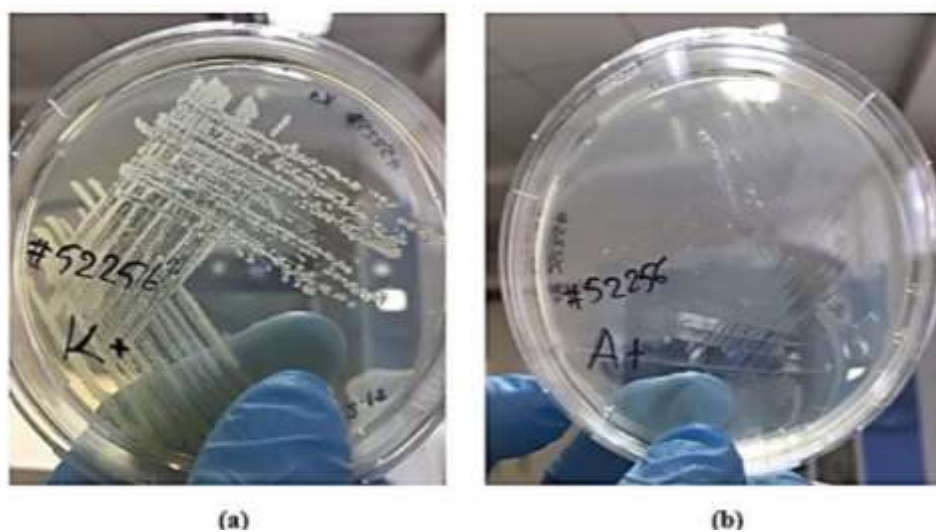


Figure 4.5. a) Colonies of pFGC-pcoCas9 growing on media containing kanamycin selection; b) Colonies of pFGC-pcoCas9 that cannot develop in a control environment with ampicillin selection (Adapted from C. Simsek, 2021).

Two enzyme restriction areas (EcoRI and NcoI) found on the pFGC-pcoCas9 plasmid itself were chosen for the digestion procedure in order to establish the existence of the two pFGC-pcoCas9 plasmid DNAs that had been isolated. By seeing two expected bands of 4931 bp and 8399 bp, it has been confirmed that the pFGC-pcoCas9 plasmid will be employed as a vector (Figure 4.6).

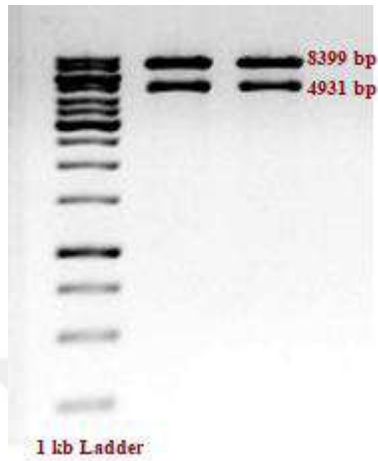


Figure 4.6. The outcome of the cut reaction between pFGC-pcoCas9 (# 52256) 1-2 plasmids and the 1 kb GeneRuler Ladder (Thermo Scientific) (Adapted from C. Simsek, 2021)

4.5. Digestion and ligation

It is important to first digest the vector in a combination with restriction enzymes in order to produce a vector containing the intended gRNAs. The pTZ57R plasmids produced fragments with sizes of 726 bp for SlCCD8-gRNA1 and 721 bp for SlCCD8-gRNA2 (Figure 4.7). These bands were excised from the gel and purified.

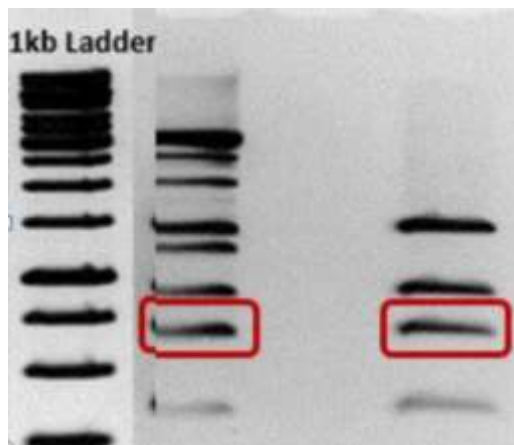


Figure 4.7. Digestion of pTZ57R-SlCCD8-gRNA1 and pTZ57R-SlCCD8-gRNA2 with the help of AclI and PacI enzymes (Adapted from C. Simsek, 2021)

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

TAATATTGGTTTATCTCATCGGAACTGCAAACTCAACTAACTGAAAAGTACAAAACCACTGAATCATATTATTTGAGATT
 TTTTITAGGTCAAATTTAGGTTTCAGTTACAGAAAACGAAGAGAAAACCCAGAAATTGAACGCCGAAGAACAGAGGAA
 GAAGAAATCGATCTGGAAAATTTGCAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTA
 TTTAACTTGCTATTTCTAGCTCTAAAACCTCAACGACGAGTTCTCCTTCAATCACTACTTCGACTCTAGCTGTATATAAACTCA
 GCTTCGTTTTCTTATCTAAGCGATGTGGGACTTTTGAAGATTGTTTTCAACTTAAATGGGCCTATATAAGAAATACTATTGTT
 CTTTCCCATATAAATGGGCCTGCTTCTCTTCTTTCAGATTCCCAGGGGCCTTTTGAAGATTATCTTCATATCTTAAGAATGAA
 GATGTTTTATTCAATCAAATCTTGAAGGTTTCGATGCCTAATCATTCTAATCCTGGGACAACTATGAAACAAGATACAAAA
 ACTCGAATGGAAGTTAAAAAGAAGAAAACGAAAGCTACGGTTCAAGAAAATGTAAGCTGATAAACAAAAAAAACCTG
 TATGAACGAAGAAGAAGAAAAAAGCTAAGAAGAAATGATGTATTGTGCGGAAGGCAAGTCGACTAGTAAAA**AA**

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

CGTTTATTGGTTTATCTCATCGGAACTGCAAACTCAACTAACTGAAAAGTACAAAACCACTGAATCATATTATTTGAGATT
 TTTTITAGGTCAAATTTAGGTTTCAGTTACAGAAAACGAAGAGAAAACCCAGAAATTGAACGCCGAAGAACAGAGGA
 AGAAGAAATCGATCTGGAAAATTTGCAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTT
 ATTTAACTTGCTATTTCTAGCTCTAAAACGGATACGGTGGTGAGAATAATCACTACTTCGACTCTAGCTGTATATAAAC
 TCAGCTTCGTTTTCTTATCTAAGCGATGTGGGACTTTTGAAGATTGTTTTCAACTTAAATGGGCCTATATAAGAAATACTATT
 GTTCTTCCCATATAAATGGGCCTGCTTCTCTTCTTTCAGATTCCCAGGGGCCTTTTGAAGATTATCTTCATATCTTAAGAAT
 GAAGATGTTTTATTCAATCAAATCTTGAAGGTTTCGATGCCTAATCATTCTAATCCTGGGACAACTATGAAACAAGATACA
 AAAACTCCGAATGGAAGTTAAAAAGAAGAAAACGAAAGCTACGGTTCAAGAAAATGTAAGCTGATAAACAAAAAAA
 CTGTATGAACGAAGAAGAAGAAAAAAGCTAAGAAGAAATGATGTATTGTGCGGAAGGCAAGTCG**TAAAT**

Figure 4.8. Sequences of SICCD8-gRNA1 and gRNA2 in the pTZ57R plasmid and in bold font are sequence information for restriction regions of the AclI and PacI enzymes

The PacI restriction site on the pFGC-pcoCas9 (# 52256) plasmid was digested to prepare it for the ligation of the gRNA products. After the enzymatic digestion was scraped from the agarose gel with a scalpel and purified. By adding rSAP (Shrimp Alkaline Phosphatase), which dephosphorylates the 5'-ends, the cut vector is prevented from self-ligating.

Using *E. coli* competent cells (DH5 α strain), the resultant vectors were then transformed using the heat-shock procedure.

T4 ligase is used to join the pFGC-pcoCas9 (#52256) digested vector and gRNAs together. The gRNAs will be introduced into the digested vector as a result of this ligation process. A phosphodiester link is created between the 3' hydroxyl group of one fragment and the 5' phosphate group of another fragment thanks to the T4 ligase.

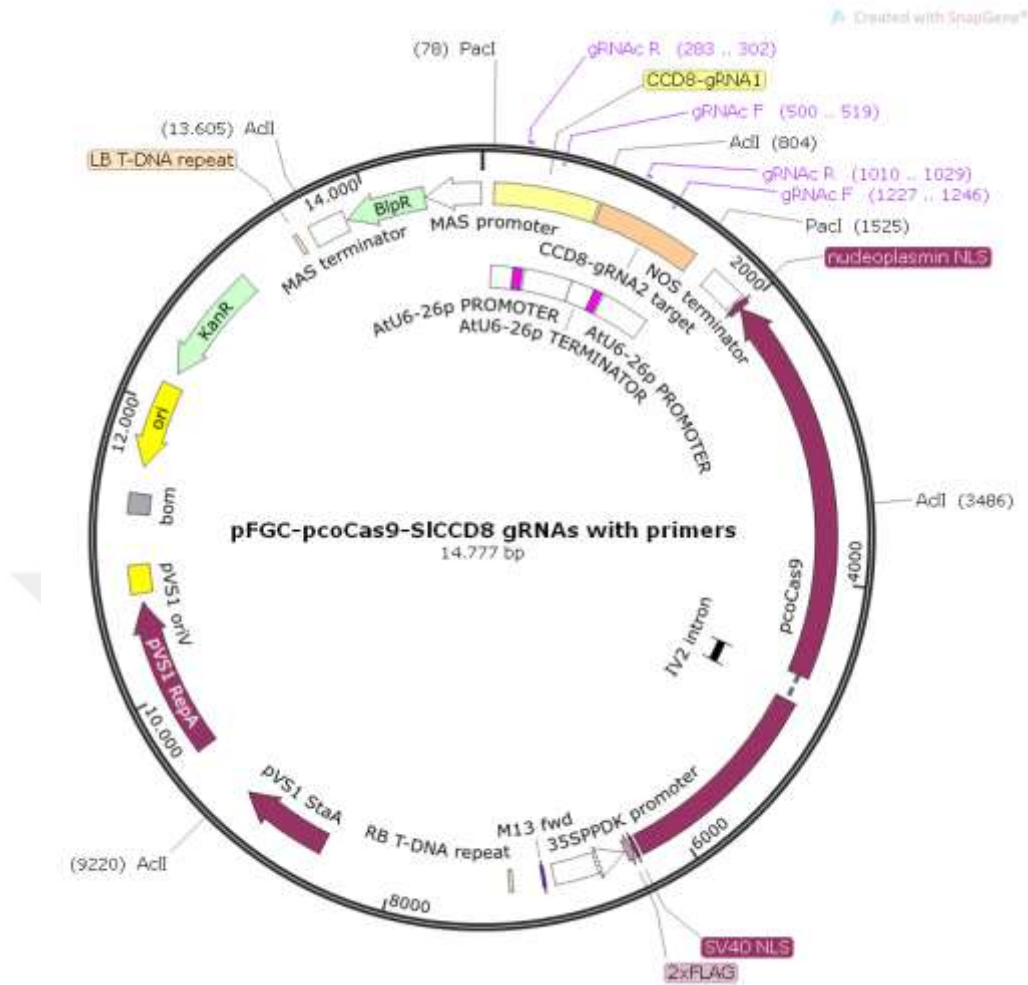


Figure 4.9. Following the ligation of the SICCD8 gRNA1+gRNA2 to the pFGC-pcoCas9 vector, a representative plasmid map was generated. The locations of the gRNAs primers on the plasmid are also shown (Adapted from C. Simsek, 2021)

4.5.1. Confirmation of gRNAs insertion

Five colonies were chosen from both media and utilized as template DNA for colony PCR using gRNAc-F/R primers to assess the accuracy of the colonies chosen for plasmid isolation following transformation (Figure 4.10).

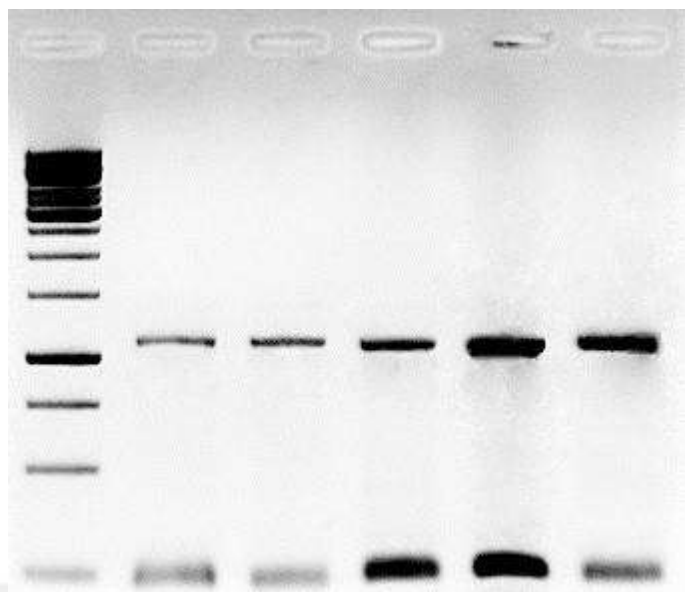


Figure 4.10. Results from PCR colony where 5 colonies were selected as a result of ligation of CCD8 gRNA1+gRNA2 to the pFGC-pcoCas9 (#52256) plasmid, it was used GeneRuler Ladder (Thermo Scientific) (Adapted from C. Simsek, 2021).

It was apparent during the ligation procedure that the gRNAs selected in accordance with the SICCD8 gene were effectively ligated to the pFGC-pcoCas9 vector.

4.6. Bacterial Transformation

Using electroporation the ligated product, was transformed with the *Agrobacterium tumefaciens* EHA105 strain. After transformation, cells were cultured in a medium containing 25 µg/ml Rifampicin and 50 µg/ml Kanamycin (a bacterial selection agent for the pFGCpcoCas9 plasmid). The empty competent EHA105 strain was used as a control. Due to the absence of Kanamycin resistance, the empty competent EHA105 strain cells were unable to survive, while gRNAs were colonized in the selection medium (Figure 4.11).



Figure 4.11. Colonies formed after transformation and empty EHA105 strain (Adapted from C. Simsek, 2021)

One more time PCR was performed to check the correctness of transformation with the *A. tumefaciens* EHA105 strain (Figure 4.12).

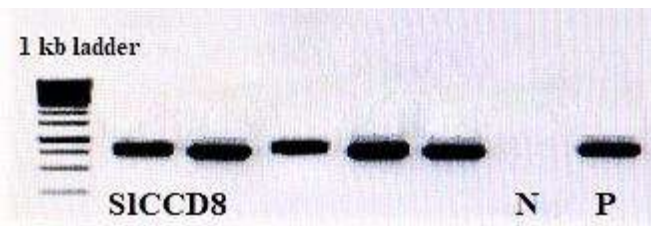


Figure 4.12. Results from colony PCR for CCD8-gRNA F/R primer pairs confirmed the correctness with expected band at 755bp

4.7. Confirmation of similarity sequences

We confirmed the sequence by alignment before we can begin the transformation of the construct to the eggplant genome. Coding sequences of CCD8 *Solanum lycopersicum* L. and *Solanum melongena* L. have 90.88% identity (Figure 4.13). Sequences were identified in 8 chromosome of *Solanum melongena* L. (Figure 4.14).



Figure 4.13. Coding sequences between CCD8 *Solanum lycopersicum* L. and *Solanum melongena* L matched 90.88%

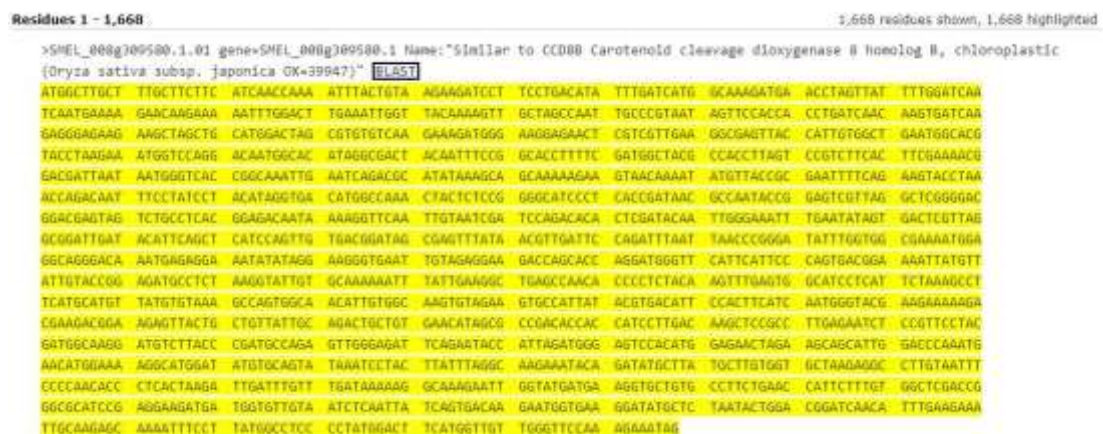


Figure 4.14. Information of *Solanum melongena* L. sequence similar to SICCD8

Next, using the Clustal Omega tool, we aligned the tomato CCD8 construct's exons 1 and 2, from which two gRNAs were created to see whether they matched eggplant. Without any mismatches, the gRNA1 sequences designed in exon 1 of the tomato genome were similarly identified in the genome of the eggplant (Figure 4.15).

CLUSTAL O(1.2.4) multiple sequence alignment

```

solanumlyco_exon1      ATGGCTTCTCTTGCTTCTTCAACAACCAAAATTTATTGTAACAAGATCCTTCTGACATG 60
SMEL_008g309580.1.01  ATGGCTTGCTTTGCTTCTTCAACAACCAAAATTTACTGTAAGAAGATCCTTCTGACATA 60
*****

solanumlyco_exon1      TTTGATCATGGCAAACATGAATCTCATCTTGGATCAAAGTTGAAAAACAACGAAAAAAC 120
SMEL_008g309580.1.01  TTTGATCATGGCAAAGATGAACCTAGTTATTTTGA-----TCAATCAATGAAAAGAAC 114
*****

solanumlyco_exon1      AAGAAAAAATTGGACTTGAAATTGGTTACAAAAGTTGCTAGCCAATTGCCTGTAATAGTT 180
SMEL_008g309580.1.01  AAGAAAAATTTGGACTTGAAATTGGTTACAAAAGTTGCTAGCCAATTGCCCGTAATAGTT 174
*****

solanumlyco_exon1      CCACCACCAGATCAAGAGGTGATTAGTAAGGAGAAAAAGCTTGCTGCATGGACTAGCGTA 240
SMEL_008g309580.1.01  CCACCACCTGATCAACAAGTGATCAAGAGGGAGAGAAGCTAGCTGCATGGACTAGCGTG 234
*****

solanumlyco_exon1      CGCCAAGAAAGATGGGAAGGAGAACTCGTCGTTGAAGGCGAGTTACCATTTGTGGCTG--- 297
SMEL_008g309580.1.01  TGTCAAGAAAGATGGGAAGGAGAACTCGTCGTTGAAGGCGAGTTACCATTTGTGGCTGAAT 294
*

solanumlyco_exon1      ----- 297
SMEL_008g309580.1.01  GGCACGTACCTAAGAAATGGTCCAGGACAATGGCACATAGGCGACTACAATTTCCGGCAC 354

solanumlyco_exon1      ----- 297
SMEL_008g309580.1.01  CTTTTCGATGGCTACGCCACCTTAGTCCGCTTCACTTCGAAAACGGACGATTAATAATG 414

solanumlyco_exon1      ----- 297
SMEL_008g309580.1.01  GGTCACCGGCAAATTGAATCAGACGCATATAAAGCAGCAAAAAGAAGTAACAAAATATGT 474

```

Figure 4.15. gRNA1 was identified in eggplant coding sequences without any mismatch

Sequences of gRNA2 created in tomato exon 4 showed mismatches when compared to gRNA1, indicating that these gRNA2 may not function in the eggplant genome (Figure 4.16).

solanumlyco_exon2	-AATGGCACGTACCTAAGAAATGGTCCAGGACAATGGCACATAGGTGACTACAATTTTCG	59
SMEL	GAATGGCACGTACCTAAGAAATGGTCCAGGACAATGGCACATAGGCGACTACAATTTCCG	60

solanumlyco_exon2	TCACCTTTTCGATGGCTACGCTACCTTAGTCCGTCTTCATTTGAAAATGGACGATTAAT	119
SMEL	GCACCTTTTCGATGGCTACGCCACCTTAGTCCGTCTTCACTTGAAAACGGACGATTAAT	120

solanumlyco_exon2	CATGGGTATAGACAAATCGAATCGGACGCATATAAGCAGCAAAAATCAGTAAGAAAAT	179
SMEL	AATGGGTACCCGGCAAATGAATCAGACGCATATAAGCAGCAAAAAGAGTAACAAAAT	180

solanumlyco_exon2	ATGTTACAGAGAAATTTTCAGAAAGTACCTAAAGTAGACAATTTCTTATCTACATAGGTGA	239
SMEL	ATGTTACCGCGAAATTTTCAGAAAGTACCTAAACCAGACAATTTCTTATCTACATAGGTGA	240

solanumlyco_exon2	CATGGCAAAATTAATCTCTCCGGTGCATCCCTAACCGATAATGCTAACACTGGAGTCGTTAA	299
SMEL	CATGGCCAAACTACTCTCCGGGGCATCCCTCACCGATAACGCCAAATACCGAGTCGTTAG	300

solanumlyco_exon2	ACTTGGGGATGGACGCGTAGTCTGCTTAACTGAGACGATAAAAGGTTCCATTGTAATTGA	359
SMEL	GCTCGGGGACGGACGAGTAGTCTGCCTCAGCGAGACAATAAAAGGTTCAATTGTAATCGA	360

solanumlyco_exon2	TCCGAACACCCTAGATACAATTGGGAAATTTGAATATAGTGACTCGTTAGGTGGATTGAT	419
SMEL	TCCGAGACACCTCGATACAATTGGGAAATTTGAATATAGTGACTCGTTAGGCGGATTGAT	420

solanumlyco_exon2	TCATTGAGCTCATCCAGTGGTTACGGACAGTGAGTTTATAACGTTGATTCGGGATTTAAT	479
SMEL	ACATTGAGCTCATCCAGTGGTACGGATAGCGAGTTTATAACGTTGATTCAGATTTAAT	480

solanumlyco_exon2	GAACCCGGGATATACGGTGGTGAGAATGGAGGCAGGGACAAATGAGAGGAAGTATATAGG	539
SMEL	TAACCCGGGATATTTGGTGGCGAAAATGGAGGCAGGGACAAATGAGAGGAAATATATAGG	540

solanumlyco_exon2	GAGAGTGAGTTGTAGAGGAGGACCAACGACGAGATGGGTTCAATTCCTGTTACAGA	599
SMEL	AAGGGTGAATTGTAGAGGAGGACCAACGACGAGATGGGTTCAATTCCTGTTACAGA	600
	**	
solanumlyco_exon2	AAATTATGTTATTGTGCTGAGATGTCACTAAGGTATTGTGCAAAAAATTTGTTGAAGGC	659
SMEL	AAATTATGTTATTGTACCGGAGATGCCCTCAAGGTATTGTGCAAAAAATTTGTTGAAGGC	660

solanumlyco_exon2	TGAGCCAACACCACTGTATAAGTTTGAGTGGCATCCTGATTCTAAAGCATTTGTACATGT	719
SMEL	TGAGCCAACACCCCTCTACAAGTTTGAGTGGCATCCTGATTCTAAAGCCTTCATGCAATGT	720

solanumlyco_exon2	TATGTGTAAAGCCAGTGGCAACAT---	743
SMEL	TATGTGTAAAGCCAGTGGCAACATTGT	747

Figure 4.16. Mismatches of gRNA2 in *Solanum melongena* L.

4.8. Transformation

Many scientists have sought to create stable transgenic plants for use in breeding programs as well as to design the transformation technique. However, there is still a need for a thorough analysis to improve the influencing common elements, such as genotype, explants, *Agrobacterium* strains, selection markers, and culture conditions. Effective transformation techniques fundamentally involve direct or indirect organogenesis to have effective control over the plant regeneration from the infected explants.

For the transformation of the Yamula variety, a total of 160 cotyledon explants were used, and 100 cotyledons for the Aydin Siyahi variety. Plant regeneration and callus induction were carried out from cotyledons. Co-cultivated leaf explants from in vitro pre-cultures were permitted to develop a white friable callus. The shoots were formed from the calli in a selection medium. Yamula was able to successfully undergo this transformation, however Aydin Siyahi was unable to do so due to the fact that the callus of Aydin Siyahi does not regenerate transformed shoots (Figure 4.17 and Figure 4.18).

Transformation of recombinant DNA and the regeneration of plantlets need an effective protocol. Ineffective transformation and regeneration techniques may limit the delivery of desired genes for overexpression or desired gene editing components, like Cas9 and guide RNA, for agronomically important traits (Khatun and et al., 2022). It was found that MS+1 mg/ml ZEATIN and 1 mg/ml IAA are ideal hormone supplements for optimum cell division and shoot production in the variety of Yamula used in the study.

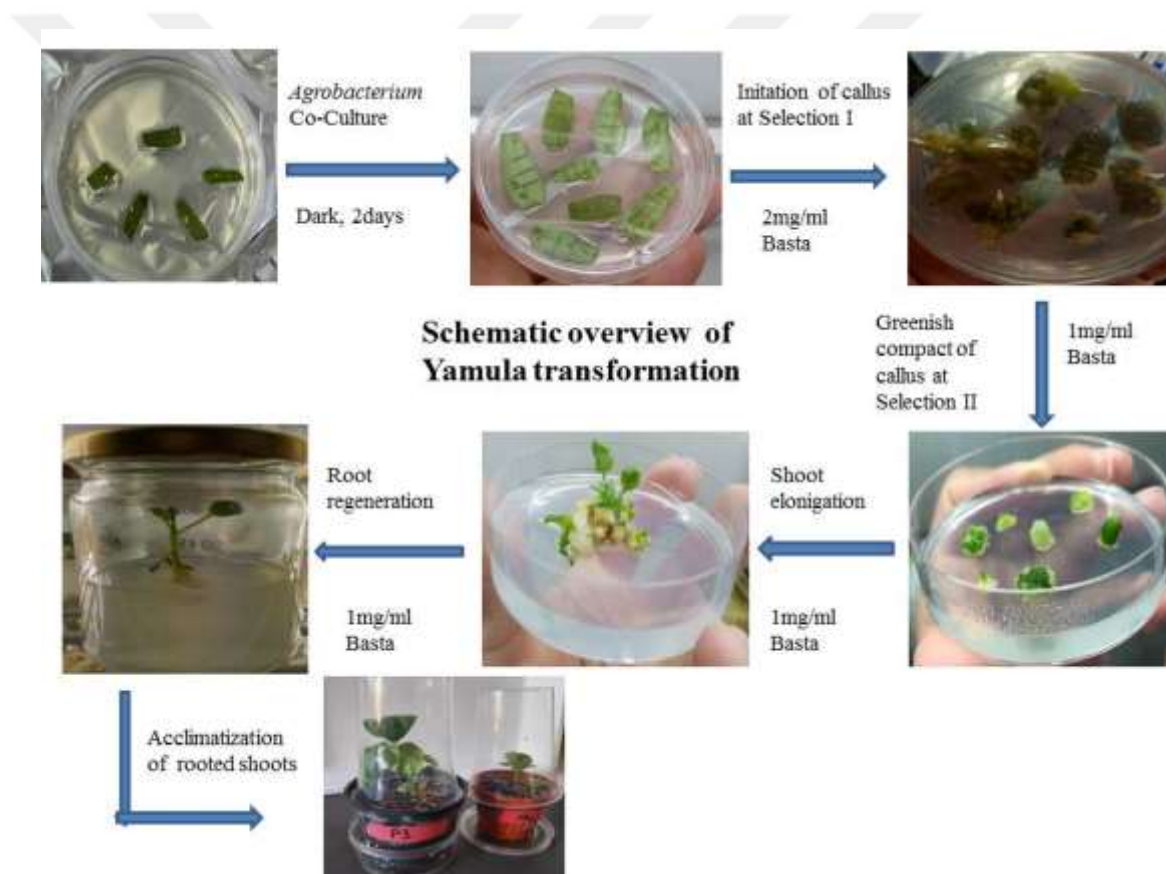


Figure 4.17. Transformation on Yamula eggplant was successfully completed

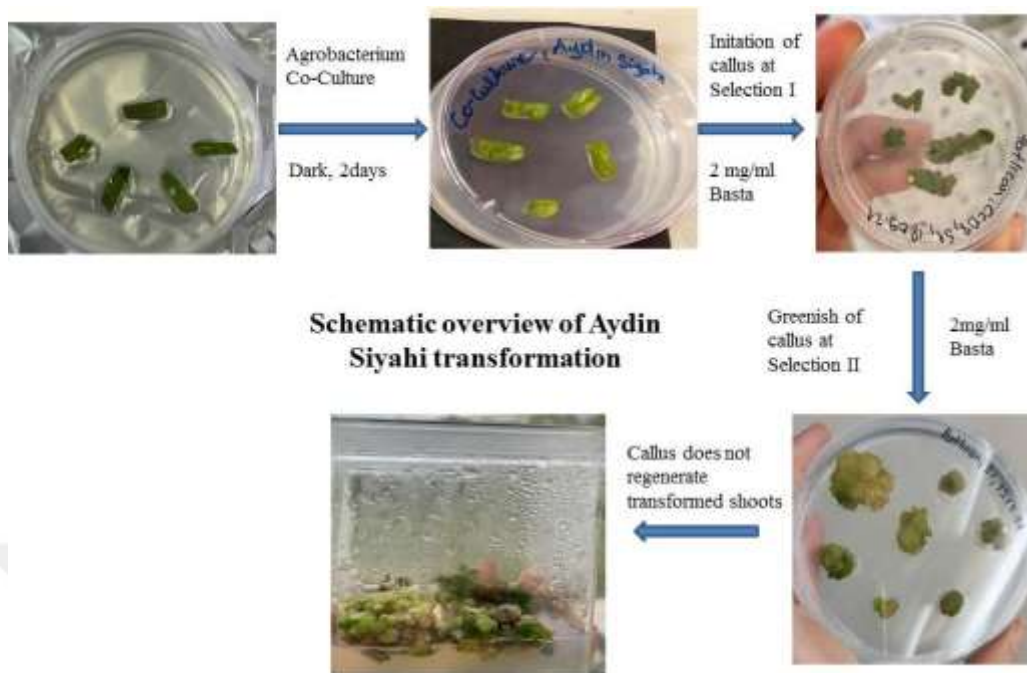


Figure 4.18. Aydin Siyahi's transformation resulted in calluses that did not regenerate on the transformed shoots

4.8.1. Evaluation of eggplant transformation efficiency

The formula for calculating transformation efficiency is $\frac{np}{ni} \times 100\%$, where **np** is the number of transgenic seedlings that are positive and **ni** is the number of explants that were first infected. On the basis of this formula, we performed the calculations necessary to determine the transformation efficiency for the Yamula genotype. We ended up with 16 positively mutated plants out of a total of 160 explants, bringing our success rate to 10%.

$$\text{Percentage of transformation efficiency} = \frac{np}{ni} \times 100 = \frac{16}{160} \times 100 = 10\%$$

With a transformation efficiency of 10% overall, 21,4% of the infected in vitro leaves developed resistant callus and 55% of the callus generated shoots (Table 4.2). In the presence of 1 mg/L Basta, the regenerated shoots were capable of forming roots and grew normally in the greenhouse. Aydin Siyahi did not respond to transformation as Yamula did. From Aydin Siyahi, 55% of the explants revealed callus development, however these calli did not show any shoot initiation.

Table 4.2. Percentage of eggplant explants that survived transformation

A stage of development	Transformed Yamula		Transformed Aydın Siyahi	
	Survived total	Percentage	Survived total	Percentage
Explants	160	100%	100	100%
Number of explants after co-culture	140/160	87,5%	100/100	100%
Explants developing calli	30/140	21.40%	55/100	55%
Shoot initiation	75/30	250%	0	0
Number of rooting shoots	67/75	89.30%	0	0

4.9. Molecular characterization

We looked for mutational events in the T0 generation to help us decide which T0 lines to go on with for further screening. This is how we advise testing the plants for mutations.

DNA extraction

First, DNA was isolated from eggplant young leaves using the CTAB method in order to test for mutations. As a PCR template, the extracted DNA was used. Specific primers were used in the PCR reaction, 52256-2 and gRNA CCD8 primers.

PCR

In order to produce CCD8 mutants with expected indels at positions 40bp and 60bp in the *SmCCD8* gene, two gRNAs was developed. Using particular primers, PCR analysis of T0 transgenic plants demonstrates positive amplification of anticipated bands (Figure 4.19).

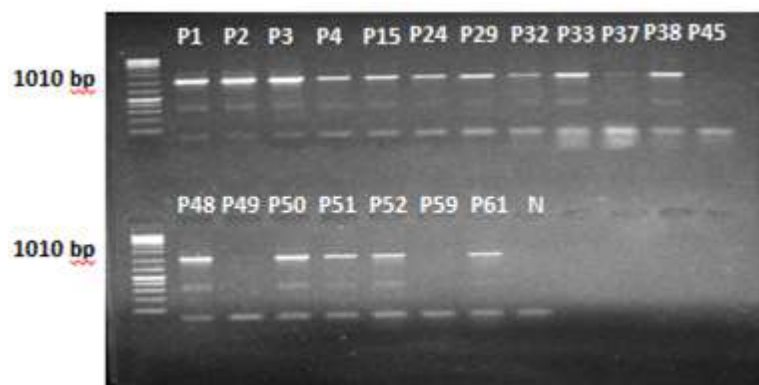


Figure 4.19. The results of the PCR showed the predicted bands at 1010bp for gRNA1 for 15 plants.



Figure 4.21. Chimeric mutations are presented in P3

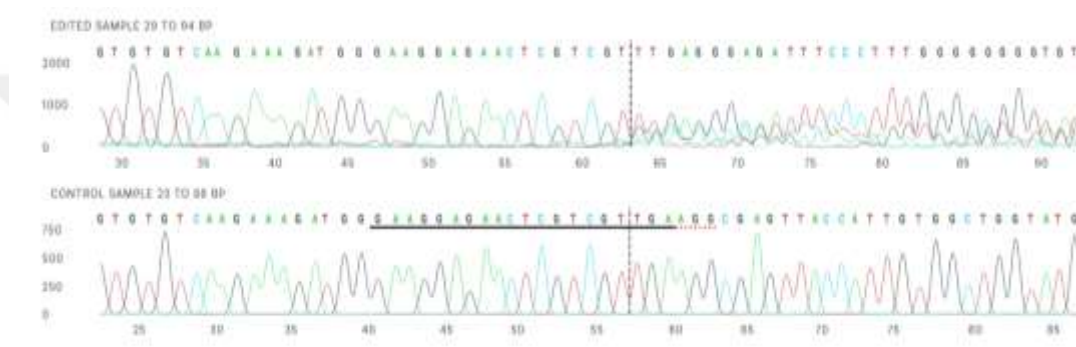


Figure 4.22. Image shows mutant P3 and wild-type (control) sequences in the guide target sequence area.

Insertion of 1 nucleotide in P4 resulted in formation of stop codon and confirmation of gene knockout (Figure 4.23). In wild type, normally after the amino acid valine (GTT), another amino acid glutamate comes next (GAA). However, following the valine GTT there came a 1bp insertion T, which resulted in the stop codon TGA in the mutant P4 (Figure 4.24).



Figure 4.23. Gene editing efficiency show respectively a nucleotide insertion 96% (Adapted from <https://ice.synthego.com>)

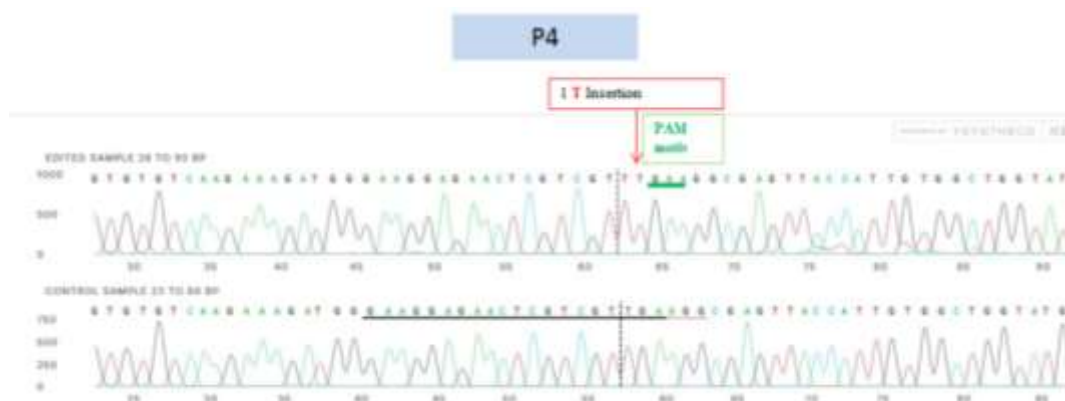


Figure 4.24. Insertion of one nucleotide (T) is present in P4 (Adapted from <https://ice.synthego.com>)

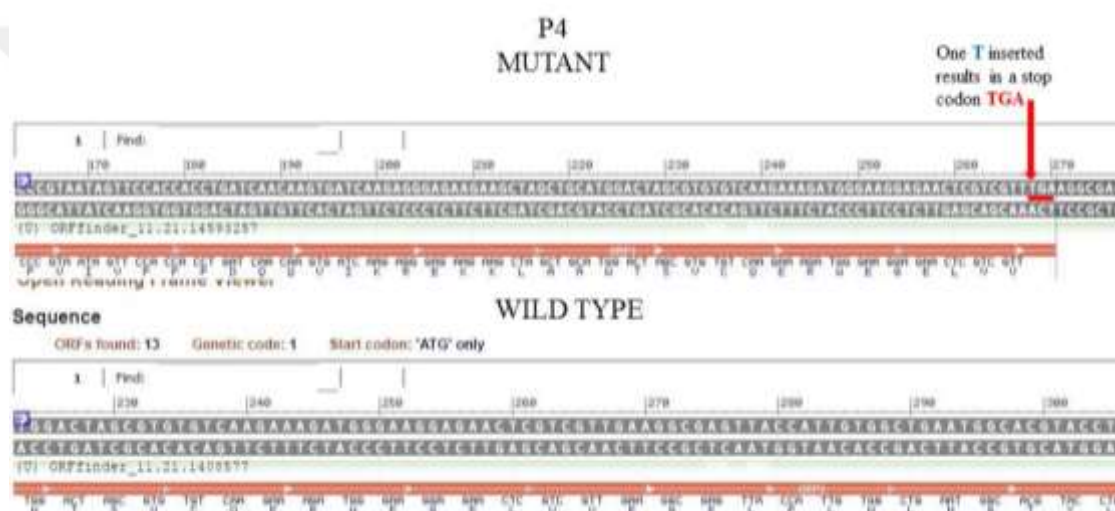


Figure 4.25. Insertion of 1 nucleotide in P4 resulted in formation of stop codon and confirmation of gene knock-out (Adapted from <https://www.ncbi.nlm.nih.gov>.)



Figure 4.26. Protein alignment between P4 mutant and wild type (Adapted from <https://www.ebi.ac.uk>)

T1 Generation

From T0, was obtained 16 positive mutants. However, we only used three of them in T1 because we lost other lines due to hot temperatures that caused fruit abortion (P1, P3, P38, and P4). In T1, we received 1 event from P1, 49 events from P3, 36 events from P38, and 6 events from P4.

P1 was sequenced using the Sanger method during the T1 generation, and the results showed that it had two insertions that caused it to have a homozygous knockout mutant, which was confirmed by single peaks at the mutation site. As result of two (T) insertions framshift it happened from where 5 aminoacids changed, due to this it came formation of stop codon and confirmation of CCD8 knockout gene for P1 (Figure 4.27 and Figure 4.28).

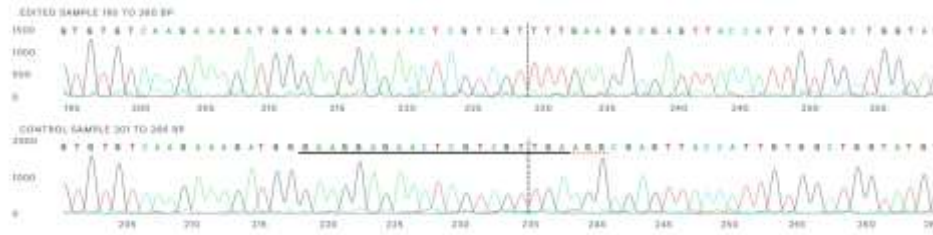


Figure 4.27. Insertion of two (T) nucleotides in P1 (Adapted from <https://ice.synthego.com>)

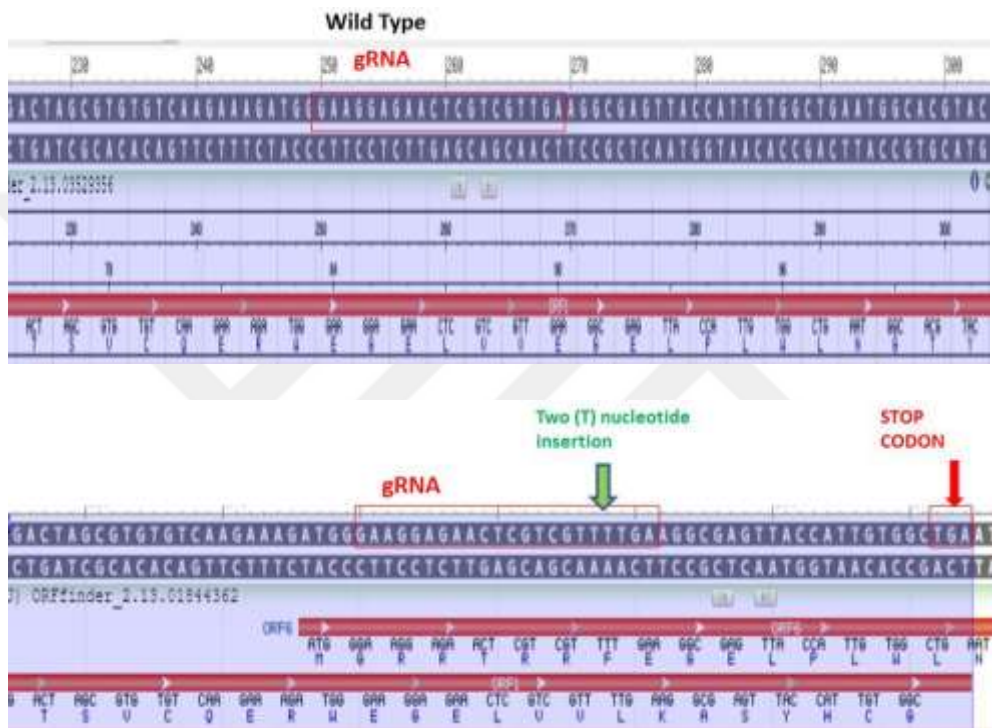


Figure 4.28. Confirmation of stop codon and knockout of CCD8 in P1 mutant

The lack of viable pollens is what causes fruit abortion since, at high temperatures, pollen production is severely decreased, resulting in fewer fruits per plant and poorer output overall. The ideal temperature range for fruit development and production, specifically for eggplant, is between 22 and 32°C. The natural dwindling of daylight and the high temperature of the night (30°C) encourage flower abortion, and temperatures over 32°C have a significant negative impact on production (RNV and et al.). Plants were tested with CCD8 gRNA1 primer and with the use of 52256-2 primers, the T1 seeds (obtained from T0 plants) were once again examined for the presence of the Cas9 protein. Cas9-free seeds are chosen to produce stable mutant plants devoid of unwanted wild-type allele mutations and off-target mutations, the transgenic free lines are highlighted in red in the lines (Figure 4.29).

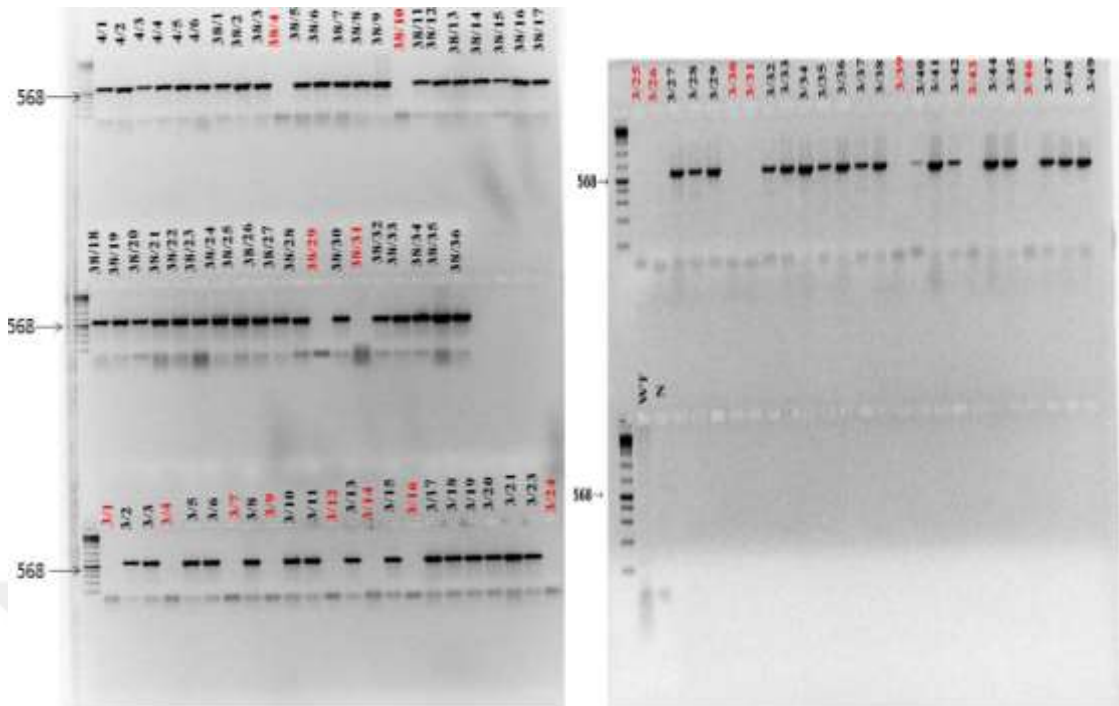


Figure 4.29. T1 generation showing transgene free lines which are highlighted in red

Plants of the T1 generation are often chimeras because the Cas9 editing mechanisms work separately in each cell of the plant. This results in different genotypes being present in the various cells of the plant. It is only possible for progeny plants to develop with an uniform genotype if a mutation first occurs in the germline cells. Because of this, screening techniques for the discovery of mutations should also be used in T2.

T2 Generation

T2 generation was achieved by selfing of T1s. To learn how CRISPR/Cas9-mediated alterations are transmitted, the genotypes at the target sites T2 progeny were analyzed. As expected, P4 progeny of gRNA1- CCD8 were each knockout in T0, demonstrating that the mutations were carried down to the following generation when we saw homozygous knockout once more in T2.

When we tested the P3 (Chimeric) and P38 progenies' plants in a hydroponic system, they demonstrated resistance to *P. aegyptica*. However, we used the roots of these plants to measure their biomass. And for Sanger Sequencing, abscisic acid and gene expression analysis we used other seeds from P3 and P38. From Sanger Sequence results we understand that mutation of P3 (chimeric) and P38 was not inherited in these plants. Next, to do, it's to test one more time these genotypes on hydroponic way and in resistant ones to search for mutation. P1 and P4 homozygous knockout mutants, found to be carrying the transgenes associated with CRISPR-Cas9.

4.10. Phenotypic profile in lines with ^{CCD8}Cas9 mutations and resistance to *P. aegyptiaca*

Eggplant CCD8 T-DNA insertion line were isolated and cultivated in parallel with eggplant WT plants to provide a visual reference of the predicted phenotype. In T2 generation we observed variations between mutants and wild type in terms of morphology. P1 and P4 lines with CCD8 gene knockout exhibited a significant difference, reduced plant height, and increased branching compared to P3 lines with chimeric mutations, which showed a little drop in plant height (Figure 4.30 and Figure 4.31).



Figure 4.30. Phenotypic profile in lines with CCD8Cas9 mutations



Figure 4.31. Enhancement of branching in the *SmCCD8* eggplant lines: **a)** internode length on wild type eggplant; **b)** *SmCCD8* knockout mutant internode distance was shorter, and the plant had more branches than the wild type; **c)** increased shoot branch number on the *SmCCD8* P1 knockout mutant

P. aegyptiaca seeds were tested with mutant eggplant plants from T2 lines in hydroponic way. This was done to see if the CRISPR/Cas9-generated mutations in the *CCD8* gene make the plant resistant to *P. aegyptiaca*. Because *P. aegyptiaca* germination surrounding the roots of the mutants was much lower than that of the WT, the roots of the mutants had less *P. aegyptiaca* infection. Due to this, the ^{CCD8}Cas9 mutant lines had considerably less connected parasite tubercles in comparison to the wild-type. Although it was less severe than what was shown for lines P3 and P38, *P. aegyptiaca* decreased drastically in some of the knock out mutant line P4. Because there was no parasite seed germination in the P4 mutant, no tubercle development was observed (Figure 4.32).

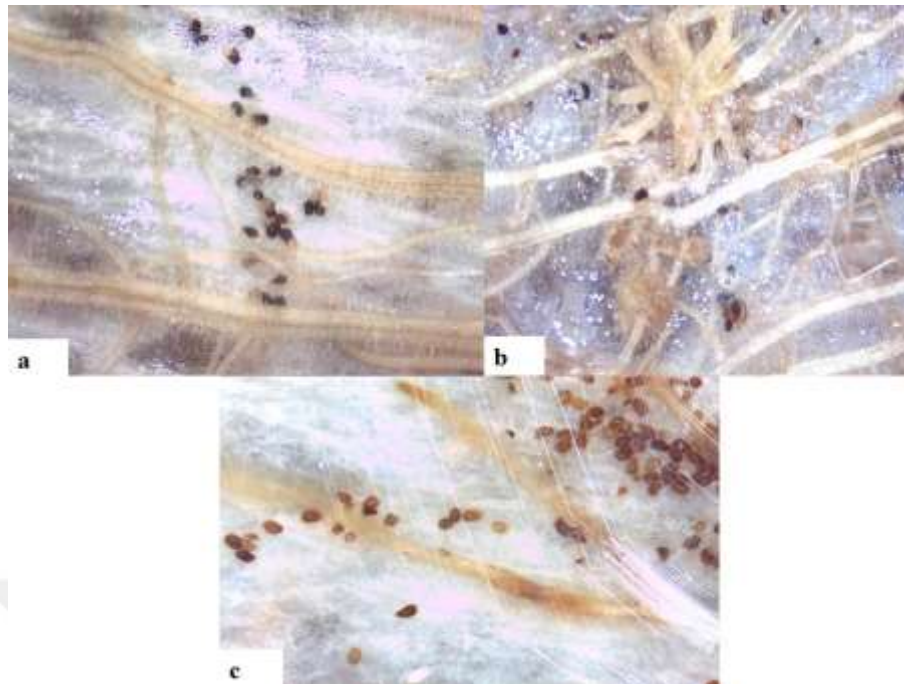


Figure 4.32. Differences that occurred throughout the infection process between wild-type eggplant plants and mutant P4 plants were observed under the microscope while employing the rhizotron system; **a)** germination of *P. aegyptiaca* near of wild type roots; **b)** on the wild-type plant's roots the parasite was able to successfully penetrate its host and cause the development of tubercles; **c)** no tubercle formed because no parasites germinated close to P4-2 mutant roots

Similar findings in tomato were also reported by Kohlen et al. (2012), who discovered that interestingly, compared to wild-type plants, the infection of all *SICCD8* knock-down lines by *P. ramosa* was decreased by 90%. Our results are also in line with those of a previous study by Hasegawa et al. (2018), in which the *SICCD8* gene was mutated in the Micro-Tom tomato using the TILLING method. When mutants were compared to the wild type, it was found that *Phelipanche aegyptiaca* infection was less common in the *SICCD8* mutants than in the wild type due to low germination of *P. aegyptiaca*.



Figure 4.33. Number of attached tubercles of *P. aegyptiaca* to the was significantly reduced in the P3-28 mutated lines compared to the wild type



Figure 4.34. Tubercule formation on wild-type roots of the eggplant and no tubercule formation on the roots of the P3-47 mutant



Figure 4.35. Massive *P. aegyptiaca* infection of wild-type roots during a one-month hydroponic root screening system test

According to the findings of our research, the total number of attached parasite tubercles saw a considerable reduction in comparison to the non-transgenic plants: (Figure 4.36).). In addition to lower infection rate, the fresh weights of parasite tubercles connected to lines P3, P38, and P4 were much lower than those of parasite tubercles attached to the control plants. This difference was statistically significant (Figure 4.37).

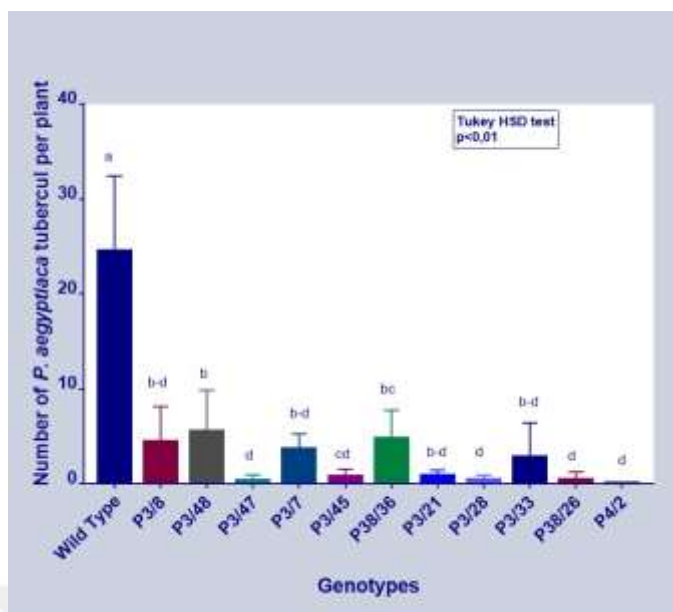


Figure 4.36. Distribution of the tubercle/rhizotron for 12 lines and wild type. The mean of each experiment is shown by a bar in the graph.

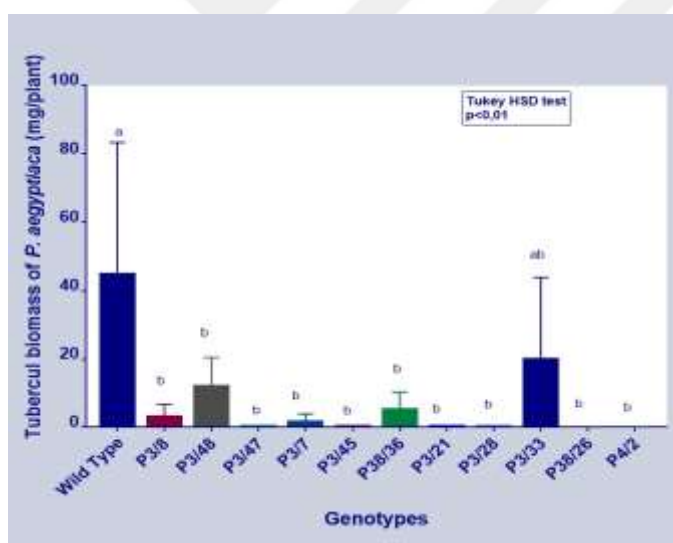


Figure 4.37. The bars show the average results of tubercle biomass of *P. aegyptiaca* using 12 separate line plants in T2 generation



Figure 4.38. Mutants and wild type tested with *P. aegyptiaca* seeds in pots

4.11. Real-Time Quantitative PCR for CCD8

In order to determine the stability of expression of the selected CCD8 gene, mRNA expression levels were measured in eggplant young leaf. Before we performed qRT-PCR, first as a control, we carried out PCR using Actin primers with the parameters outlined in the (Table 3.4 and Table 3.5), to be sure if samples aren't contaminated with genomic DNA (Figure 4.39.)

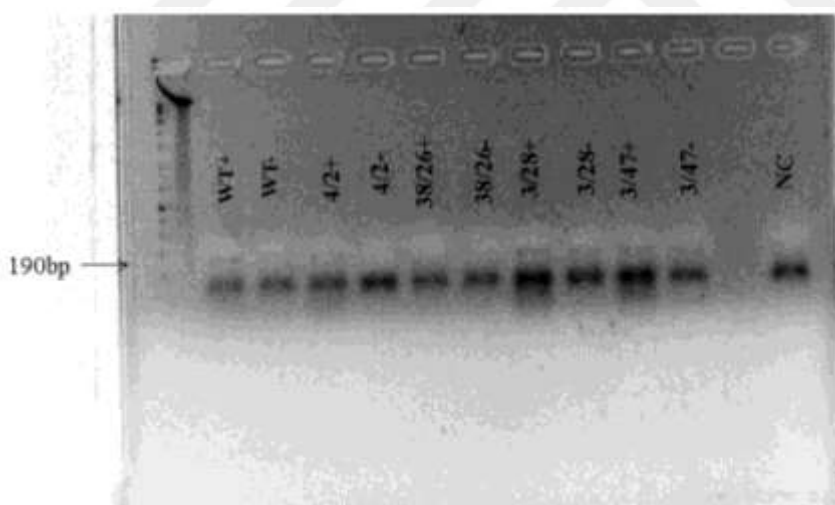


Figure.4.39. RNA samples tested with Actin primers to be sure if samples aren't contaminated with genomic DNA

To perform qRT-PCR we used reference gene and target gene, which in our case was CCD8 gene. Reference genes are internal controls whose expression must be steady across samples and experimental circumstances. The expression of these "housekeeping" genes might vary by sample species, genotypes, tissues, developmental stages, treatments, or experimental settings (Lacerda and et al., 2015).

Ct mean values were further used for the expression stability calculation. The quantitative real-time PCR method was used to analyze the level of CCD8 expression,

with Actin serving in the role of an internal control. The levels of CCD8 expression that were found in P1, P4-2, P38-26, P3-28, P3-47 mutant plants were analyzed compared with wild type. According to the results, the quantity of the CCD8 transcript was higher in mutants than it was in the wild-type plants (Figure 4.32). This suggests that the focused mutagenesis seen in CCD8 resulted in the increased levels of the transcript in th mutants. The experiment was performed with three independent biological replicates, and for each biological replicate two technical replicates were done. The results are presented with the mean $\pm$ SD. The significance of differential expression levels was analyzed using the Student's t-test (Figure 4.40).

Table 4.3. Data of Ct value for CCD8

Table format: Grouped		Group A	Group B	Group C	Group D	Group E	Group F
		WT	P4-2	P38-26	P3-28	P3-47	Title
		Y	Y	Y	Y	Y	Y
1	ccd8	4.365	3.330	1.005	5.125	4.860	
2	ccd8	4.460	3.330	1.805	6.335	6.795	
3	Title						

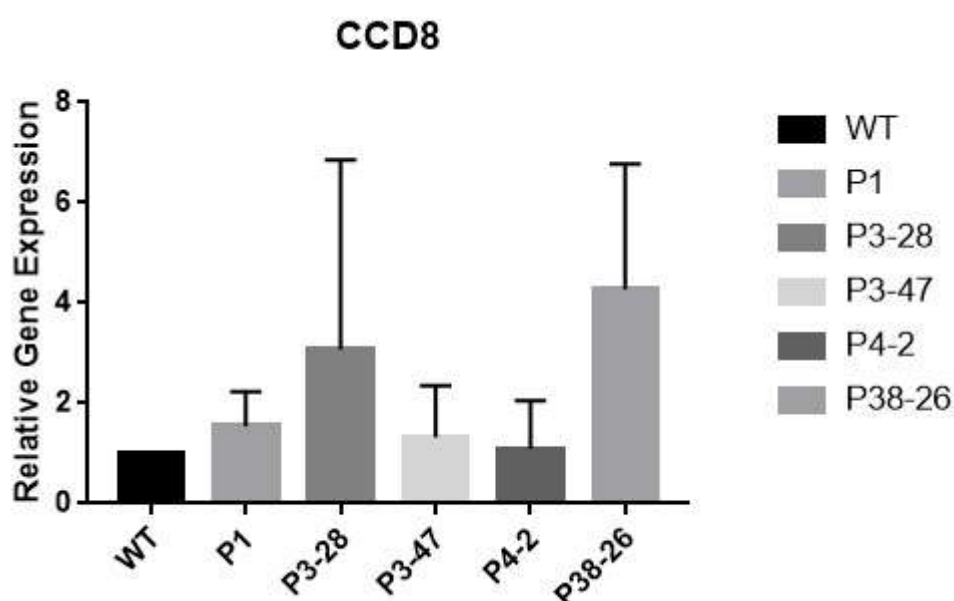


Figure 4.40. CCD8 expression profiles in mutant plants show increased transcript levels compared to wild-type plants

4.12. Analyzing the amount of abscisic acid in the leaves of mutant CCD8Cas9 eggplant lines

Research conducted by López-Ráez et al. (2010) was the first study to demonstrate a relationship between the levels of the phytohormone ABA and the newly discovered class of phytohormones known as strigolactones. Findings using tomato

ABA-deficient mutants stopped at various points along the ABA biosynthetic pathway and the use of specific NCED and CCD inhibitors imply that ABA is one of the regulators of strigolactone production. Real-time qPCR analysis of LeCCD7 and LeCCD8 expression revealed a significant decline in both genes' expression in all three mutants, which also showed reduction in ABA content.

The Calibration Curve of the ABA Standard

The standards for calibration were made with different ABA concentrations of 10ppm, 20ppm, 50ppm, 75ppm, 100ppm, and 150ppm, and the right results of area were produced (Table 4.4). From the observed peaks and concentrations of the ABA standard, the standard curve equation in (Figure 4.41) was developed as follows: $Y = a + bx$, where Y is the concentration. The link between peak area and ABA concentration as seen in is represented by this equation (Figure 4.41).

Table 4.4. Various ABA concentrations gave accurate findings in terms of area

Concentration (ppm)	Area		Concentration (ppm)	Area
10	26.2		10 abs -1	26.8
20	53.1		10 abs-2	26.1
50	129.5		10 abs-3	25.7
75	186.4		10 abs-4	26.9
100	255.1		10 abs-5	26.2
150	363.4		10 abs-6	25.7
	area	ppm	Wavelength(290nm)	
Mean (area)	26.23333333	8.618873333		
S. Deviation (SD)	0.474926895	-2.00904516		
S.Error (SE)	0.158308965	-2.13968172	ppm	
LOD	1.424780685	-1.61713549		
LOQ	4.74926895	-0.24545163		

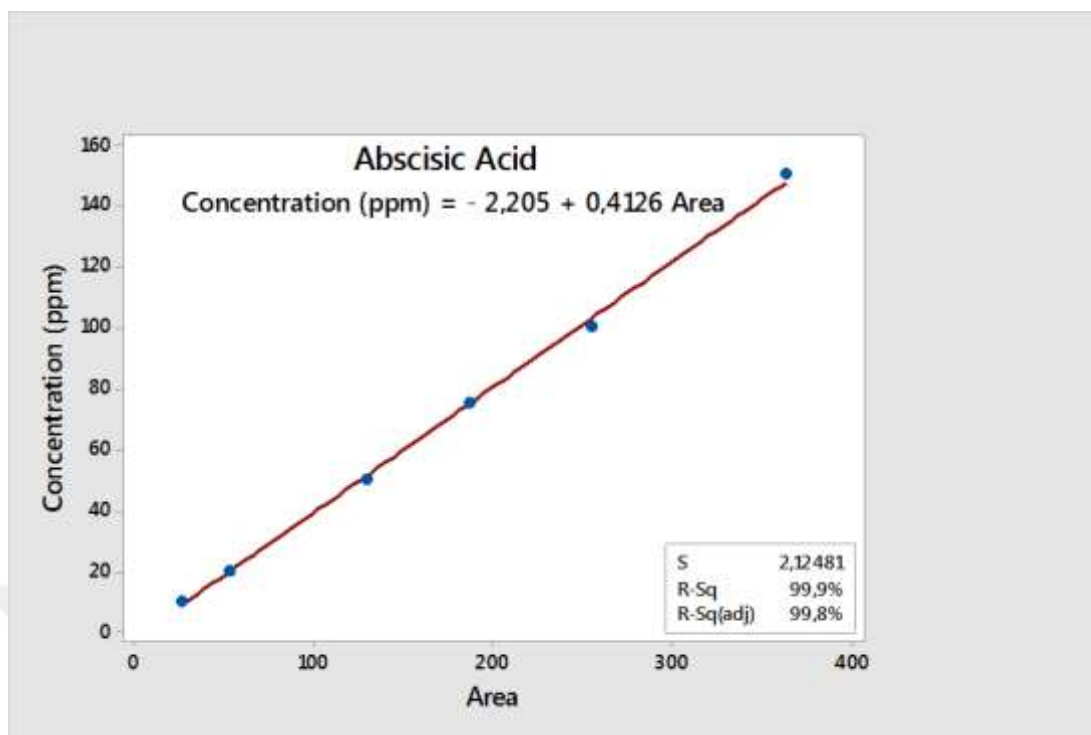


Figure 4.41. ABA standard solution calibration curve

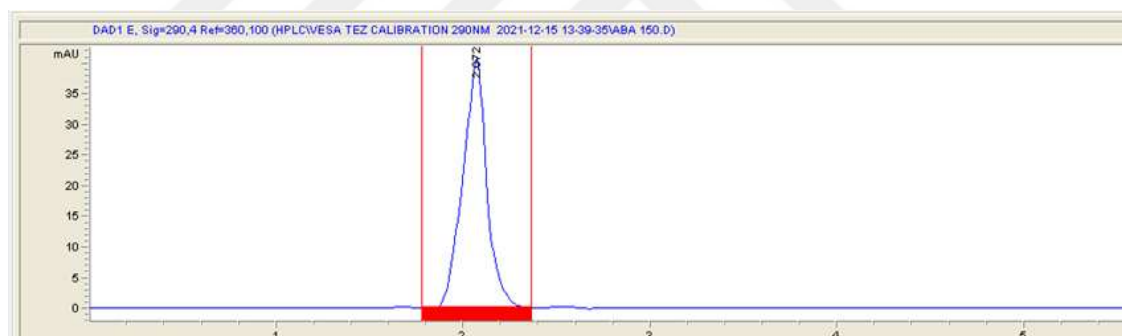


Figure 4.42. Naturally occurring free abscisic acid is separated using HPLC from eggplant leaves

We conducted an HPLC analysis of the total abscisic acid content in the leaves of wild-type and ^{CCD8}Cas9-altered T2 lines to investigate the relationship between SL biosynthesis in the ^{CCD8}Cas9 mutants and their resistance to *P. aegyptiaca* infection. Final concentration of ABA on mutant plants and wild type was calculated according to dilution factor. Absciscic acid levels were much lower in the ^{CCD8}Cas9 mutant lines than they were in the wild-type lines (wild-type resulted in 275.93 ppm; homozygous knockout P4-2 abscisic acid was 204.45 ppm, P3-28 had 112.65ppm, abscisic acid was not decreased in lines P3-47 and P38-26 but resulted in higher levels of ABA) (Table 4.5). It seems reasonable, given this information, that lines P4-2 and P3-28 had the highest level of resistance to *P. aegyptiaca*. Because the HPLC analysis was only done once per replicate, we are not able to draw any conclusions from these results and must repeat the process two or three more times. Abiotic factors used to indicate the presence of abscisic acid too. Due to this experiment should be repeated to form a stronger

conclusion with more statistical significance

Table 4.5. Absciscic acid levels decreased in the ^{CCD8}Cas9 mutant lines P4-2 and P3-28 compared to the wild type

Final concentration	Sample name
204.4	P4-2
112.6	P3-28
361.7	P3-47
377.1	P38-26
275.9	WT

Our results on eggplant mutant plants, which were developed by manipulating *SmCCD8* gene involved in the production of SL, seem to be similar with those of a number of other research studies.

The first research about tomato strigolactone-deficient mutant (*Sl-ORT1*) was by Koltai, LekKala, et al. (2010). The mutant showed a variety of characteristics, including enhanced lateral shoot branching and resistance to the parasitic plant *Orobancha*. There is a link between *Sl-CCD7* transcription levels and strigolactone production in tomato and a decreased level of strigolactone synthesis in the *Sl-ORT1* mutant relative to the WT

Kohlen et al. (2012) used RNAi-mediated silencing to create transgenic *SlCCD8* tomato plants. In these lines, strigolactone levels were decreased, and *Phelipanche ramosa* infestation was reduced by 90%. Increased shoot branching, decreased plant height, more nodes, and excessive adventitious root formation were all observed.

Gao et al. (2018) used CRISPR/Cas9 method to introduce the mutations *NtCCD8A* and *NtCCD8B* into tobacco. Insertion of one nucleotide and deletion of three nucleotides led to the production of premature stop codons and mutant alleles of *ntccd8a* and *ntccd8b* were created. As a result and comparison to wild-type plants, mutants displayed greater shoot branching, decreased plant height, more leaves and nodes, and lower total plant biomass; nevertheless, the root-to-shoot ratio remained unaffected. Additionally, compared to wild type, mutant lines showed more lateral roots and shorter main roots.

Bari et al. (2019) studied in tomato (*Solanum lycopersicum* L.), the second exon of *CCD8* was targeted using Cas9/single guide (sg) RNA constructs. With no off-targets found, mutated tomato lines with varying insertions or deletions in *CCD8* were generated. In comparison to wild type, mutant plants significantly reduced parasite infection. ^{CCD8}Cas9 mutated tomato lines produced noticeably more fruits with smaller sizes as compared to the non-mutated wild-type. Mutant had morphological changes that included dwarfing, excessive shoot branching, and adventitious root formation. Orobanchol, a significant SL in tomato root exudates and a germination stimulator for *P. aegyptiaca*, was measured in ^{CCD8}Cas9 mutant roots. ^{CCD8}Cas9 mutant lines have lower orobanchol levels than the wild type. Mutant line 5, the most resistant to *P. aegyptiaca* infection, was found not to have orobanchol.

Previous gene silencing studies on tomato *SlCCD8* knockdown plants have showed increased shoot branching, changed lateral adventitious root development, and decreased plant height. Our findings somewhat matched those of prior research; we found that ^{CCD8}Cas9 eggplant plants showed a higher number of shoot branches, there was a difference in mutant plant height compared to the wild type. When tested with *P. aegyptiaca* seeds, mutants showed resistance and significantly fewer parasite tubercles than the wild type.

We used quantitative real-time PCR to conduct an analysis of the relative transcript abundance of *CCD8* so that we could determine its level of expression. It is interesting to note that the level of the *CCD8* transcript was significantly increased in the transgenic *CCD8*Cas9 mutated eggplant plants in comparison to the wild-type plants. Previous studies had similar findings; one study by Bari et al. (2019) found that the amount of *CCD8* transcript present in the transgenic *CCD8*Cas9 mutated tomato plants (lines 1a, 2a, 5a, and 11a) was significantly higher than that of the wild-type plants.

This research involved the use of the CRISPR/Cas9 system to precisely edit the *CCD8* gene in eggplant. This mutation, which confers broomrape resistance in eggplant lines P1, P4, can be passed on to any variety of eggplant through the use of the conventional backcrossing technique, and is expected to be effective against other *Phelipanche* and *Orobanche* species

5. CONCLUSION

Many CCD8 mutants have been described before in literature and the role of CCD8 enzymes in the biosynthesis of strigolactones have been reported for several plant species, but this study is the first to describe how we created mutant eggplants that are resistant to parasitic weeds using the CRISPR/Cas9 technology in eggplant. We created an effective resistance to *Orobanch* spp. Parasitic weeds mutation in eggplant using CRISPR/Cas9 that can be used in breeding resistant varieties. With the help of this technology, we were able to manipulate the *SmCCD8* gene in eggplant, one of the plants with a significant economic impact and susceptibility to parasitic weeds.

Using the *Agrobacterium*-mediated technique with the EHA105 strain, a construct that was intended for tomato and had two gRNAs from different exon regions, was introduced into eggplant. This construct showed a high level of homology to the eggplant genome, and through it different mutation were obtained.

Our CRISPR/Cas9 experiments resulted in two successfully mutated eggplant plants. Only one (gRNA1) out of two designed gRNAs led to gene editing. Two homozygous knockout plants, P1 and P4-2, were obtained. In P1, a 2-bp insertions caused a frameshift resulting in a change of 8 amino acids, and this led to a stop codon, confirming the knockout of CCD8. In P4, a 1-bp insertion also caused a stop codon to appear early, confirming the knockout of CCD8.

Yamula and Aydin Siyahi, two different genotypes of eggplant, were employed for the transformation process. However, the same protocol of transformation was used for both genotypes of eggplant. Aydin Siyahi did not generate any transgenic plant. And based on our findings, we came to the conclusion that this genotype did not respond to transformation. On the other hand, due to the ability of Yamula eggplant to respond well in tissue culture, the use of the biotechnology approach CRISPR/Cas9 has been made possible for the improvement of eggplant.

According to the results of RT-PCR, the quantity of the the transgenic ^{CCD8}Cas9 mutant showed a substantial increase in CCD8 transcript levels. We also analyzed the total abscisic acid content in wild-type and ^{CCD8}Cas9-altered T2 lines using HPLC to determine the link between SL production in ^{CCD8}Cas9 mutants and their resistance to *P. aegyptiaca* infection. Abscisic acid levels were reduced in ^{CCD8}Cas9 mutant lines P4-2 and P3-28, compared with wild type but not in P3-47 and P38-26. According to the results of our study, some of the transgenic lines had a lower level of ABA. This result indicates that CCD8 affects the level of endogenous hormone.

P4-2, P38-26, and P3-28 appear to have the most resistance against *P. aegyptiaca*. According to our research, the production of SL, carotenoid biosynthesis, and the resistance mechanism are closely related.

SmCCD8 target gene knockout has an impact on the number or size of parasite tubercles that appear on the tested plants. Because of CCD8 knockout, these host plants had much fewer and smaller tubercles than those seen for the control treatment (WT). The parasite tubercles that appeared on the roots of the mutated plants were much smaller compared to that of wild type plants.

Also, more branches were seen in the *Smccd8* mutants as compared to the WT. Especially in homozygous knockout lines P1 and P4-2, these mutants result in shorter internode distance too.

P1 and P4 homozygous knockout mutants, found to be carrying the transgenes associated with CRISPR-Cas9.

By using the traditional backcrossing technique, this mutation, which confer broomrape resistance in eggplant lines P1, P4, may be passed on to any eggplant variety. The use of genetic resistance created in frame of this doctoral study is expected to reduce yield and quality loss caused by the parasitic weed in eggplant production areas in Turkey and in the world.



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EUICATION INFORMATION

Doctorate	Akdeniz Univeristy
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Publications

1- Pakashtica, V., Yigit, M., Uysal, E., Bulbul, C., Simsek, D., Pinar, H. (2022). New Source for High Anther Culture Response Turkish Local Eggplant (*Solanum melongena* L.) Genotype. Fresenius Environmental Bulletin, vol.31, no.5, pp.5082-5086, 2022.

2- Uysal, E., Yigit, M., Pakashtica, V., Hasan, P. (2023). Molecular and Morphological Characterization of Turkish Local Eggplant (*Solanum melongena* L.) Populations. Türk Tarım ve Doğa Bilimleri Dergisi , 10 (1) , 167-179 . DOI: 10.30910/turkjans.1101258.

Papers presented at national scientific meetings and published in proceedings

1- Pakashtica, V., Mutlu, N., Pinar, H., Soylu I., Bulbul, C., Dogan, S., Karabeniz, A., Akcakale, F., Kaba, A., Muratbek Kyzy, A., Zonon, M. and Bostan, A.B. Eggplant Genome Editing Using CRISPR/Cas9 Technology. IV. International Plant Breeding Congress, November 21-25, 2022. Poster presentation.

2- Dođan, S., Soylu, I., Őimőek, C., Kaba A., Akçakale, F., Karabeniz, A., Pakashtica, V., Mutlu, N., Sırık Domateste CRISPR-Cas9 Teknolojisi ile Old-Gold Crimson Mutasyonun Oluőturması. XIII. Sebze Tarımı Sempozyumu 21-23 Eylöl 2022. Oral presentation.

