GENETIC MODIFICATION OF GLYCOALKALOID CONTENT IN EGGPLANT BY *AGROBACTERIUM*-MEDIATED TRANSFORMATION TECHNIQUE

A Thesis Submitted to
the Graduate School of Izmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Molecular Biology and Genetics

by

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June 2022
İZMİR

ACKNOWLEDGMENTS

First of all, I would like to thank Anne Frary for her interest, patience, support and feedback during the thesis writing and laboratory studies. I would like to thank Sami Doğanlar for providing and supporting the opportunity to work in their laboratory.

Thank you to the Doğanlar & Frary laboratory members for their help, support and ideas during difficult times. Sincere thanks to Hatice Selale for her deep knowledge and guidance during my master.

To my friends, thank you for listening, advice and supporting me under every condition. I would like to thank all my close friends such as Gökalp Bayram, Olca Çalhan Cansu Dilege, Can Göktaş and Hakan Sevgili for supporting me emotionally during this thesis.

Finally, I would like to thank very much to my family who are my father Okan Gültekin, my mother Serap Yılmaz my sister Ece Demircioğlu Uğuz and Zeynep Lümalı, my brother Serkan Uğuz and other family members for always supporting and encouraging me to do this project. Without their support, I would not become the person I am now.

ABSTRACT

GENETIC MODIFICATION OF GLYCOALKALOID CONTENT IN EGGPLANT BY *AGROBACTERIUM*-MEDIATED TRANSFORMATION TECHNIQUE

Eggplant is one of the first plants that was cultivated in the Old World. It is economically and agriculturally important for many countries such as Turkey. In addition, eggplant has been used medicinally since ancient times. The medicinal effects of eggplant come from its secondary metabolites. One of the most useful secondary metabolites is steroidal glycoalkaloids (SGA). SGAs have been used in cancer treatment research. The biosynthesis of SGAs in plants is controlled by enzymes and transcription factors. GAME 9 is a transcription factor that has been investigated in tomato and potato and has been shown to be an important element in the production pathway. In this research, a copy of eggplant's own GAME 9 transcriptional factor was transformed to the eggplant genome with *Agrobacterium*-mediated transformation. To this end, the GAME 9 gene was amplified from the eggplant genome and inserted into the pSoup/pGreen0029 vector system. This vector system was inserted into *Agrobacterium.* Eggplant seedling explants were incubated with transgenic *Agrobacterium*. The transformation of regenerated plants was confirmed with PCR and efficiency was found to be 1.3%. RNA expression levels were checked by RT-QPCR for 12 confirmed transgenic plants. On the metabolite level, the leaves of transgenic plants were assayed for SGA content using Dragendorff's reagent method. The transgenic plants' mRNA and metabolite levels showed plant to plant variation due to the random nature of transgene insertion. Of the 12 transgenic eggplant, nine plants had significantly increased production of both mRNA (at least 2-fold difference) and metabolite levels. In addition, two plants did not show any difference in mRNA level but their SGA amount was significantly increased. The remaining plant had a 0.16-fold decrease in mRNA level but a significantly greater level of SGA than control plants. In the future, these transgenic plants can help illuminate the SGA production pathway of eggplant and be used for the production of economically and medicinally valuable SGAs.

ÖZET

AGROBACTERİUM ARACILI TRANSFORMASYON TEKNİĞİ İLE PATLICANDA GLİKOALKALOİD İÇERİĞİNİN GENETİK MODİFIKASYONU

Patlıcan eski dünyadan kültüre alınan ilk bitkilerden biridir. Türkiye'de patlıcanın üretimi ekonomik ve tarımsal açıdan büyük önem taşımaktadır. Ayrıca eski çağlardan beri tıbbi alanda kullanılmaktadır. Patlıcan tıbbi açıdan etkili olmasının nedeni ikincil metabolitleridir. En etkili metabolitlerden biri steroidal glikoalkaloidlerdir (SGA). SGA'lar kanser tedavisi araştırmalarında kullanılmıştır. Bitkilerde SGA'ların biyosentezi, enzimler ve transkripsiyon faktörleri tarafından kontrol edilir. GAME 9, domates ve patateste araştırılmış ve üretim yolunda önemli bir unsur olduğu gösterilmiş bir transkripsiyon faktörüdür. Bu araştırmada, patlıcanın kendi GAME 9 transkripsiyon faktörünün bir kopyası, Agrobacterium aracılı transformasyon ile patlıcan genomuna transfer edilmesi amaçlanmıştır. Bu amaçla, GAME 9 transkripsiyon faktörü pSoup/pGree0029 vektör sistemine transfer edildi ve vektör sistemi *Agrobacterium*'a yerleştirildi. Patlıcan eksplantları transgen içeren *Agrobacterium* ile inkübe edildi. Rejenere bitkilerin transformasyonu PCR ile doğrulandı ve verim %1,3 olarak bulundu. RNA ekspresyon seviyeleri, 12 doğrulanmış transgenik bitki için RT-QPCR ile kontrol edildi. Metabolit seviyeleri için, aynı transgenik bitkilerin yaprakları, Dragendorff'un reaktif yöntemi kullanılarak SGA içeriği için test edildi. Transgenik bitkilerin mRNA ve metabolit seviyeleri, *Agrobacterium*'un transformasyonu rastgele ekleyen doğası nedeniyle bitkiden bitkiye değişiklik göstermiştir. 12 transgenik patlıcanın dokuzu, hem mRNA ekspresyonunda (en az 2 kat fark) hem de metabolit seviyelerini önemli ölçüde artığı gözlemlenmiştir. Ek olarak, iki bitkinin mRNA seviyesinde herhangi bir farklılık göstermemesine (1 kat fark) karşın SGA miktarları önemli ölçüde arttığı gözlemlendi. Bir diğer bitkide ise mRNA seviyesinde 0,16 kat azalmaya gözlenirken, kontrol bitkilerinden önemli ölçüde daha yüksek bir SGA seviyesine sahipti. Gelecekte, bu transgenik bitkiler, patlıcanın SGA üretim yolunu aydınlatmaya yardımcı olabilir ve ekonomik ve tıbbi açıdan değerli SGA'ların üretimi için kullanılabilir.

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CHAPTER 1

INTRODUCTION

1.1. *Solanum melongena L.*

Solanum melongena L. is a plant that belongs to the Solanaceae family. It is one of the few species in this family that was first cultivated in the Old World. While this species is known as eggplant in America, it is known as aubergine in England and France and brinjal in India. The first known country to cultivate eggplant was India and from there the crop spread to Africa, Asia and the Middle East (R.Gowda 2016). Eggplant is currently cultivated worldwide (Agnieszka Sekara 2007). According to the Food and Agriculture Organization, eggplant was grown on 1.85 M hectares and total production was 55.2 M tonnes in 2019 (FAO-STAT, 2019). Although eggplant prefers to grow in a warm climate, it is not extremely affected by temperature changes and can be grown in the greenhouse. This means that the expense of eggplant in everyday life does not alter much with season (Wenquan Sun 1990).

Eggplant, like the other Solanum family members tomato, pepper and potato, has 12 chromosomes and is a diploid autogamous plant (2n=24). Eggplant fruit is classed as a berry. The form and color of the fruit can vary depending on the eggplant cultivar; for example, the shape might be round, long, or intermediate, and the color can be white, green, yellow, red, purple, or near to black. There are three main eggplant species: brinjal (*Solanum melongena*, scarlet eggplant (*Solanum aethiopicum*) and gboma eggplant (*Solanum macrocarpon*). Scarlet and gboma eggplants are cultivated in a limited area, mostly in Africa. In contrast, brinjal is cultivated worldwide. There are three main botanical varieties of brinjal: *S. melongena* var. *esculentum* is the most well-known eggplant variety and includes many cultivar groups, *S. melongena* var. *depressum* is dwarf eggplant and *S. melongena* var. *serpentium* is also called snake eggplant, because the shape of its fruit is long and thin, resembling a snake (Gowda 2016). There are also several wild relatives of *S. melongena* such as *Solanum linnaeanum* and *Solanum incanum.* Although wild eggplant fruits are not suitable for eating, they can be useful for the manufacture of medicine due to their phytochemical content (Sandra Knapp 2013).

Eggplant is a dietary food with strong nutritional content and a low price which makes it highly valuable and popular. The low-calorie content of eggplant makes it ideal for diet meals (Table 1.1.). Also, it has a major role in vegan/vegetarian cuisine due to its excellent nutritional content and ability to be prepared using practically any cooking technique. Even though its vitamin A and E levels are modest, eggplant has high phytochemical levels, making it beneficial to human health. Because of these favorable qualities, interest in eggplant is growing day by day (Chapman 2019).

Table 1.1. Nutritional value of 100 g eggplant
(Source: USDA, 2019)

Proximate		**Vitamins**	
Water	92.3 g	Vitamin C	2.20 mg
Energy	25.0 kcal	Thiamin	0.039 mg
Protein	0.98 g	Riboflavin	0.037 mg
Total lipid	0.18 g	Niacin	0.649 mg
Carbohydrate	5.88 g	Vitamin B6	0.084 mg
Fiber	3.00 g	Folate, DFE	22.0 µg
Sugar	3.53 g	Vitamin B12	0.00 µg
Minerals		Vitamin A, RAE	1.00 µg
Calcium	9.00 mg	Vitamin A, IU	23.00 IU
Iron	0.23 mg	Vitamin E	0.30 mg
Magnesium	14.00	Vitamin D	0.00 µl
Phosphorus	24.00	Vitamin K	3.50 µl
Potassium	229.00	**Lipids**	
Sodium	2.00	Fatty acids, total saturated	0.034 g
Zinc	0.16	Cholesterol	0.00 mg

The phytochemicals found in eggplant and its wild relatives have long been used in traditional medicine. As a result, eggplant has been used to treat asthma, skin infections, and rheumatic illnesses (Ceylan Dönmez 2020). Its usage in traditional medicine caught the interest of scientists, leading them to study eggplant's metabolites. According to this

research, the types and quantities of phytochemicals in eggplant are scientifically linked to its use in the treatment of certain disorders (O.A.A. Eletta 2017).

1.2. Plant Metabolism

The chemical processes that occur within a living creature in order to sustain life are referred to as metabolism. Metabolites are chemicals with molecular weights less than 1500 Daltons (Da) that are produced by these processes. They can be products or substrates. Metabolites are divided into two categories: primary metabolites and secondary metabolites (Samuel 2004).

Primary metabolites are in charge of fundamental cellular functions such as growth and development. The majority of primary metabolites are quite similar across all plant species. They are organic substances that are either the substrates or products of central metabolism pathways like photosynthesis and biosynthetic activities. Carbohydrates, amino acids, fatty acids, and organic acids are types of primary metabolites. These chemicals are involved in plant growth and development, respiration/photosynthesis, hormone and protein synthesis. While carbohydrates such as glucose and sorbitol promote plant development, amino acids such as arginine and tryptophan are present in the structure of the plant's key enzymes. Other examples include oleic acid which is a useful fatty acid in the development of cell membranes, and citric acid an organic acid which is used in the plant's roots to maintain homeostasis (N. Hounsome 2008).

Secondary metabolites are substances that have no influence on the plant's primary metabolism. The reason for the synthesis of secondary metabolism is plants' sessility. Many plants rely on animals for pollination and dispersal. Therefore, having multicolored flowers is one of plant's reproductive strategies. Colorful blooms make the plant more appealing to pollinators and can increase the speed and efficiency of reproduction. Likewise, colorful fruit attract dispersers. These pigments are often secondary metabolites produced by the plant. Plants are able to manufacture a variety of metabolites to survive because they cannot otherwise escape stresses in nature. Secondary metabolites are important when plants encounter both abiotic and biotic stress (Murray 2017). Plants can respond to biotic stress in two ways: they can either activate a cellular defense system or they can limit their growth

rate to neutralize parasites. Secondary metabolite synthesis can be altered by various pathogens. Plants can synthesize metabolites with antiviral, antibiotic, and antifungal effects. It is also known that when plants are exposed to biotic stress, they communicate with one another by secondary metabolite secretion (Alan Crozier 2006). Secondary metabolites secreted to communicate are generally volatile and are detected by the green leaves of plants. Examples of these volatile compounds are geraniol, α-carene and limonene (Angelika Böttger 2018). In addition, plants produce secondary metabolites to protect themselves from abiotic environmental changes such as UV-irradiation and drought. Drought has an impact on phytohormone production. Increasing quantities of phytohormones, in turn, trigger secondary metabolic pathways for compounds like shikimic acid, melonic acid, and mevalonic acid. Capsaicin production in chili peppers is a notable example of this. Pepper generates more capsaicin during drought, a change that increases pepper's pungency (Abhimanyu Jogawat 2021).

In terms of chemical structure, secondary metabolites are quite similar to primary metabolites. This is because secondary metabolites are essentially methylated, glycosylated, and hydroxylated forms of primary metabolites. Plant secondary metabolite synthesis can be influenced by cell differentiation. Although there is no universal categorization for secondary metabolites, they are commonly classified as phenolics, terpenes and alkaloids (El-Anssary 2017).

Terpenes are the most abundant and diverse category of secondary metabolites. Fruits and vegetables produce a wide range of terpenes. Isoprenoids are another name for this class. Terpenes are generated by combining the mevalonate pathway with the methylerythritol 4-phosphate (MEP) pathway. Their chemical structures consist of cyclic unsaturated hydrocarbons. Terpenes are all generated by deviating from the isopentane structure. Terpenes function in plants as sterols, hormones, aromatic compounds and carotenoids. Pro vitamin A is one example. It is formed by the combination of the aforementioned pathways in the form of β-carotene. Thus, while many of these secondary metabolites are utilized by plants to provide protection from stress, flavor and attractants, they are also used by humans as essential vitamins (Al-Taweel 2018).

Phenolics are aromatic organic molecules that contain several hydroxyl groups. Combination of the shikimate and mevalonate pathways are responsible for phenolic

synthesis which begins with phenylalanine. The main effect of phenolics is their antioxidant activity because of their radical scavenging capability. Moreover, they have antifungal and antibacterial action. In addition, plant color is affected by phenolics. Drugs for cancer, diabetes, and stroke have been developed using phenolic compounds' chemical features (Nwokeji Paul Anulika 2016).

Alkaloids are formed from cyclic hydrocarbons with a nitrogen group. They are produced by the merging of the shikimate and pyruvate pathways. Since alkaloids are nitrogen-based and the amount of nitrogen is limited in plants, alkaloids are rare compounds in plant vegetative tissue. Nevertheless, alkaloids have benefits for both plants and human beings. For plants, they are an important part of the defense mechanism against fungi, bacteria and herbivores. Alkaloids are used as narcotics, poisons and also pharmaceuticals. Nicotine and caffeine are examples of widely used alkaloids. Morphine is an important medicine, while berberine is used in cancer research because of its anti-cancer activity (Robert Verpoorte 1991).

1.3. Eggplant Secondary Metabolism

The major secondary metabolites in eggplant are phenolics, carotenoids and glycoalkaloids (Muhammad Yasir Naeem 2019). Eggplant contains high concentrations of phenolics. Although phenolics are beneficial in the pharmaceutical industry, they are a major cause of concern in the food industry since they promote browning. The total phenolic content of eggplant ranges between 23 mg/100 g dry weight and 1168 g/100 g dry weight (Patricia Garcia-Salas 2014). While the amount of phenolics in wild species like *S. incanum* is greater than cultivated eggplant, the content profile remains constant. The majority of this profile is composed of anthocyanin, tannins, and phenolic acids. Anthocyanin gives eggplant its purple color. The amount of anthocyanin is altered by temperature, light intensity and storage conditions (Florina Dranca 2015). Tannins impart a bitter flavor to foods such as eggplant. Tannins are also important due to their significant antioxidant capabilities. The most abundant phenolic acid in eggplant is chlorogenic acid (CGA). CGAs have strong antioxidant capacity. They have been shown in studies to enhance the quantity of hyaluronic and collagen in fibroblasts cells, and are of great medicinal value (S. U. Nergiz Gürbüz 2018).

Carotenoids are photosynthetic pigments found in yellow and orange colored plants. These pigments are critical in the food sector. Although the first carotenoid that springs to mind, β-carotene is not abundant in *S. melongena,* it does contain lutein and zeaxanthin. These two compounds' beneficial effects on aging and cataracts are being studied (Schenk 2014). Other cultivated eggplants, such as *S. aethiopicum* and *S. macrocarpon*, have a high concentration of carotenoids with orange and red fruit. Under stress conditions such as drought, the amount of carotenoids in leaves and fruit rises, thereby increasing the color of fruit (Elias K. Mibei 2017).

Glycoalkaloids are secondary metabolites produced by Solanaceae family members. Each member of this family contains a unique set of glycoalkaloids. Glycoalkaloids help these species defend themselves against pests and pathogens. They also have the potential to be treatments for various types of cancers (Prasanta Dey 2019). Furthermore, investigations demonstrated that glycoalkaloids have antiparasitic properties against *Leishmania mexicana, Leishmania amazonensis*, and *Trypanosoma cruzi* (Mariza Abreu Miranda 2013). Although their anti-cancer and anti-parasite properties are well recognized, the amount of glycoalkaloid used in such treatments should be optimized and carefully chosen because over-consumption of glycoalkaloids can result in death (CONTAM 2020). The properties of steroidal glycoalkaloids are described in more detail in section 1.4.

1.4. Steroidal Glycoalkaloids (SGA)

Steroidal glycoalkaloid (SGAs) production occurs in the leaves, stems, roots, flowers, and fruits of Solanaceae family members. SGAs consist of a combination of an aglycone and varied sugar molecules. Modification of SGAs occurs via transamination and glycosylation. The alteration of a SGA's chemical structure can affect its toxicity to animals, anti-cancer and anti-microbial properties. These qualities are related to the sugar molecules connected to the aglycone and their type influences these capabilities. SGAs and their features differ amongst the members of the Solanaceae (Kati Helmja 2017).

Tomato generally produces α-tomatine and esculeaside as SGAs. The quantity varies depending on plant growth stage and cultivar. α-tomatine is found in immature tomatoes due

to the integration of tomatidine and lycotetraose. On the other hand, esculeaside has a greater abundance in ripe fruit of some tomato cultivars (Jaana Laurila 1999).

Potatoes produce more than 50 kinds of SGAs. However, in terms of content, 95% of potato SGAs are α-solanine and α-chaconine. α-solanine was one of the first SGAs to be discovered and isolated. It contributes to the plant's natural defense system against pests. α-chaconine is a naturally occurring toxin that is typically found in green potatoes. It is one of the chemicals responsible for the bitter flavor of the tuber. Because SGAs are toxic, they protect the crop from fungus and animals until it ripens (Pinchas Krits 2007).

α-solamargine (SM) (Figure 1.a) and α-solasonine (SS) (Figure 1.b) are the two primary SGAs produced by eggplant. In 1987, the plant-based SM and SS were examined, as well as their anti-cancer potential (Cham B. E. 1987). These chemicals cause cancer cells to enter programmed cell death and do not cause apoptosis in healthy cells (S.S.S. Al Sinani 2017). Drug trials have been conducted for a variety of cancers. SM, in particular, and the combination of SM and SS have potential to contribute to cancer research. SM has a stronger cytotoxic effect than several chemotherapeutic drugs, including cisplatin (cDDP), epirubicin (EPI), and cyclophosphamine (CP) (L.Y.Shiu 2007). The combination of SM and cisplatin is lethal for cisplatin-resistant cancer cells, particularly in lung malignancies and breast cancer (Chia-Hua Liang 2004). BEC is one example of how eggplant SGA might be used in cancer treatments. BEC is a mixture of eggplant SGA compounds. It is composed of 34% mono and diglycosides of SS, 33% SM, and 33% SS. BEC has been licensed as a topical therapy for the treatment of human skin malignancies with a curative outcome in a cream formulation called Curaderm (B. E. Cham 2020). The studies conducted by Cham in 2017 show that, while BEC causes cancer cells to enter apoptosis, it has no effect on healthy cells. The rhamnose group present in eggplant SGAs is the primary cause of this. Healthy human cells have a low level of rhamnose binding protein (RBP), but cancer cells have a high level of RBP. The affinity and quantity of receptors varies with the type of cancer cell, which alters the efficiency of BEC. Since the rhamnose group is the primary cause of apoptosis, its quantity influences the effectiveness of its anticancer function. While the SM molecule has two rhamnose groups, SS only has one, this makes SM preferable for cancer research. SS, on the other hand, has the ability to penetrate through the cell membrane and induce apoptosis. This indicates the significance of SS. Although BEC has high potential as a monotherapy,

the majority of current trials use it in combination with cisplatin or against cisplatin-resistant cancer cells. Cancer cells can develop resistance to chemotherapy medications, which is known as multi-drug resistance (MDR). MDR causes chemotherapy used in cancer therapies to fail. BEC inhibits MDR in cancer cells and is effective against a variety of drug-resistant malignancies. SM promotes death in MDR tumor cells and increases the expression of the MDR 1 gene which is responsible for actin disruption. The therapeutic benefits of eggplant SGA on cancer cannot be overlooked, and research into this area is ongoing (B. E. Cham 2017).

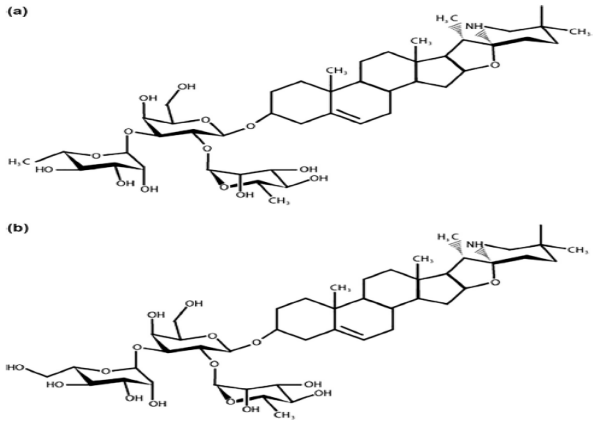

Figure 1.1. Chemical structures of the eggplant glycoalkaloids (a) solamargine, (b) solasonine

(Source: Nergiz Gürbüz, 2018)

1.5. SGA Biosynthesis

The SGA pathway begins with acetyl CoA in the mevalonate pathway, and cholesterol is the precursor molecule that starts SGA synthesis. Later, cholesterol is transformed to SGA by a series of hydroxylation, oxidation, transamination and glycosylation reactions. Because not all of the genes and transcription factors in this pathway have been fully elucidated, this area is still under investigation and research (Prashant D. Sonawane 2017). The known genes and metabolites of the pathway are shown in Figure 1.3.

As cholesterol is the primary precursor of SGA synthesis, the enzymes involved in its synthesis influence SGA synthesis. The enzyme responsible for converting 3-hydroxy-3-methylglutary-CoA (HMG-CoA) to mevalonic acid is 3-hydroxy-3-methylglutaryl-CoA

reductase (HMGR). HMGR does this conversion with the help of two triphosphopyridine nucleotides (NADPH) and is the rate-limiting enzyme in the mevalonic acid pathway. Since mevalonic acid is a precursor to cholesterol, it is essential in the synthesis of SGA (Hui Wei 2019). 2,3 oxisqualene is generated after mevalonic acid synthesis, and the enzyme that converts it to cycloartenol is cycloartenol synthase (CAS). This enzyme is necessary for the synthesis of cholesterol (Elisabet Gas-Pascual 2014). Sterol side chain reductase 2 (SSR2) is also involved in the synthesis of cholesterol by reducing cycloartenol to create cycloartanol. In order to form cholesterol, Δ(7)-sterol-C5(6)-desaturase (C5-SD) reacts with cholesta7-enol to form 7-dehyrocholesterol. (Pablo D. Cárdenas 2016). When the genes considered to be responsible for cholesterol synthesis in tomato were transferred to Arabidopsis, these plants generated 15 times more cholesterol than usual. As a result, it was shown that these genes are active in the cholesterol pathway (Satoru Sawai 2014). After cholesterol is created, it must be hydroxylated twice in order to form SGA products. GAME 7 is the first enzyme to accomplish this, followed by GAME 8. The product formed as a result of hydroxylation is 22,26 dihyroxycholesterol. This product is then converted to the E form using GAME 4, GAME 11, and GAME 12 enzymes. The resulting molecule is known as 26-amino-16-beta-22-dihyroxycholesterol. Later, the GAME 6 enzyme closes the E shape in this structure, resulting in the cyclo structure. This is known as 26-aminodihyrodiosgenin. Finally, water is removed to generate the aglycone form of the molecule, which results in the production of solasodine, the main precursor of eggplant SGAs. After undergoing glycosylation reactions with the enzymes GAME 1, GAME 2, GAME 17 and GAME 18, solasodine can be transformed into SS or SM, depending on the type of added sugar molecule (Lorenzo Barchi 2019), (Prashant D.Sonawane 2020).

In addition to pathway enzymes, the role of APETALA2/ERF transcription factors (TF) in the biosynthesis of structurally complex alkaloid metabolites has been demonstrated. For instance, ORCAs are responsible for the production of monoterpenoid indole alkaloids in *Catharanthus roseus*, whereas ERF189 and AaORA in play the same role in tobacco and *Artemisia annua*, respectively (Priyanka Paul 2020). GLYCOALKALOID METABOLISM 9 (GAME 9) is a jasmonic acid (JA)-responsive ERF4 in the Solanaceae family (JRE4). The majority of work on this TF has been conducted in potato and tomato. This TF is located on chromosome 1 in the tomato genome. GAME 9's C-terminal domain is rich in the amino acid

serine. The ethylene hormone prevents the synthesis of JRE TF. Again, research in tomato and potato revealed that suppressing GAME 9 dramatically decreased alkaloid synthesis. Overexpression, on the other hand, increased the quantity of SGA (Masaru Nakayasu 2018). Cardenas et al. (2016) demonstrated a significant effect of GAME 9 on the SGA synthesis pathway in *Solanum tuberosum* and *Solanum lycopersicum.* Furthermore, GAME 9 can regulate the expression of the HMGR1, CAS, SSR2, and C5SD2 genes, which all play roles in cholesterol production in the upper pathway. In the research of Gang Yu et al. (2020), GAME 9 was altered by 1 bp and the effects on *Solanum pimpinellifolium* and *S. lycopersicum* plants were studied. The binding ability of GAME 9 to the GC-rich region of GAME 17 was reduced as a result of this experiment. GAME 9 has been demonstrated to increase the expression of enzyme synthesis genes, particularly after cholesterol, either directly or by co-binding with SlMYC2 (Gang Yu 2020). MYC2, a bHLH-type transcription factor, is important for proteins upstream of this pathway. The GCC- and GC- boxes are the targets for MYC2 and GAME 9. When MYC2 joins with GAME 9, they improve the amount of binding. As a result, the GAME 9/SlMYC2 complex can stimulate HMRG1 SSR2 and C5-SD2. This impact increases output in the downstream pathway. GAME 9's effect in *S. tuberosum* and *S. lycopersicum* suggests that the same gene may be active in other Solanum family members as well. One of these members is eggplant, and until now, the influence of GAME 9 on SGA production in eggplant has not been studied (Pablo D. Cárdenas 2016).

1.6. Plant Transformation

The insertion of a gene of interest into a host plant genome is referred to as plant genetic transformation. Some of the potential motives for such genome alteration are to increase the plant's agricultural value, to increase the manufacture of medicinal compounds from the plant, or to explore unknown pathways. There are two types of plant transformation methods: non-biological based transformation (direct method) and biological gene transfer (indirect method) (G. Keshavareddy 2018).

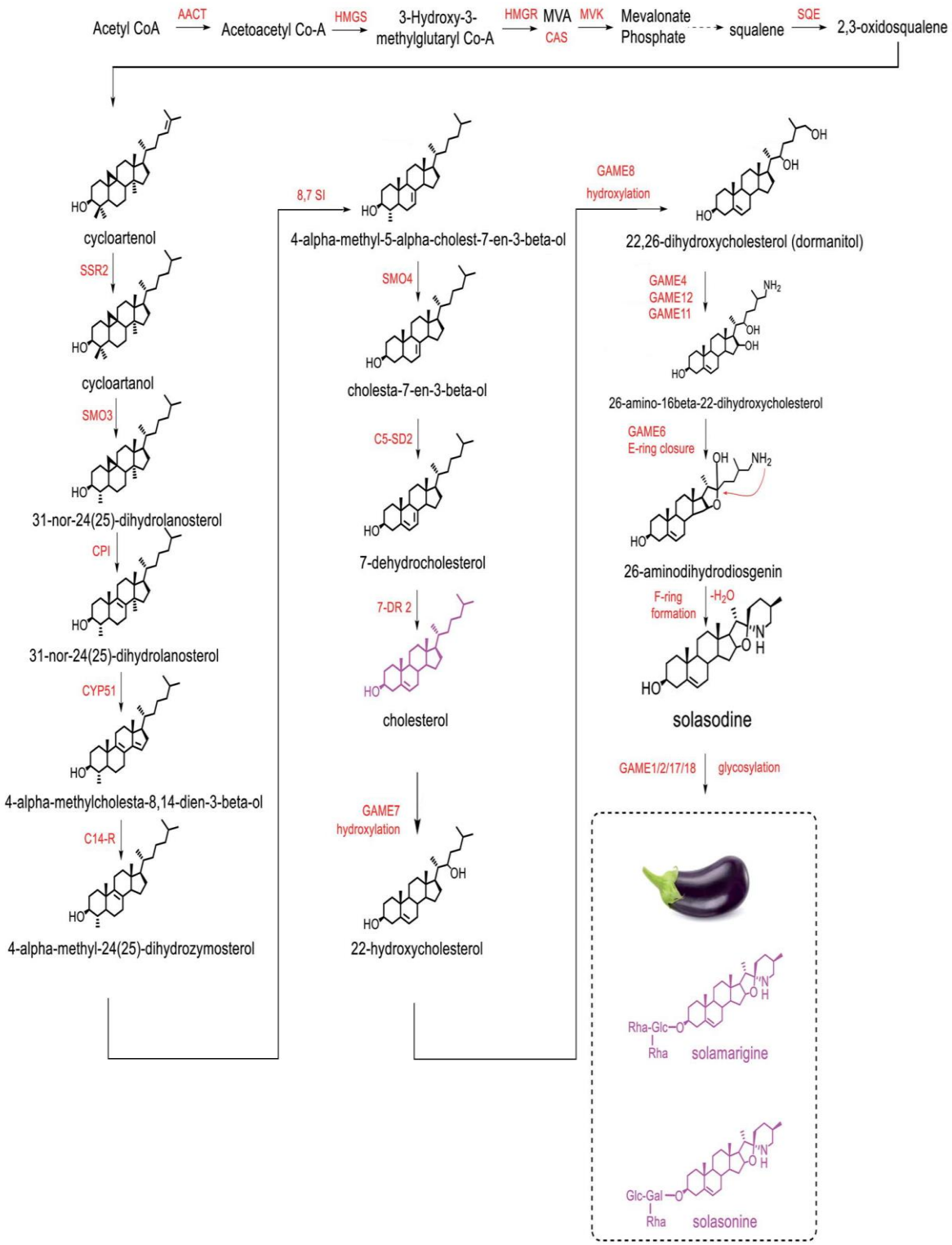

Figure 1.2. Biosynthesis of eggplant SGAs

(Source: Da-Ke Zhao, 2021)

The direct method is applicable to both protoplasts and plant tissues. Methods for manipulating protoplasts include electroporation, lipofection, microinjection, and sonication. Electroporation makes cells permeable to exogenous molecules by providing a high-voltage electric shock (K D'Halluin 1992). In lipofection, negatively charged DNA is encapsulated by positively charged liposomes. In this manner, the DNA-containing capsule penetrates through the cell's membrane and nucleus to reach the genome (Behrooz Darbani 2008). In the microinjection procedure, a glass micro capillary-injection pipette is used to deliver DNA to a protoplast (Gunther Neuhaus 1990). Sonication is a technique that is similar to electroporation. With sound waves, it allows the cell to be permeable to exogenous molecules (Morten Joersbo 1992).

Particle bombardment (biolistic) and silicon carbide fiber mediated gene transfer are alternative direct methods of plant transformation. One of the most often utilized methods in this field is the biolistic technique. Wrapping DNA molecules around particles of noble metals (gold, tungsten) creates micro bullets. A gene gun is used to deliver these bullets to the cell tissue. Although this approach is highly efficient, it is costly due to the usage of gold and tungsten (Sanford 1990). The silicon carbide fiber mediated gene transfer technique is commonly used on plant callus in liquid culture. With the aid of silicon carbide fibers, DNA penetrates the vortexed cell (Madhumita Sarangi 2008).

The indirect method of plant transformation involves the usage of an intermediate to transmit the gene. Viruses and *Agrobacterium* are examples of intermediary organisms. The procedures are known as viral vector-mediated transformation and *Agrobacterium*-mediated transformation. For viral-based transformation, a viral vector carrying the target gene is required. First, the virus is modified to become transgenic by introducing the gene of interest in an appropriate plasmid vector. The transgenic virus is then used to infect the plant, and the plant begins to express the gene of interest. There are two restrictions to this sort of transformation. First, the transformation/expression period is restricted to the time the virus functions. Therefore, it is a transient transformation system. Second, this approach is only effective in plants which can act as a host for the virus (Keshavareddy 2018). The most extensively used method for producing stable transgenic plants has been *Agrobacterium*-mediated transformation. The vector containing the gene of interest is first prepared and then transformed into *Agrobacterium*. The plant genome incorporates the gene(s) in the vector

after inoculation with the bacterium. This approach is highly efficient and produces stable transformants meaning that the gene of interest is inserted into the plant nuclear genome. It is applicable to many plants' species in the plant kingdom, particularly dicots (Behrooz Darbani 2008).

1.7. *Agrobacterium*-Mediated Transformation

Agrobacterium is a soil pathogen that causes crown gall, cane gall, and hairy root diseases in plants by transmitting transferred DNA (T-DNA) into the plant's wounded tissue. The most essential aspect of *Agrobacterium* is its ability to transfer genetic information to the plant genome via T-DNA (Figure 1.4.). The *Agrobacterium* genes that cause these diseases have been revealed over time. Early studies on *Agrobacterium* were conducted to better understand the diseases they cause. The transfer of oncogenes and opines from bacteria to the plant genome via T-DNA is the cause of these diseases. Cells transformed by the T-DNA proliferate and develop and creating opines that *Agrobacterium* can digest. Uncontrolled production of phytohormones occurs as a result of gene transfer leading to cell proliferation and tumor development. The T-DNA is delivered by the tumor-inducing (Ti) plasmid in *Agrobacterium tumefaciens*, which causes tumor growth (Barbara Schrammeijer 2000). In contrast, *Agrobacterium rhizogenes* (syn. *Rhizobium rhizogenes*) contains a root-inducing (Ri) plasmid which induces the plant to generate roots from wounded tissue (Keiichirou Nemoto 2009).

The T-DNA region of *Agrobacterium* contains the opine region, and the oncogene region which contains auxin and cytokinin production genes. These sequences are positioned between the right and left borders. This region between the right and left borders is known as the T-DNA region. The plasmid's remaining components include the opine catabolism genes, virulence region, and replication origin. Opines are unique compounds generated by the reaction of amino acids with ketoacid or sugar molecules. Opines were discovered the 1950s. This research revealed that *Agrobacterium* can catabolize opines, opines are only formed in tumors, and opines are only synthesized at the location of interaction with *Agrobacterium*, not in all plant parts. Opines differ according to the type of *Agrobacterium*, and their catabolism operates correspondingly with catabolism genes not located in the T-

DNA region. These catabolism genes are in charge of the bacteria's uptake of opines, conversion of these opines into digestible molecules, and creation of specific regulatory proteins. In this way, *Agrobacterium* can continue to develop by feeding on the opines which are generated by plant metabolism (I. A. Vladimirov 2015). The disease symptoms are mostly caused by the oncogenes in *Agrobacterium*'s T-DNA. These genes promote excessive cell proliferation by altering the rate at which plant hormones are produced. IaaH (indole-3-acetamide hydrolase) and IaaM (tryptophan monooxygenase) genes in the T-DNA region are important for auxin synthesis, whereas Ipt (isopentenyl transferase) produces cytokinin (Yi Zhang 2015).

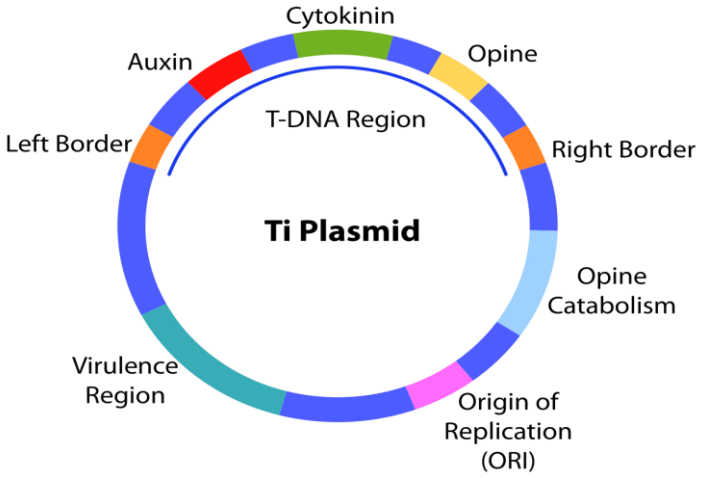

Figure 1.3. Circular map of the Ti-plasmid.

A. tumefaciens needs two things in order to perform genetic transformation: T-DNA and virulence (vir) genes. As mentioned previously, the T-DNA is delimited by conserved 25 base pair repeats situated at each end of the T-region. There are at least eight main loci in the virulence (vir) region (vir A, vir B, vir C, vir D, vir E, vir F, vir G and vir H) which is located outside of the T-DNA (S. Gelvin 2006). The transformation process begins with a plant cell sending phenolic molecule signals to the bacterium to migrate to the plant cell. The bacterium then performs chemotactic motions to migrate towards the plant cells. Acetosyringone is the most common plant phenolic compound that leads to chemotactic motions in bacteria. The bacterium then adheres to the plant via adhesion. This is caused by unipolar polysaccharide (UPP)-dependent polar attachment and UPP-independent adhesion. Transmembrane protein receptors (vir A) in the bacterium recognize the host plant's signals and phosphorylate vir G in the periplasmic space. Next, vir G is phosphorylated and activated

by Chromosomal virulence protein G/Chromosomal virulence protein I (Chv G/Chv I) proteins. Chromosomal virulence protein E (Chv E), on the other hand, maintains the signaling pathway by interacting with plant monosaccharides and vir A (S. B. Gelvin 2017).

The process then continues with the double-stranded unwinding of the T-DNA by vir D1 starting at the 25 base pair left and right border repeat sequences of the Ti plasmid. The 3’ to 5’ strand is then cleaved by the nuclease vir D2. vir D2 caps the 5' end of the T-DNA with covalent bonds, and the 3' end becomes the priming site. Through its C-terminus, vir C1 binds the right border sequence of the T-DNA, increasing the amount of T-strand molecules. The direction of DNA transfer is from the right to left border sequences of the T-DNA (Lan-Ying Lee 2008). In addition to its role as a nuclease, vir D2 has a nuclear localization signal (NLS) that caps the 5' end of the T-DNA. This signal allows the complex to pass through the Ti-pilus type IV secretion system (T4SS) and nuclear pore. Vir E2 proteins in the bacteria enter the host cell via clathrin-mediated endocytosis through the use of vir E1 proteins. The vir D2/T-DNA complex is surrounded by vir E2, which permits it to reach the nucleus from the endoplasmic reticulum (Tzvi Tzfira 2004).

The T-DNA integration mechanism is still not fully elucidated; however, it is known that it occurs in random chromosomal locations. Vir E2 interacting protein 1 (VIP1) interacts with the T-DNA coated by vir E2. Vir F proteins are in charge of vir E2 degradation. According to one hypothesis, the host genome facilitates the integration of the T-DNA. The T-DNA is replicated and made double-stranded by the host DNA polymerase. Then, the T-DNA is integrated into the plant’s DNA at sites with double stranded breaks, non-homologous end joining (NHEJ) completes repair of the region to finish the process. As integration occurs at random, a few things can happen after the bacterium has transmitted the T-DNA to the host plant. The T-DNA may be integrated and expressed to varying degrees, or it may be integrated but not expressed due to its position. That means, that the genes on the T-DNA might be overly expressed or entirely silent. This circumstance, called position effect, may differ depending on the plant and bacterium species (S. B. Gelvin 2000).

Agrobacterium's gene transfer mechanism aroused the scientific community's curiosity, and it was hypothesized that it could be used as a transformation system. To this end, genetic engineers modified the Ti plasmid. The oncogenes, the bacteria's disease-causing genes, were removed from the plasmid in order to prevent disease. In addition, the

opine genes were also removed from the T-DNA while the 25-bp repeat sequences were preserved. For transgenic selection, an antibiotic resistance gene was inserted into the plasmid. This modified Ti-plasmid is utilized in conjunction with a helper plasmid that contains the vir genes. This is referred to as a binary vector system. The Ti-plasmid of *Agrobacterium* is now used routinely as a method for gene transfer in plants (Ramón Penyalver 2000).

The most significant advantage of *Agrobacterium*-mediated transformation is its high efficiency of yielding stable transformation. In addition, it is a method that can be applied easily in many monocot and dicot plants as it is not host specific. Another benefit is that this approach can be used to transform various plant tissues, whether or not the host material is in tissue culture. The disadvantage is that transformation is random in terms of region and number of insertions. Despite these problems, this method is preferred since the benefits exceed the drawbacks (Surya 2020). Transformation efficiencies in monocot plants such as Oryza and Brachypodium has been demonstrated by many studies. For example, Vogel et al. 2007 showed that transformation efficiency was 41% for transferring the Agro Bd21-3 gene to *Brachypodium distachyon* (John Vogel 2007). On the other hand, it has been shown that the transformation efficiency of the Oryza plant, which does not respond very well to *Agrobacterium*, can be as high as 25% (Sadiye Hayta 2019). On the other hand, the transformation efficiency of dicots is usually higher than monocots as was demonstrated by experiments performed by V. J. Chetty et al. (2013). In this article, it was shown that the transformation efficiency of dicot *S. lycopersicum* can vary between 30 and 65% in varying conditions (V. J. Chetty 2013). In another study on *S. tuberosum*, it was shown that the *Agrobacterium*-mediated transformation yield was between 50-60% under varying conditions (Melanie Craze 2018).

1.8. Applications of *Agrobacterium*-Mediated Transformation

Gene transfer preserves the host genome while transferring the target gene and is critical for molecular biology and genetics research. Studies in this area may be categorized into three types: increasing product yield and quality, bioremediation applications, and pharmaceutical applications (Kedong Xu 2014).

Plants' nutritional value can be increased by genetic manipulation. Examples include increasing the quantity of beta-carotene in canola, soybean, and maize, as well as the amount of starch generated by potatoes (Ziemienowicz 2014). *Agrobacterium*-mediated transformation, for example, can provide legumes with amino acids that they normally lack such as methionine, lysine, and tryptophan. Increased synthesis of these amino acids will play a significant role in malnutrition in developing countries (Dung Tien Le 206).

Plant transformation can also be applied to improve a plant's tolerance to biotic and abiotic stress. Cotton was made resistant to insects by introducing a toxic gene. This gene is the *Bacillus thuringiensis* (Bt) toxin gene, and it has enhanced cotton yield by protecting it against cotton-eating insects (Nehanjali Parmar 2017). In addition to the Bt gene, proteases, trypsin inhibitors, and lectins genes have been found to be beneficial in conferring insect and pest resistance in various plants. For example, introducing a trypsin inhibitor gene into strawberry resulted in resistance to *Otiorhynchus salcatus,* a pest known as vine weevil (Cesare Gessler 2007). With *Agrobacterium*-mediated transformation, it is possible to protect the plant from abiotic stresses such as drought, heat, and salinity. For instance, tomato, which is not very drought resistant, was transformed with the Myb transcription factor gene to protect it from stress, and it was discovered that they emerged with reduced water damage in addition to drought tolerance (Gemma Pasquali 2008). Furthermore, the Cu/Zn SOD gene for synthesis of Cu/Zn superoxide dismutase was introduced into potato to remove reactive oxygen species produced in the plant at high temperatures and prevent them from damaging the plant. Potatoes with this gene were able to endure temperatures as high as 40°C without being impacted by heat (Li Tang 2006). Plant production and storage conditions can also be improved by transformation. Polyphenol oxidase (PPO) is the gene that causes fruit browning, and silencing or lowering its expression can increase the storage life of fruits and vegetables. González et al. (2021) aimed to silence the StPPO gene in potatoes and reduce the darkening of potatoes. In the study, PPO enzyme activity showed a significant decrease of 50%, while darkening of the potato was also significantly reduced (Matías Nicolás González 2021).

Bioremediation and biomonitors are another area in which plants may be used by altering their genetic code with *Agrobacterium*. Plants engineered in this way can transform extremely poisonous compounds into volatile or less harmful molecules by utilizing enzymes

that they produce. This process is called bioremediation. Biomonitors convert toxic or explosive compounds in dangerous areas. According to research, transgenic tobacco plants can detoxify soil contaminated with 2,4,6-Trinitrotoluene (TNT) by converting it to hydroxyamino dinitrotoluene (HADNT) (Nerissa Hannink 2001).

Gene transfer with *Agrobacterium* can be used in the pharmaceutical industry to produce valuable proteins such as edible vaccines and recombinant antibodies. A genetically modified plant can produce proteins at low cost, with a high production rate, and with minimal risk of contamination by human pathogens. Plants can also perform post-translational modification that are not possible using bacteria or yeast for such production. Edible vaccines are appropriate for immunizations that are given orally. Because it is difficult to deliver vaccinations to countries such as Africa, researchers aimed to develop products that might be produced in that region and utilized as vaccines. Banana is an excellent host for an edible vaccine, and research in this field is continuing. In addition, *Agrobacterium*-mediated transformation was used to transfer the proteins that are essential for the hepatitis B vaccination (G. B. Sunil Kumar 2005). Plants are also utilized in the production of anticoagulants, growth factors, and interferon. Human growth factor is obtained by genetic modification of the tobacco plant. In this way, proteins with high efficiency and a low chance of contamination are produced and utilized in the pharmaceutical industry (Oranicha Hanittinan 2020). Furthermore, *Agrobacterium*-mediated transformation can enhance the plant's own potency by increasing the production of a metabolite that the plant normally generates. Some examples of this method’s use to enhance different plant metabolites are improvement of the beta-carotene content of canola, the oil content of rice, and the quantity of vitamin A in rice. Scientists inserted a beta-carotene production pathway into rice in order to fortify the diet and reduce the incidence of diseases caused by vitamin A deficiency in underdeveloped countries. Because the color of the rice produced is similar to gold, it is known as golden rice. When golden rice was initially developed, vitamin A levels were 4-5 times greater than normal rice. This amount was increased by further research. (Swapan K. Datta 2007).

1.9. Aim of The Study

Eggplant is a valuable commercial and agricultural plant. This plant is an important crop in many countries, including Turkey. Furthermore, its secondary metabolites are utilized in the pharmaceutical industry. SGAs are significant secondary metabolites of eggplant and have been found to be effective as a cancer treatment. By introducing an additional copy of the eggplant's own transcription factor, GAME 9, to eggplant, using *Agrobacterium*-mediated transformation, we intended to upregulate the SGA pathway. Therefore, the purpose of this project was to obtain transgenic eggplants with stable insertion of the GAME 9 gene and to investigate the effects of this addition on transgenic plants at the DNA, RNA, and metabolite levels. Transformation was verified with PCR experiments and efficiency was calculated. To analyze the transformant plants, RNA expression levels were examined via RT-QPCR. Then SGA amounts were determined with the Dragendorff's reagent method. The results are useful for understanding the effects of GAME 9 overexpression on eggplant's SGA production pathway. In the future, since SGA is a valuable metabolite in medicine, the transgenic plants can be used as a plant factory to produce more SGA from eggplant for medical usage. Moreover, further insight on the SGA pathway can be gained by study and manipulation of the transgenic plants produced in this work.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Biological Materials

Antalya Agriculture, Inc. provided the *Solanum melongena* cv Kemer seeds. Kemer was chosen because it is a popular Turkish cultivar and regenerates well in tissue culture. *Agrobacterium tumefaciens* strain LBA4404 was used for plant transformation.

2.2. Methods

2.2.1. Preparation of Plasmid

The GAME 9 gene was described in tomato by Cardenas et al. (2016). The gene information found in this article was used to find its ortholog in eggplant. The ortholog gene was identified in the Kazusa database which contains the eggplant genomic sequence http://eggplant.kazusa.or.jp/ (Hirakawa H 2014). Sme2.5 forward and reverse primers: (5'ATGAATATTCCATATGATGATGA3') and (5'TCACATCTGTATCAACAT TTCT3') were designed to amplify the whole coding sequence to clone the gene from the cDNA, using the Kazusa gene entry for eggplant Game 9 gene coding sequence (Sme2.5_05213.1_g00003.1). Total mRNA was isolated from the fruit peel of Kemer eggplant. Then, cDNA was synthesized from the isolated mRNA. In this way, the SmGAME 9 gene was cloned. The gene was amplified by PCR from the cDNA and cloned into the pTZ57R/T vector for amplification. From there it was cloned into the 35S cassette and transferred into the pGreen0029 vector for plant transformation. The PGreen0029 vector was used with the pSoup vector which is a helper vector. The pGreen0029 vector contains the kanamycin resistance gene as a selective marker for bacteria and plants. This part of the

experiment was conducted by Hatice Şelale. The pSoup/pGreen 0029 vector containing the GAME 9 gene was transferred to *E. coli* DH5α cells by heat shock method (E. A. Roger P. Hellens 2000). Bacteria were inoculated in LB medium (10 g/L peptone, 5 g/L yeast extract and 5 g/l sodium chloride) containing 50 mg/l kanamycin, 5 mg/l tetracycline. Following growth of bacterial colonies on selective medium, colonies were inoculated to a 2 ml volume of liquid LB medium for plasmid isolation. Plasmid DNA was extracted using the PureLink HiPure Plasmid Miniprep Kit (ThermoFisher). PCR was performed with GAME 9 specific Sme2.5 forward and reverse primers: (5'ATGAATATTCCATATGATGATGA3') and (5'TCACATCTGTATCAACATTTCT3'). PCR conditions were: one step of 5 min at 95°C, 30 cycles of 1 min at 95°C, 1.5 min at 60°C, and 1 min at 72°C followed by one cycle 5 min at 72°C. PCR products were separated by 1% agarose with Tris-acetate-EDTA (TAE) buffer (48.5 g Tris, 11.4 mL glacial acetic acid and 0.5M EDTA (pH 8.0)) gel electrophoresis.

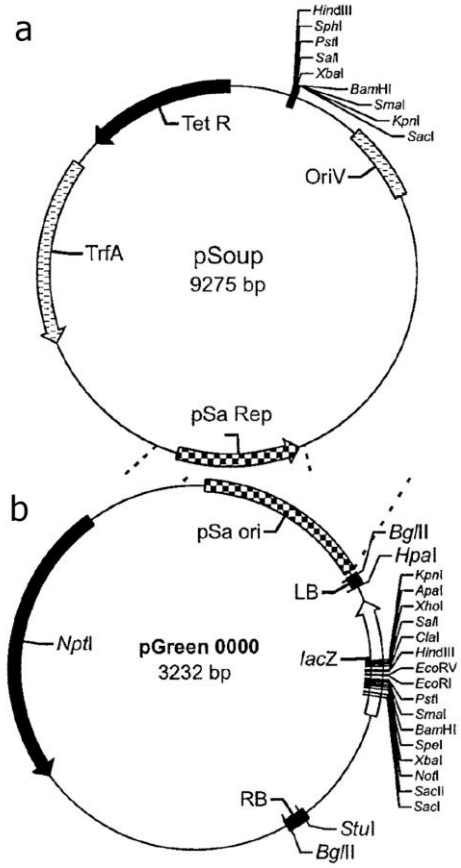

Figure 2.1. Vector system pSoup/pGreen0029

(Source: Roger P. Hellens E. A., 2000)

2.2.2. Preparation of Transgenic *Agrobacterium tumefaciens* Strain

The pSoup/pGreen 0029 vector system containing GAME 9 was transferred to *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (G. Jyothishwaran 2007). For selection, bacteria were plated on LB medium (10 g/L bacto-peptone, 5 g/L yeast extract, 10 mg/L NaCl, 1.5% agar) with 50 mg/ml kanamycin, 15 mg/L rifampicin, and 10 mg/L tetracycline. Following selection, the presence of the plasmid containing the target gene was confirmed by conducting PCR with Sme2.5_05213.1 reverse (5'ATGAATATTCCATATGATGATGA3') and forward (5'TCACATCTGTATC AACATTTCT3') primers. These primers were developed specifically for the target gene to confirm its existence in the *Agrobacterium tumefaciens* cells. PCR conditions were: one step of 5 min at 95°C, 30 cycles of 1 min at 95°C, 1.5 min at 58°C, and 1 min at 72°C followed by one cycle 5 min at 72°C. Bacteria that were confirmed to be transformed with the vector containing the target gene were frozen in 10% glycerol solution and kept at -80°C.

2.2.3. Seed Sterilization and Germination

S. melongena cv Kemer seeds were soaked in 70% (v/v) ethanol for one minute then ethanol was removed. The seeds were then left in sterilization solution [50% (v/v) bleach) plus 250 µl of Tween 20] for 25 minutes. The seeds were rinsed three times with autoclaved distilled water to eliminate the sterilization solution. Lastly, seeds were planted on ½ MS medium (hormone-free MS medium with 3% sucrose and 0.8% agar) in 500 ml jars with 20 seeds per jar (Toshio Murashige 1962). Two sets of transformations were made by planting 500 seeds each. 25 jars with 20 seeds were used for each set. The total number of seeds used was 1000. A total of 50 jars were planted, with each jar containing 20 seeds. Cultures were maintained at 25°C with a light/dark cycle of 16 h light/8 h dark.

2.2.4. Plant Transformation

For transformation, the protocols of Van Eck et al. (2006) and García-Fortea et al. (2020) were modified and used. After 3 weeks, hypocotyls from germinated seeds were collected for transformation. In preliminary research, cotyledons were used, but the eggplant regeneration yield was low. Thus, it was decided to proceed with hypocotyls. With a scalpel, each hypocotyl was cut into approximately two sections and then these explants were incubated with *Agrobacterium* solution for 10 minutes before being placed in a pressure chamber until -0.08 MPa. To prepare the *Agrobacterium* solution, YEP medium (10 g/L bacto-peptone, 5 g/L NaCl, 10 g/L yeast extract, 1.5% agar) with the required antibiotics (50 mg/L kanamycin, 15 mg/L tetracycline, 10 mg/L rifampicin) was inoculated with the bacterium and incubated at 28°C overnight. Then, a single colony from the plate was inoculated into liquid YEP medium (10 g/L bacto-peptone, 5 g/L NaCl, 10 g/L yeast extract) with the required antibiotics (50 mg/L kanamycin, 15 mg/L tetracycline, 10 mg/L rifampicin) and cultured overnight on an orbital shaker at 28°C and 120 rpm. The bacteria were then separated from the medium using a centrifuge at 14000 rpm for 10 minutes. The bacteria were resuspended in liquid MS medium (MS, 3% sucrose, 100 mg/L myo-inositol, pH: 5.6) with addition of 100 M acetosyringone. The bacterial solution was diluted in medium until the optical density was between 0.4 and 0.6 at 600 nm. After treatment with *Agrobacterium*, 262 explants from the first transformation set and 649 explants from the second transformation set were incubated on a total of 26 plates for 2 days on MS solid medium (MS, 3% sucrose, 100 mg/L myo-inositol, 0.8% agar, pH: 5.8) containing 2 mg/L zeatin hormone. After 2 weeks, explants were then put on solid MS medium that also included 75 mg/L kanamycin and 250 mg/L timentin. Explants were subcultured in every 2 weeks. The kanamycin concentration was lowered to 30 mg/ml after three subcultures, and the timentin was withdrawn from the medium. The shoots formed from hypocotyl explants were then excised and transferred to rooting medium (RM) (MS, 3% sucrose, 100 mg/L myo-inositol, 4 mg/L IAA, 0.8% agar, pH: 5.6) for root formation. Plants with sufficient rooting were planted in autoclaved soil, covered with bags to enhance humidity and facilitate adaptation, and incubated in a light environment at 24°C. Within a week, the bag was gradually opened, and the plant adjusted to its new environment.

2.2.5. Transformation Verification

PCR was used for initial analysis of the transformed plants. The CTAB DNA extraction technique was used to extract the genomic DNA of such plants (C. N. Stewart 1993). DNAs were loaded to 1% agarose gel to verify that the DNAs were high quality, then PCR was carried out using 35SP341 reverse (5'GGGCTGTCCTCTCCAAATGA3') and forward (5'TCAACAAAGGGTAATTTCGGGA3') primers, which amplify the promoter region of the T-DNA. The PCR mixture was 25 µl in volume and contained 2.5 µl 10X PCR buffer, 1 mM dNTPs, 1 mM MgCl2, 400 ng DNA template, and 1 U TAQ DNA polymerase. In PCR tests, the plasmid construct pSoup/pGreen 0029-GAME 9 was used as a positive control. The reaction began with a 5 min incubation at 94 °C, followed by 30 cycles of 60 sec at 94 °C, 60 sec at 59 °C, 60 sec at 72 °C, and a final 5 min extension at 72 °C in a thermocycler. PCR products were loaded on 1% agarose gels stained with ethidium bromide. Visualization under UV light was used to determine if the samples contained the expected amplification product.

2.2.6. RNA Expression Analyses

For mRNA expression level measurements, Real-time Quantitative polymerase chain reaction (RT-qPCR) was used. Total RNA was isolated by Plant/Fungi Total RNA Purification Kit (Norgen, Canada) according to the manufacturer's instructions from plants which were confirmed as transgenic. DNase was used to treat RNA samples (NEB, UK). A Nanodrop spectrophotometer was used to quantify RNA concentration (MultiskanGO Microplate Spectrophotometer, Thermo Scientific, USA). According to the procedure of the GoScriptTM Reverse Transcription System (Promega), 1.5 µg of total RNA was allocated for cDNA synthesis from each sample. The cDNA was synthesized from the isolated RNA using oligo$(dT)_{18}$ primers. RT-qPCR was carried out on a Light Cycler 480 using cDNA templates and the GoTaq® qPCR Master Mix (Promega). In addition to the mix and cDNA, Sme2.5_05213.1_RT reverse (5'TTCATAAGATCCCAGCCACAG3') and forward (5'AAGATTGCAGGCGGTTCATAG3') and Cyclophilin_RT reverse (5'ACAAAGGGCA GGGACGTAGTCAA3') and forward (5'CGCGCGCTACACTGATGTATTCAA3')

primers were utilized. Each reaction was made in 20 µl volume. The reaction began with initial denaturation by 2 min incubation at 95 °C, followed by amplification using 40 cycles of 15 sec at 95 °C, 60 sec at 60 °C, 10 sec at 72 °C, then melting 5 sec at 95 °C, 60 sec at 65 °C, 10 sec at 97 °C and a final cooling step of 10 sec at 40 °C in the Light Cycler 480 II. RNA isolation was done twice from each plant to yield two biological replicated. RT-qPCR reactions were done twice on each RNA sample yielding two technical replicates for each biological replicate. The Livak method was used to determine the relative expression levels of each sample (J. Livak and Thomas D. Schmittgen 2001). The ΔCT value was computed using the formula provided in the article. In the formula, the average Ct value of the target gene, GAME 9, was subtracted from the average reference gene, Cyclophilin [Equation 1]. The ΔΔCT value was obtained from the transgenic ΔCT by subtracting the non-transgenic ΔCT [Equation 2]. Finally, the relative expression was calculate using Equation 3.

$\Delta\Delta\mathrm{Ct} = \mathrm{Ct}(\mathrm{Target\ gene, Game\ 9}) - \mathrm{Ct}(\mathrm{Referenece\ gene, cyclophilin})$[Equation1]

$\Delta\Delta Ct = \Delta Ct(non - transgenic) - \Delta Ct\ (trangenic)$ [Equation 2]

Relative expression = $2^{-\Delta\Delta CT}$ [Equation 3]

2.2.7. Metabolite Expression Analyses

For total SGA analysis, the Dragendorff's Reagent (DR) technique was applied (Mehrotr 2003). Metabolite extraction from each plant was done twice yielding two biological replicates and the procedure was applied twice on each extract to provide two technical replicates. For DR procedure, 0.25 g of plant leaf tissue was incubated for 3 h in 98% acetic acid: Methanol (v:v). As indicated in the published protocol, the resulting liquid was centrifuged at 14000 rpm, and supernatant was filtered and used. Then the filtered liquid was mixed with 2 ml DR reagent (0.8 g bismuth nitrate in 50 ml acetic acid: water (5:1) and 8 g potassium iodide in 20 ml water) and vortexed. Samples were centrifuged at 14000 rpm for 5 min and supernatant was removed. The pellet was then washed with 98% ethanol two times. The pellet was then treated with 2 ml disodium sulfide (1%) before being dissolved in nitric acid (65%). Then samples were diluted with water up to 10 ml. Finally, 2 ml of each sample was taken and mixed with 5 ml thiourea (3%). UV visible spectrophotometer was

used to measure the resultant combination at 435 nm. The total SGA standard curve was created using SM standard solution (0.1-0.7 mg/mL).

2.2.8. Statistical Analysis

Using Microsoft Excel, all data were gathered and analyzed as mean ± S.D. The student's t-test was then used to compare means. P values less than 0.01 were regarded as indicating statistically significant differences between means. The data were also statistically examined using one-way ANOVA.

CHAPTER 3
RESULTS AND DISCUSSION

3.1. Verification of Plasmid

For verification that the correct plasmids were inserted in *E. coli*, PCR was performed with Sme2.5 GAME 9 specific primer after plasmid isolation. This PCR was replicated twice for 13 plasmid samples in total. In Figure 3.1, the PCR products in the wells labelled 15 were negative controls, contained only the PCR mix and yielded no PCR product as expected. Duplicated samples in wells 4 and 9 to 14, have the expected band at 673 bp. The samples in wells 1 to 3 and 5 to 8 have no amplification which indicated that there was no plasmid transformation in these clones. As a result of this experiment, seven positive plasmids were selected and further experiments were continued with them.

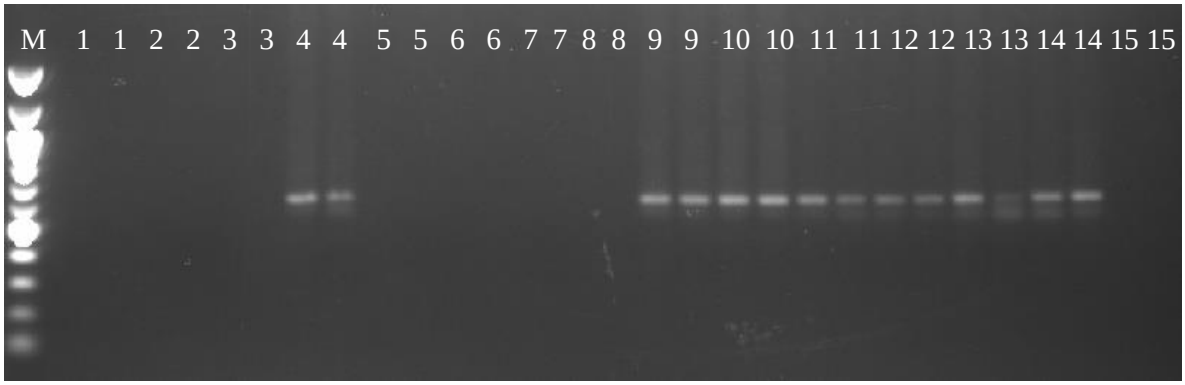

Figure 3.1. Verification of GAME9 gene in plasmids by PCR. M-marker DNA 100bp ladder, 1- 14 are independent colonies tested for GAME9 gene (673 bp), 15- negative PCR control. Each colony was tested in duplicate.

3.2. Verification of Transgenic *Agrobacterium tumefaciens* Strains

Agrobacterium tumefaciens transformation was done with the verified plasmids to obtain *A. tumefaciens* strains to use for transformation of the GAME 9 gene into eggplant. Transformed bacteria were inoculated on plates containing kanamycin and rifampicin. The

colonies that multiplied on these plates were collected and colony PCR was applied to them using the same primers as used in section 3.1 to confirm that they contained the GAME 9 gene. Gel electrophoresis of the amplification products (Figure 3.2.) indicated that except for samples 2 and 7, the band appeared where it should be, as desired, at 673 bp. This result indicated that most of the *Agrobacterium* clones contained the desired plasmid.

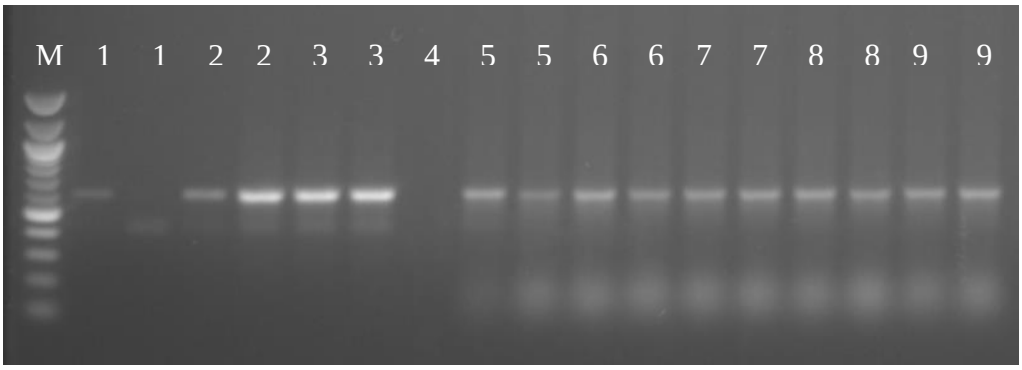

Figure 3.2. Confirmation of Agrobacterium transformation with the GAME 9 plasmid by PCR. M-marker, 1 tested sample, 2-3 positive control for PCR, 4 negative control for PCR, 5-9 tested samples for GAME 9 gene (673 bp).

3.3. Seed sterilization and germination

Two sets of transformation were done with each set starting with hypocotyl explants from 500 seeds. Over both experiments, 465 of the 1000 sown seeds germinated, a germination efficiency of 46.5%. Figure 3.3. shows the germinated seedlings in plant tissue culture. Eggplant seeds are easy to germinate, however, when seeds are taken from field-grown fruit, they sometimes contain a variety of microorganisms. The seeds provided for this thesis, however, did not include any contaminating organisms that could cause problems. The sterilization and germination efficiencies were compared with other articles and were found to be similar to previous work (Edgar García-Fortea 2020).

Medium selection is a critical factor in plant tissue culture. Seeds have an endosperm to germinate easily but providing a suitable environment increases the germination efficiency. For germination, moisture of the magenta boxes and light cycle were optimized and half-strength MS medium was suitable for seedling growth after germination. Seeds

reached the seedling stage in 21 days. At this point, cotyledons emerged and the seedlings were ready for transformation.

Figure 3.3. Germinated aseptic eggplant seedlings in plant tissue culture.

3.4. Plant Transformation Efficiency and Verification

Explants were first incubated on medium which contained zeatin. Zeatin is a cytokinin that encourage the explants to produce shoots. Trans-zeatin was used as the cytokinin of choice in the experiments since it provides better results than other cytokinin in eggplant regeneration (Edgar García-Fortea 2020). Two days after exposure to *Agrobacterium*, explants were transferred to medium containing zeatin and antibiotics. Antibiotics were used for elimination of excess bacteria in the media and to select for the regeneration of transformed cells. Every 2 weeks, explants were transferred to new medium in order to maintain the medium's nutritional quality and a suitable level of selection. At the beginning of the 3rd week, shoots began to form on hypocotyl explants. In the 4th week, shoots that had elongated were taken from petri dishes to jars. The total number of shoots reached its maximum in the 6th week. From transformation set 1, 110 shoots were obtained from 262 explants, a 42% regeneration efficiency. From transformation set 2, 576 shoots were obtained

from 649 explants, a 89% regeneration efficiency. After 6 weeks, some shoots which were non-transgenic turned yellow and withered due to the presence of antibiotics.

At the beginning of the 12th week, the shoots that reached 3 cm in length were removed to rooting medium which contained IAA. IAA is an auxin which induces root formation. Roots formed by the 22nd week, at which time plantlets were transferred to soil for acclimatization. Figure 3.4. shows the regeneration of the explants to plants in 40 weeks. Since the environment outside plant tissue culture is not sterile and has lower humidity, plants needed to undergo an acclimatization process. Soil may contain fungus and bacteria; therefore, soil was autoclaved. Also, plants were covered with a plastic bag to increase humidity. After a week, the plastic bag was opened and removed slowly over several days and plants were adapted to their new environment. Then, after 8-10 weeks plants were transferred to bigger pots to accommodate their growth.

The experiment, which started with 911 explants, ended with the adaptation of 19 transgenic candidate plants to the soil at the end of the 30th week. Thus, regeneration efficiency was calculated as the number of verified transgenic plant divided by total explant number, resulting in an efficiency of 2.1%.

A PCR test was performed on the DNA samples taken from the candidate transgenic plants. The 35S341P primers used for this test were expected to yield a product of 341 bp. The primers were designed to amplified the promoter region of the gene construct. The results showed that 12 out of 19 (63%) plants tested for PCR were transgenic because the expected bands were obtained. There was no band for the negative control which contained only master mix. In addition, no band was observed in the gel image of the PCR test performed on DNA taken from three non-transgenic plants which were used as controls. Based on the PCR results, transformation efficiency was 1.3%.

In the literature, transformation efficiency varies between 10-50%. However, in our experimental setup, this value was lower because transformation was performed with a binary vector. This binary vector is present at a low copy number which reduces the probability of transformation of both plasmids into a given cell. It is well known that binary vectors can reduce transformation efficiency (Roger P. Hellens, 2000).

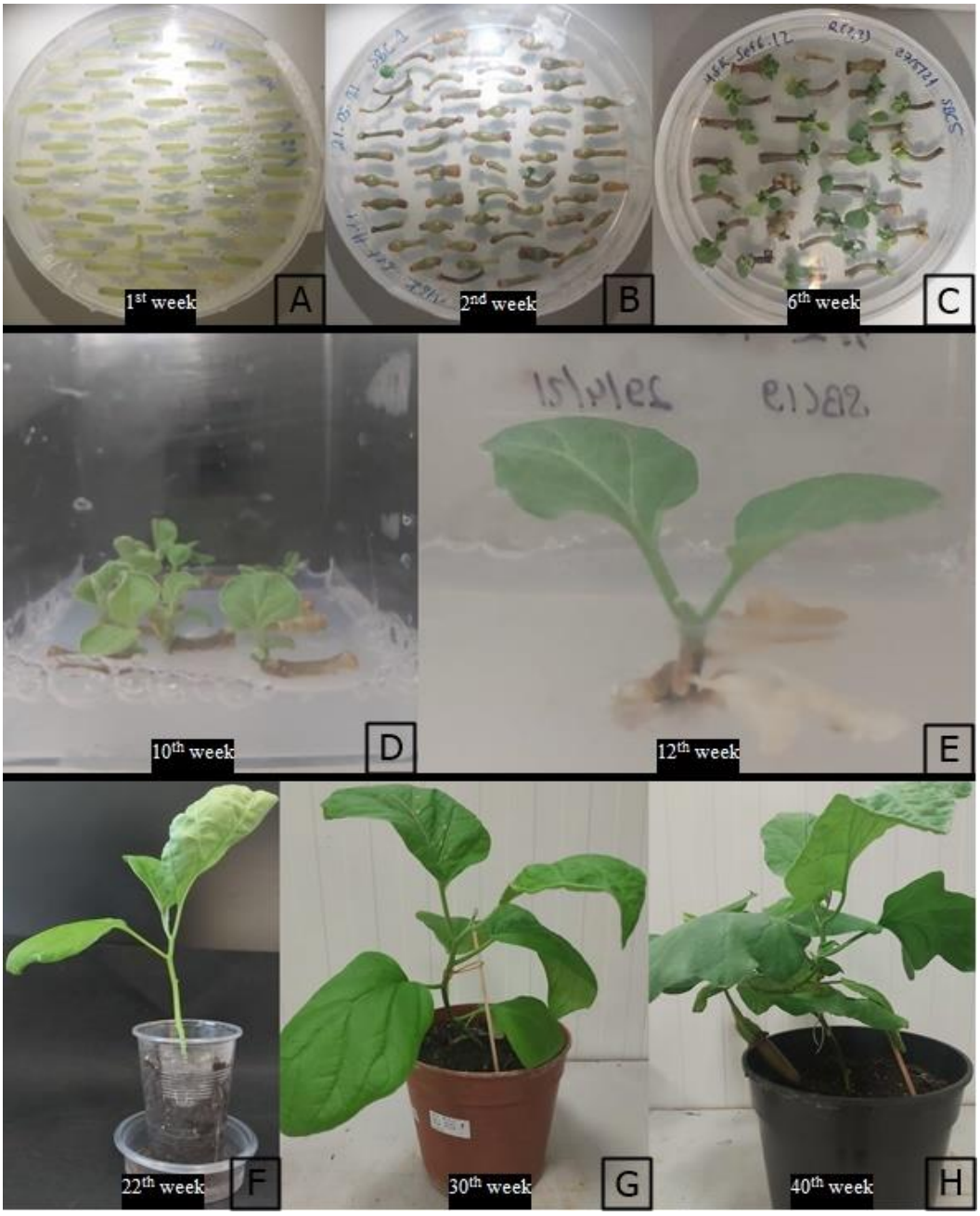

Figure 3.4. Regeneration of transformed plants over a 40-week period. (A) Explants were cut from seedlings and incubated in antibiotic-free medium, 1st week, (B) 2nd week, explants were transferred to antibiotic-containing medium, (C) first shoot formation at 6th week, (D) 10th week, shoot has grown large enough to transfer to RM, (E) 12th week, plant is now 3-4 cm in RM, (F) 22th week plant transfer to autoclaved soil and covered with plastic bag for acclimatization, (G) 30th week, plant transplanted to larger pot, (H) 40th week fruit development is seen.

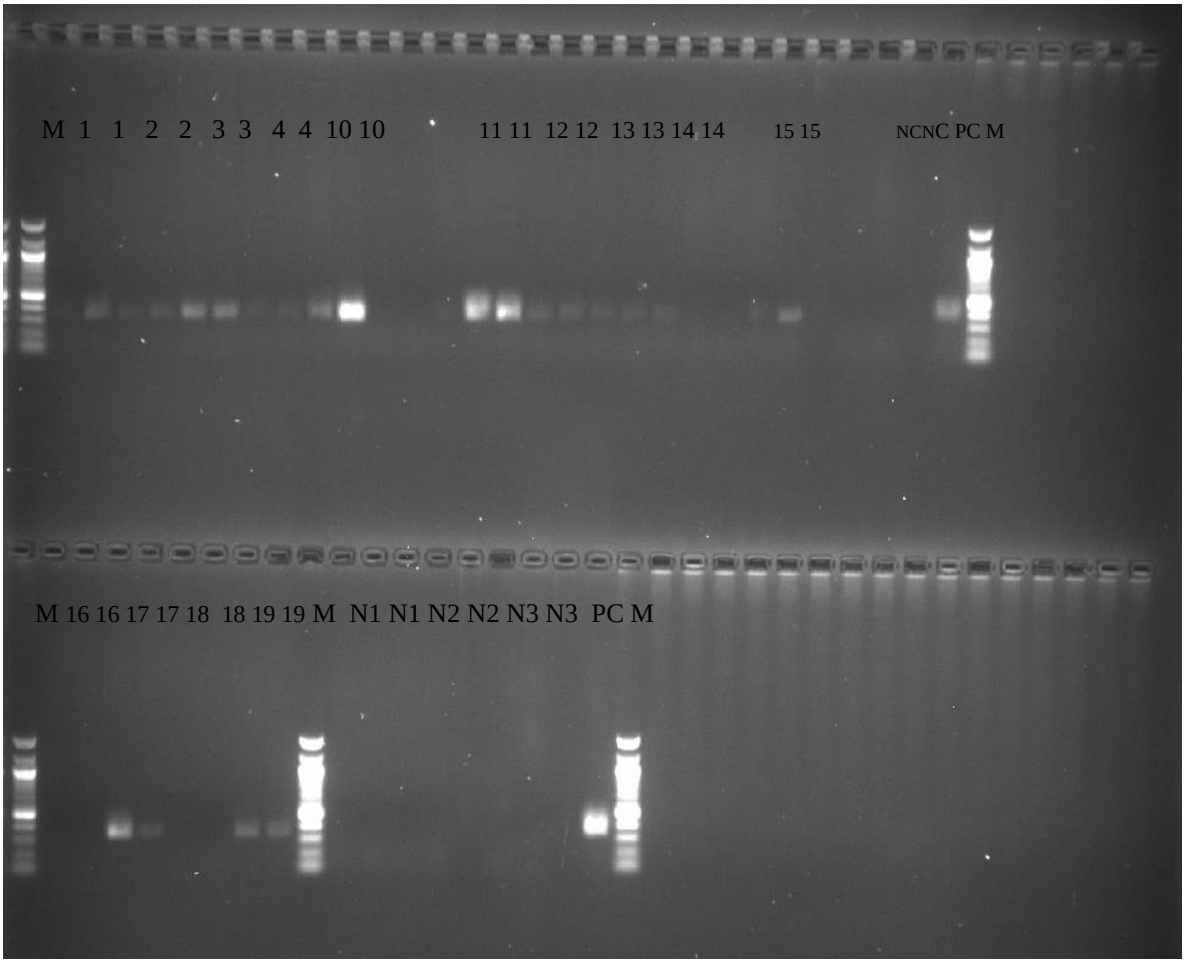

Figure 3.5. Verification of transformation with 341P primer. Each sample is duplicated. M- marker DNA 100bp ladder, 1, 2, 3, 4, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 are the samples tested for vector promoter region (341bp), NC- negative control of PCR, PC positive control, N1, N2 and N3 non-transgenic plant samples. Unlabeled lanes are empty.

In addition, another article indicated that the antibiotic concentration has a serious negative effect on yield of transformed plants. Concentrations of 5-25 mg/l kanamycin affect regeneration negatively in eggplant. Although the plants are transgenic and resistant to antibiotics, the degree of resistance they carry may be low when they are in the explant state, thus reducing the amount of regeneration. However, antibiotics are important in this study as they enable us to select for only regeneration of transgenic cells (Mahmoud Bagheri 2017). Eggplant genotype also plays an important role in transformation efficiency. In the light of the preliminary studies carried out by the laboratory, Kemer provided higher regeneration efficiency than Topan and Halep, which are cultivars grown in Turkey.

3.5. mRNA Expression Analyses

For mRNA expression analysis, RNA extraction was performed and RT-QPCR was applied with Sme 2.5 RT primers which are specific for the GAME 9 gene. Because the plants were transformed with a gene that is already present in the eggplant genome, the expression of both the endogenous and transgenic GAME9 copies are observed and cannot be distinguished by the RT-PCR primers. To try to gauge the effect of the transgene alone, the same experiment was applied to non-transgenic plants. Comparison of the results for both transgenic and nontransgenic individuals allows calculation of relative mRNA expression. Data from this experiment are shown in Figure 3.6. RT-QPCR results analyzed by the Livaak method provide a relative analysis. Overall gene expression of the transgenic plants ranged from 0.16 to 9.02-fold that of the non-transgenic control. Plant samples 1, 2, 3, 4, 11, 12, 13, 14 and 17 showed at least twice the expression level of the non-transgenic plant sample. On the other hand, plant 19 had a lower level of expression of the gene (0.16) which may mean that the transgene and endogenous copy were partially silenced in this plant.

As is evident from the results, the expression of the GAME 9 gene differed from plant to plant. These results are expected because many factors affect gene expression. One explanation for this is that *Agrobacterium* transferred the gene of interest randomly into the plant genome. As a result, the gene may have been transferred to a heterochromatic region and its expression level affected by nearby silencing. Alternatively, insertion into a highly active region could be associated with increased activity of the transgene. The number of transgene copies may also differ among plants which can also increase or decrease expression depending on whether or not silencing occurs. Copy number of the transgenic plants should be assessed using Southern hybridization and study of the progeny from self-pollination.

Variability in expression levels can also result from interaction of the endogenous and introduced copies which can lead to silencing. In the literature, overexpression of GAME 9 has shown different results in different tissues and plant species from the same family as eggplant. While statistically significant overexpression of this gene was achieved in the leaves of potato and tomato, the same cannot be said for other tissues. Potato tuber peel did not have increased GAME9 gene expression in some transformation sets (Pablo D. Cárdenas 2016).

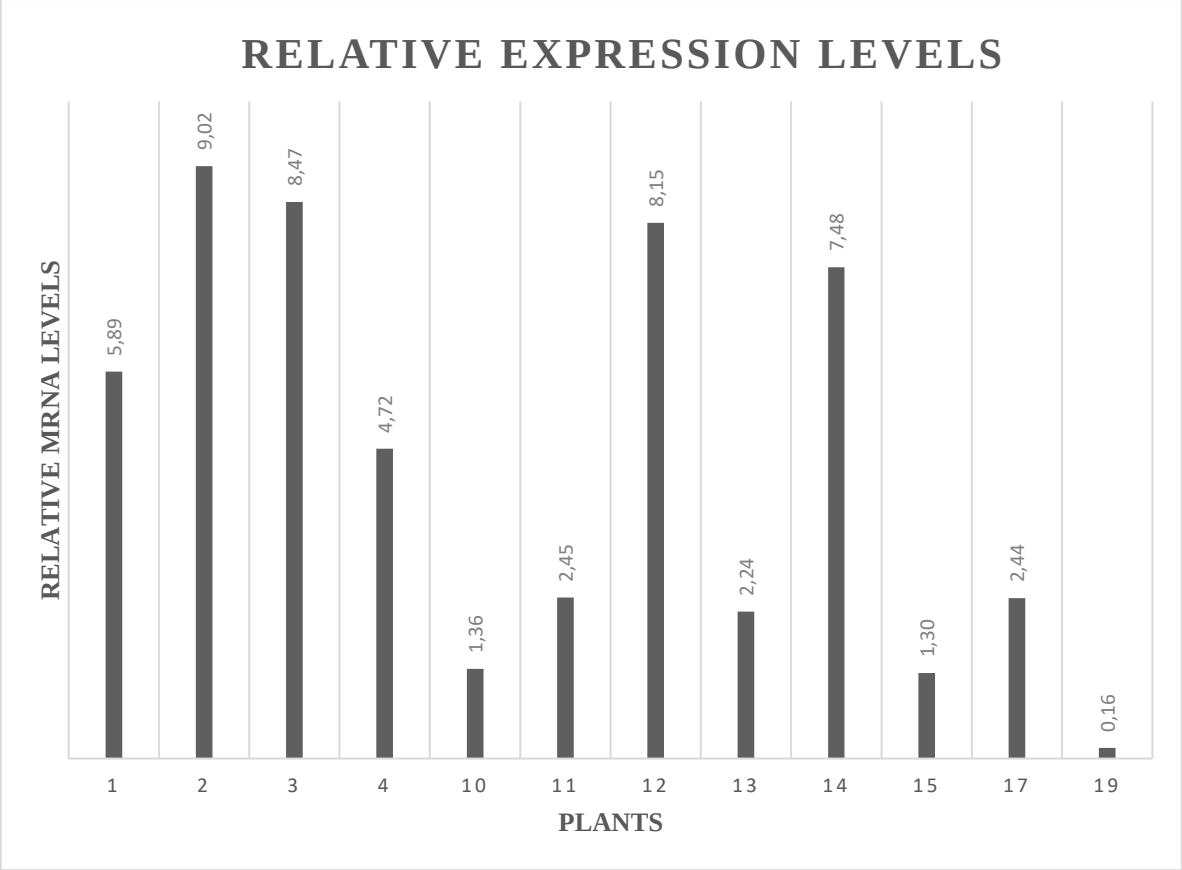

Figure 3.6. Relative mRNA expression levels of GAME 9 gene analysis via Livaak method to transgenic and non-transgenic eggplant leaf samples.

3.6. Metabolite Expression Analyses

To create a standard graphic for SGA content, 0.1 mg/ml to 0.7 mg/ml α-solamargine standards were tested with Dragendorff's Reagent (DR) method and their absorbance was determined with spectrophotometer. The results are shown in Table 3.1. There was a consistent increase in the absorbance of the standards as expected. From the data in Table 3.1. a standard curve was drawn and provided a very good fit to the data (R2 = 0.96). According to the equation of the curve in this graph (Figure 3.7.) each sample's concentration was calculated.

The SGA concentrations of leaves from the transgenic samples are shown in Figure 3.8. The average concentration of SGAs in non-transgenic samples was 0.29 mg/ml. Although there is no study with the same genotype (*S. melongena* cv. Kemer), it showed

similar results with the studies conducted in an Iranian cultivar of *S. melongena* (Mahmoud Bagheri 2017). The transgenic samples had concentrations of SGAs ranging from 0.42 to 0.86 mg/ml. They were significantly higher than the level in nontransgenic leaves. Thus, the introduction of the GAME 9 gene resulted in SGA contents that were at least 1.4-fold higher than untransformed plants. Remarkably, three of the plants had at least 2.5-fold more SGAs than the controls.

Table 3.1. Absorbance value of the α-solamargine standards at 435 nm.

Standard concentration (a-solamargine) (mg/ml)	**Absorbance value (435nm)**
0.1	0.203
0.2	0.287
0.3	0.343
0.4	0.355
0.5	0.388
0.6	0.465
0.7	0.491

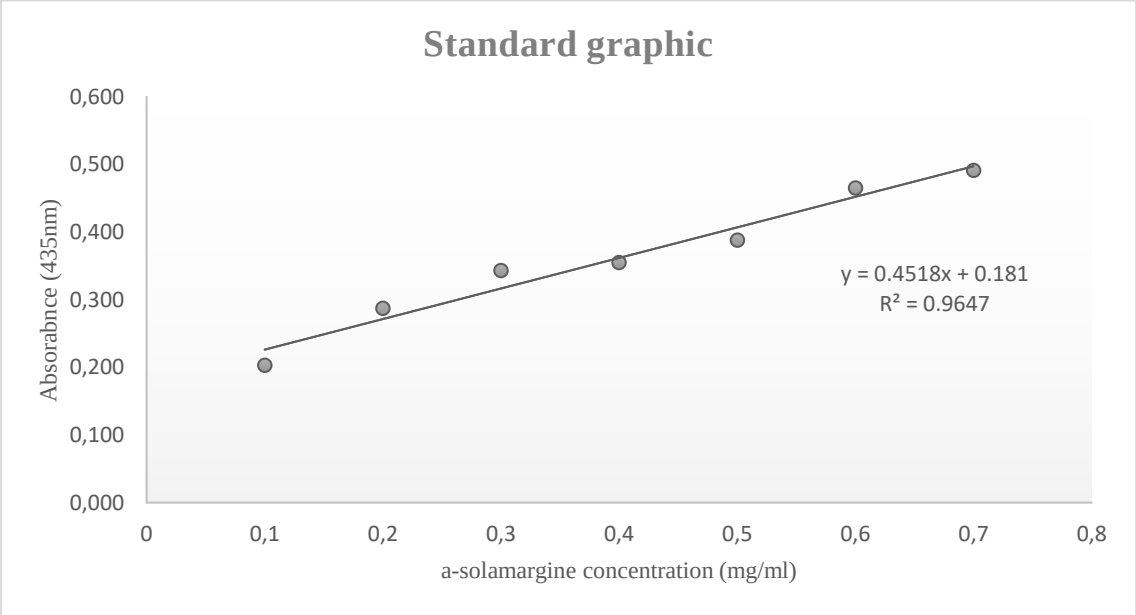

Figure 3.7. α-solamargine (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 mg/ml) standard curve, equation and R2 value.

Studies with the GAME9 gene have previously been done in potato and tomato. Studies show that a statistical increase was observed in the amount of solanine and chaconine in potatoes, and in the amount of tomatine and dehydrotomatine in tomatoes. The increase in potato leaf was 3.5-4.6 fold for chaconine and solanine. In the tomato leaf, it was stated as 1.2-2.6 fold for tomatine and dehyrotomatine. The SGA increases in this work were molecule-specific (Pablo D. Cárdenas 2016). In our experiments, the total amount of SGA was examined. A similar fold difference was found. In this study, in addition to the total SGA analysis, compound specific analyses should be performed in the future. Such work will reveal the identities and amounts of the specific SGAs that are overproduced in our plants.

SGAs are useful in the development of steroidal hormone-based therapies such as birth control pills and anti-inflammatory medications. These compounds also show anti-diabetic, antifungal, antiparasitic, antibiotic, antimicrobial, antiviral, and anticancer properties. Especially in the field of cancer, research of SGA's use to fight this disease is still being carried out due to its high cytotoxicity (S.S.S. Al Sinani 2017). Although SGAs stand out with these features, they are very expensive to manufacture and, therefore are sold at a premium price. On the market, 10 mg is sold for 528€ (Merck, 2022). Production costs can be reduced by obtaining SGA molecules from transgenic plants with increased SGA production. A wild relative of eggplant, *Solanum linnaeanum,* is known for its high amount of SGA content. *S. linnaeanum* fruits SGA production is 25.5 mg/g. It is noteworthy that this content is similar to that found in our transgenic samples. The leaves of one of the transgenic plants we produced contained 34 mg/g total SGA, with more than 25.5 mg/g in four of the detected transgenic plants. Although *S. linnaeanum* is an important plant species for SGA production, its growth is difficult due to its wild growth habit, prickles and small fruit (F. K. Nergiz Gürbüz 2015). Therefore, SGA production can be increased by using our transgenic plants. In addition to species, the amount of SGA varies with tissue. In general, the flower bud is the part of the plant that produces the most SGA, followed by the leaves and fruit (Mahmoud Bagheri 2017). SGA quantities can be examined from different tissues of our transgenic lines in future experiments. Based on these results, the transgenic lines may be preferred for production if the amount of SGA is higher in the fruit. this is because the fruit of wild eggplant is small and yield is low, while Kemer produces a good yield of large fruit.

The plant-to-plant variation in SGA content was not explained by the differences in mRNA expression level as the two values were not significantly correlated. Despite the fact that plant-19 had mRNA expression that was lower than non-transgenic plants, the amount of SGA metabolite increased. Furthermore, whereas plants-2 and 14 had enhanced mRNA expression, the expected increase in metabolites due to the increase in mRNA was not observed.

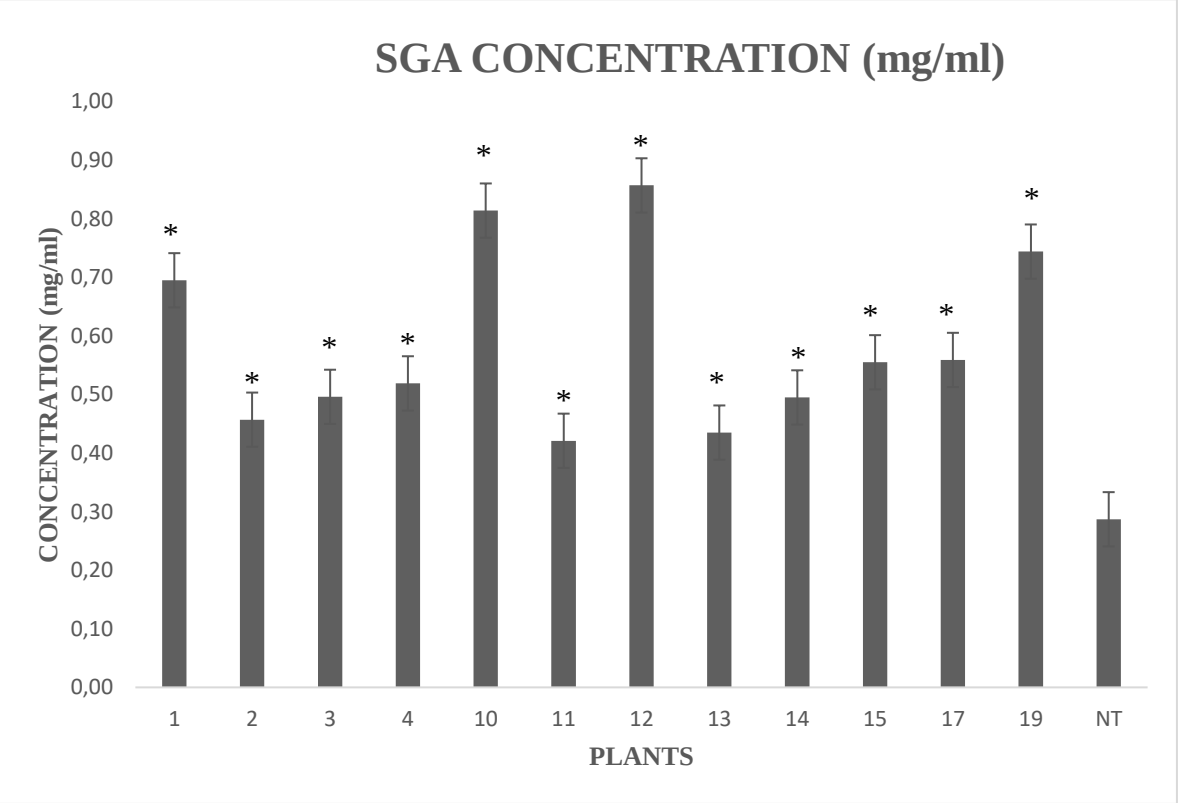

Figure 3.8. Concentration of SGAs in leaf samples from the non-transgenic (NT) and transgenic (1, 2, 3, 4, 10, 11, 12, 13, 14, 15, 17, 19) transgenic eggplant plants. *Statistically significant difference between non-transgenic control (NT) and transgenic plants ($P<0.01$).

Depite this lack of correlation, a statistically significant increase was observed relative to the non-transgenic plant. For plants-1, 10, 12 and 19, the amount of SGA increased along with the amount of mRNA. There are many reasons for this lack of correlation including post-transcriptional gene regulation, variable translational efficiency and enzyme activity. Experiments reveal that, despite being overproduced as mRNA, the amount of

metabolite produced in some situations cannot be increased. GAME 9 TF binds to the MYC gene's serine-rich region. The protein must undergo post-translational modifications in order to bind to this region and protect its stability. Post-transcriptional regulation controls most ERF TFs. Phosphorylation and ubiquitin degradation are the most common modifications. Phosphorylation provides stability and transactivation, while ubiquitin provides deactivation and destruction. Even if mRNA expression is high, inadequate or inappropriate post-translational changes may prevent the molecule from having the desired effect (Zhouli Xie 2019). Thus, it may be that, although more GAME 9 is being produced, it cannot attach to the desired place or increase production because these alterations are not being made at the appropriate level. Unfortunately, there is not yet enough information to explain the discrepancy between mRNA and SGA levels completely because GAME 9's production and modification have not yet been thoroughly studied. The difference between mRNA and metabolite levels may also be due to the different dates the samples were taken. Leaves were a bit older when metabolism analysis was performed, and there may have been a difference between the amount of SGA and the relative amount of RNA produced at that time. Such questions will be answered in future research (Yuan 2021).

CHAPTER 4

CONCLUSION

Eggplant is a valuable crop for both agricultural and medical purposes. For medicinal uses, eggplant's secondary metabolites, more specifically SGAs are quite interesting both for their pharmaceutical effects and their economic value. SGAs have been shown to have anticancer effects. Thus, it is of great importance to increase the production of these secondary metabolites. Therefore, this study aimed to increase the amount of SGA in eggplant. For this purpose, the GAME 9 transcription factor was transferred to the hypocotyls of seedlings germinated in tissue culture using the *Agrobacterium*-mediated transformation technique. Our results showed that, in the majority of cases, GAME 9 transfer to the eggplant genome was associated with increased amounts of SGA produced in leaves. Although some plants showed a decrease or little change in mRNA expression levels, an increase in SGA production of the same plants was observed. Overall, all but one of the transgenic plants showed a minimum two-fold increase in RNA expression and metabolite levels. In the future, the effect of the GAME 9 gene on SGA production can be investigated further. Variability in the amount of production can also be investigated. Plants with increased SGA production are promising for more intensive production of these valuable compounds. Synthetically obtaining 1 mg of SGA, which costs over 53 euros, is extremely challenging. As a result, alternative methods are used such as genetic modification. Wild eggplant, for example, has an abundance of SGAs, but it is difficult to grow. This makes the transgenic plants developed in this thesis valuable because they are easy to grow and manage and yield a high amount of SGA. Various purification and encapsulation processes can make these molecules more valuable in medicine. As a result, the plants produced in this research can be utilized in the research and manufacture of anticancer medications.

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