GENETIC MODIFICATION OF CHLOROGENIC ACID CONTENT IN EGGPLANT (*SOLANUM MELONGENA* L.) BY *AGROBACTERIUM*-MEDIATED TRANSFORMATION TECHNIQUE

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ABSTRACT

GENETIC MODIFICATION OF CHLOROGENIC ACID CONTENT IN EGGPLANT (*SOLANUM MELONGENA* L.) BY *AGROBACTERIUM*-MEDIATED TRANSFORMATION TECHNIQUE

Eggplant is a nutritious vegetable that is low in lipids and high in fiber, vitamins, and minerals. Eggplant is a good source of phenolic compounds, particularly chlorogenic acid (CGA), which is associated with several health benefits, such as reducing the risk of diabetes and cardiovascular diseases. Among CGA-rich fruits, eggplant has a geographically wide distribution while coffee is limited to tropical regions. For that reason, eggplant can be used as alternative to coffee for CGA production. The goal of this research was to develop transgenic eggplant that has increased CGA content using the *Agrobacterium*-mediated transformation technique. The over-expression of the *Myb1* gene, which is related to CGA synthesis and responsible for the production of a transcription factor that activates the phenylpropanoid pathway in eggplant, was used. The *Myb1* gene was isolated, amplified, and inserted into the PRI910 vector system before being transformed into *Agrobacterium tumefaciens*. After plant transformation, seven regenerated plants were verified as transgenic by PCR analysis. Young leaves from transgenic eggplants were used for mRNA expression analysis and metabolite analyses. A two-fold increase in *Myb1* gene expression levels for three of the transformed plants was observed and five plants showed a significant increase in 3-CGA content. Expression of *Myb1* correlated with CGA content measured by HPLC analysis. Specifically, the two-fold increases in *Myb1* expression of three plants corresponded with two-fold increases in their leaf 3-CGA content. In the future, these genetically modified plants could serve a crucial role in better understanding the pathway of CGA production in eggplants, and may also prove useful in generating valuable CGAs for both economic and medicinal purposes. Subsequent research can be directed towards determining the CGA content via measurement of 5-CGA and examining CGA content in different parts of the fruit such as flesh and peel. Study of the next generation (T_1 plants) will also be critical for confirming the stability of the transformation.

ÖZET

PATLICANDA KLOROJENİK ASİT İÇERİĞİNİN *AGROBACTERIUM* ARACILI TRANSFORMASYON TEKNİĞİ İLE GENETİK MODİFİKASYONU

Patlıcan, lif, vitamin ve mineral açısından zengin, besleyici ve düşük kalorili bir sebzedir. Patlıcan, özellikle klorojenik asit (CGA) olmak üzere fenolik bileşiklerin iyi bir kaynağıdır. Bu fenolik bileşikler diyabet ve kardiyovasküler hastalıkların riskini azaltmak gibi birçok faydayla ilişkilendirilir. CGA'nın iyi kaynaklarından biri olmasının yanında tropical bir meyve olan kahvenin aksine patlıcan, geniş bir iklim yelpazesinde büyütülebimektedir. Yüksek CGA konsantrasyonlu patlıcan alternatif bir ürün olarak kullanılabilir. Bu araştırmanın amacı, *Agrobacterium* aracılı transformasyon tekniği kullanarak CGA içeriği artırılmış patlıcan geliştirmektir. CGA sentezi ile ilgili olan ve patlıcanlardaki fenilpropanoid yolunun aktivasyonunu sağlayan bir transkripsiyon faktörünün üretiminden sorumlu *Myb1* geninin aşırı ifadesi kullanıldı. *Myb1* geni izole edildi, çoğaltıldı ve *Agrobacterium tumefaciens*'e aktarılmadan önce pRI910 vektör sistemine yerleştirildi. Bitki transformasyonu sonrasında, PCR ile yapılan taramada 7 bitkinin transgenik olduğu doğrulandı. Bu 7 transgenik patlıcanın genç yaprakları mRNA ifade analizi ve metobolit analizleri için kullanıldı. Bulgular, bu bitkilerin 3'ünde *Myb1* gen ifade düzeylerinde iki kat artış olduğunu gösterdi. Ayrıca, 5 bitki 3-CGA içeriğinde önemli bir artış gösterdi. *Myb1*'in ifadesi, HPLC analizi ile ölçülen CGA içeriği ile orantılılık gösterdi. Üç bitkinin *Myb1* ifadesindeki iki kat artış, 3-CGA içeriğindeki iki kat artışa karşılık geldi. Gelecekte, bu genetik olarak modifiye edilmiş bitkiler, patlıcanlarda CGA üretim yolunun daha iyi anlaşılmasında kritik bir rol oynayabilir ve ekonomik ve tıbbi amaçlar için değerli CGA'ların üretiminde faydalı olabilir. İleri araştırmalar, CGA içeriğinin 5-CGA eşdeğer ölçümüyle belirlenmesi ve meyvenin farklı kısımlarındaki CGA bolluğunun incelenmesi yönünde olabilir. Sonraki nesil T_1 bitkilerin incelenmesi, transformasyonun stabilitesinin belirlenmesi için kritik olacaktır.

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CHAPTER 1

INTRODUCTION

1.1. Importance of *Solanum melongena* L.

Solanum melongena L. (2n=24), commonly known as eggplant or aubergine, is a member of the Solanaceae family. This family also includes other economically important crops such as tomatoes, potatoes, and peppers. Eggplant is a perennial plant but is often cultivated as an annual. It is native to South Asia and was domesticated not later than 59 BC (Wang, Gao, and Knapp 2008). The plant produces large, fleshy fruits that vary in size, shape, and color, including purple, white, green, and variegated types. Eggplant is a popular vegetable crop consumed worldwide and is valued for its unique texture and mild, slightly bitter taste.

Eggplant is cultivated in various regions across the globe, particularly in Asia, the Mediterranean, and the Middle East. China and India are the world's leading producers, followed by Egypt, Turkey, and Iran. According to the Food and Agriculture Organization (FAO) of the United Nations, global eggplant production has been on the rise over the past few decades. In 2021, the total production reached approximately 58 million metric tons. Eggplants are grown in both open fields and greenhouses, with varying cultivation practices depending on the region and local preferences. Turkey is among the top eggplant producers globally. In 2021, the country produced around 832,938 tons of eggplants, accounting for approximately 1.4% of the world's total production. The major eggplant-producing regions in Turkey are the Mediterranean, Aegean, and Southeastern Anatolia regions.

Eggplants have various effects on health across different systems in the body. They offer potential benefits such as reducing liver problems, aiding in weight

management, supporting cardiovascular health, and providing antioxidants that may help protect against certain diseases (Meyer et al. 2014). The health advantages that eggplants offer can be attributed to the nutritional elements present in this vegetable. Essential nutrients found in eggplants (summarized in Table 1.1.) include carbohydrates for energy, proteins, and fatty acids that support various biological functions, dietary fiber, folate, ascorbic acid (vitamin C), vitamin K, niacin, vitamin B6, pantothenic acid, and an array of minerals such as potassium, iron, magnesium, manganese, phosphorus, and copper, as reported by the USDA (2019). It is important to note that the specific nutrient content in 100 g of eggplant can vary depending on the subspecies and growth conditions. A study done by Arivalagan et al. (2013) showed that there are significant differences in the mineral content of 32 morphologically diverse eggplant germplasm accessions with coefficients of variation of 9.95%, 25.65%, 44.33% and 30.12% for potassium, magnesium, copper and iron respectively. Eggplants generally contain a rich assortment of vitamins and minerals, as indicated in Table 1.2. Additionally, eggplants provide significant amounts of vitamin K, important for blood clotting and bone health (Pearson 2007). Vitamin B6, present in eggplants, contributes to brain development and function, as well as the reduction of high blood pressure (Hellmann and Mooney 2010).

Table 1.1. Nutritional composition of 100 g eggplant (USDA 2019)

Proximate	**Amount in 100 g**
Water	90.8 – 93.5 g
Energy	25 kcal
Protein	0.82 – 1.1 g
Total Lipid	0.1 - 0.3 g
Carbohydrate, by difference	5.88 g
Fiber, total dietary	2.2 – 3.8 g
Total Sugars	3.53 g
Fatty acids, total saturated	0.034 g

Eggplants also contain insignificant amounts of vitamins A and D (USDA 2019). Eggplants have a glycoalkaloid called solanine that can make swelling and pain worse in conditions like arthritis and rheumatism (Ionică 2020). Although eggplants may be difficult to digest for some individuals, they are a good source of dietary fiber,

which aids in digestion and promotes a healthy digestive system (Fraikue 2016). Regarding cancer, eggplants have potential positive effects in treating gastrointestinal cancers, and acting as a general anti-carcinogenic agent (Seraj et al. 2017). Eggplant is rich in a compound known as nasunin which is a derivative of the anthocyanidin delphinidin. This compound provides protection from some cancer types such as skin cancer by inhibiting the system that produces harmful hydroxyl radicals -- a primary contributor to oxidative stress within the body (Casati et al. 2013). Eggplants can be used topically as a general medicine, wound or cut healer, and burn compress (Meyer et al. 2014). Overall, the abundant bioactive compounds and nutrients in eggplant make it a valuable addition to a healthy diet and a potential resource for treating various diseases.

Table 1.2. Vitamin and mineral content in 100g eggplant (USDA 2019)

Vitamins		**Minerals**	
Vitamin B1 (Thiamine)	0.026 - 0.05 mg	Calcium, Ca	7 – 10 mg
Vitamin B2 (Riboflavin)	0.03 - 0.05 mg	Iron, Fe	0.17 - 0.27 mg
Vitamin B3 (Niacin)	0.55 - 0.803 mg	Magnesium, Mg	12 – 15 mg
Vitamin B5 (Pantothenic acid)	0.23 - 0.345 mg	Phosphorus, P	21 - 28 mg
Vitamin B6 (Pyridoxine)	0.074 - 0.097 mg	Potassium, K	201 – 259 mg
Vitamin B9 (Folate)	13 – 27 ug	Sodium, Na	1 – 3 mg
Vitamin C (Ascorbate)	1.4 - 3.2 mg	Zinc, Zn	0.11 - 0.23 mg
Vitamin E (Tocopherol)	0.3 mg	Copper, Cu	0.043 - 0.138 mg
Vitamin K (Menaquinone)	3.5 ug	Manganese, Mn	0.086 - 0.956 mg

1.2. Plant Secondary Metabolites

Secondary metabolites in plants are compounds not directly involved in growth, development, or reproduction, but they serve critical roles in defense against herbivores and pathogens, protection against environmental stress, interactions with other plants, and the attraction of pollinators or seed dispersers (Elshafie, Camele, and Mohamed 2023). For humans, plant secondary metabolites have various functions, serving as vital elements in pharmaceuticals due to their medicinal characteristics, contributing to overall health and nutrition through their antioxidant properties, adding distinctive flavors and aromas to spices and herbs, and acting as natural preservatives due to their antimicrobial qualities (Verpoorte 1998).

Based on their molecular structure, secondary metabolites are generally classified into nitrogenous compounds, terpenoids, and phenolics. Nitrogenous compounds include alkaloids which contain mostly basic nitrogen atoms. These chemicals have a wide range of pharmacological effects in humans and are found in many medicinal plants (Aniszewski 2007). Examples include caffeine, morphine, strychnine, and quinine. Terpenoids are one of the largest classes of plant metabolites that are derived from five-carbon isoprene units arranged and modified in various ways (Davies, Jinkerson, and Posewitz 2015). Carotenoids are a subclass of terpenoids. Terpenoids are required for deterring herbivores and attracting pollinators. They are found in essential oils and are often used in food additives, perfumery, and aromatherapy. Furthermore, some terpenoids have been found to have medicinal properties. For example, taxol is a diterpenoid that has been used in cancer treatment (Gershenzon and Dudareva 2007). Plant phenolic compounds, recognized for their antioxidant properties and antimicrobial activities (Park et al. 2001), have a diverse range of structures. A common feature of these compounds is the presence of one or more hydroxyl (-OH) group attached to either a single or multiple aromatic rings (Kabera et al. 2014). The antioxidant effects of phenolic compounds are strongly influenced by the number and position of these hydroxyl groups, primarily because these groups can readily donate their H^+ ions to neutralize reactive oxygen species (ROS) effectively reducing their impact (Podsędek 2007). Among the different types of phenolic compounds, those carrying a single aromatic ring are categorized as simple

phenolics and can accommodate one or more hydroxyl groups on their ring. On the other hand, polyphenols, representing a different class of phenolic compounds, are distinct in that they contain more than one phenol unit. The main classes of polyphenols are defined according to the nature of their carbon skeleton: phenolic acids, flavonoids and the less common stilbenes and lignans (Scalbert and Williamson 2000). Phenolic acids are distinguished by a carboxyl group (-COOH) in addition to the hydroxyl group(s) on the aromatic ring(s). They can be subdivided into hydroxybenzoic acids (with one aromatic ring, like gallic acid) and hydroxycinnamic acids (with an additional three-carbon chain, like caffeic acid) (Robbins 2003). Another subgroup of polyphenols is flavonoids, which are characterized by two phenolic rings connected by a three-carbon bridge, often forming a third, heterocyclic ring (Bravo 1998). They can be further divided into several groups, including flavanols, anthocyanins, and isoflavonoids, based on the degree of oxidation and pattern of substitution of heterocyclic ring (Valls et al. 2009).

Eggplants are a rich source of secondary metabolites that contribute to their nutritional and medicinal properties. For example, eggplants contain glycoalkaloids, such as α-solamargine and α-solasonine, which have shown potential in treating various types of cancers and possess antiparasitic effects (Ding et al. 2013). Despite the toxicity of glycoalkaloids at high concentrations, domesticated eggplants typically contain levels safe for consumption (Gürbüz et al. 2018). Eggplant is also known for its high content of phenolic compounds which have antioxidant properties and are mainly concentrated in the fruit peel. Among these compounds, anthocyanins, a type of flavonoid, specifically nasunin, found in the purple skin of the fruit, also have radical scavenging activity (Noda et al. 2000). The total phenolic content in eggplants can vary significantly based on numerous factors such as the variety or cultivar of the eggplant, their growing conditions, and the maturity stage at which they were harvested (García-Salas et al. 2014). Some studies have reported a wide range of total phenolic content, from as low as 23 mg/100 g of dry weight (DW) to as high as 1,168,100 mg/100 g DW, in various cultivars and wild relatives of eggplants (Gürbüz et al. 2018). This wide range suggests that there is considerable genetic diversity in eggplants with respect to their phenolic content. It is also important to note that the total phenolic content in eggplants can be affected by environmental factors such as temperature. For instance, higher temperatures have been found to negatively affect the phenolic content, with

lower levels observed in eggplants harvested in the summer compared to those harvested in the spring (García-Salas et al. 2014). Overall, the high phenolic content in eggplants is one of the reasons why they are considered a health-promoting food, as phenolic compounds have been linked to various health benefits including antioxidant, anti-inflammatory, and anticancer activities (Noda et al. 2000; Plazas et al. 2013).

1.2.1. Chlorogenic Acid

Chlorogenic acids (CGAs) are naturally occurring polyphenols that fall under the hydroxycinnamic acids, a subclass of phenolic acids. Structurally, chlorogenic acid is an ester formed from caffeic acid and quinic acid (Plazas et al. 2013). In nature, the primary categories of CGAs are the caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA), and, less frequently, feruloylquinic acids (FQAs) (Clifford and Ramirez-Martinez 1990). Each of these groups consists of at least three different isomers (Clifford and Ramirez-Martinez 1990). Three isomers of the caffeoylquinic acids are 3-CQA, 4-CQA and 5-CQA (Figure 1.1), with 5-CQA being most common isomer of CGAs in eggplants (Stommel and Whitaker, 2003; Meinhart et al. 2019). Also, 5-CQA is one of the most prevalent polyphenols in the human diet, most famously associated with coffee (Nardini et al. 2002).

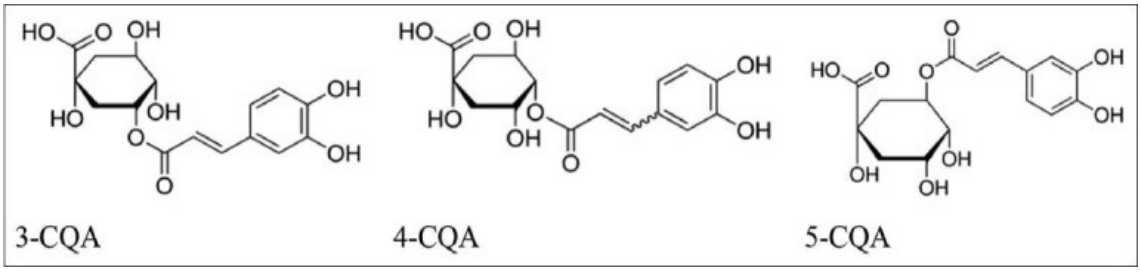

Figure 1.1. Molecular structures of different chlorogenic acid isomers (3-CQA: 3-O-caffeoyl-quinic acid; 4-CQA: 4-O-caffeoyl-quinic acid; 5-CQA: 5-O-caffeoyl-quinic acid)

In green coffee beans, CQA constitutes 76-84% of the total CGA, and can be found in regular coffee beans at about 10 g per 100 g (Tajik et al. 2017). CGAs are not exclusive to coffee and can be found in a wide variety of plants, fruits, and vegetables,

including eggplants. Table 1.3 provides the concentrations of CGAs across a range of cultivated plants.

Table 1.3. Amounts (g/kg dry weight) of CGAs in different plants species. In many cases, specific metabolites were not identified.

Plant source	Amount (specific metabolite, if known, and tissue)	Reference
Coffee	27.9-52.0 (5-CQA) 51.7-80.5 (total CGA)	Monteiro and Farah (2012)
Eggplant	14.1-28.0 (CGA, fruit) 5.0-9.3 (5-CQA, pulp) 2.6-6.7 (5-CQA, pulp) 1.4-8.4 (5-CQA, pulp)	Mennella et al. (2012) Singh et al. (2009) Luthria et al. (2010) Luthria (2012)
Mate tea	4.8-24.9 (3-CQA, leaves) 6.9-33.0 (5-CQA, leaves) 20.9-80.5 (total CGA, leaves)	Heck, Schmalko, and De Mejia (2008)
Carrot	0.3-18.8 (CGA)	Sun, Simon, and Tanumihardjo (2009)
Potato	0.01-4.60 (CGA, outer flesh) 0.002-0.09 (CGA, inner flesh)	Deußer et al. (2012)
Apple	0.1 (CGA, peel) 0.2 (CGA, flesh)	Van der Sluis et al. (2001)
Peach	0.1- 4.1 (CGA, peel) 0.1- 1.8 (CGA, pulp)	Andreotti et al. (2008)

1.2.1.1. Role of Chlorogenic Acid in Plants

CGAs are essential in plants for numerous reasons. Firstly, CGAs have significant antioxidant properties. They play a key role in protecting plants from oxidative stress caused by reactive oxygen species (ROS), which can result from environmental stresses like drought, high temperature and ultraviolet radiation

(Soviguidi et al. 2022). By scavenging ROS, CGAs prevent damage to the plant's cells and tissues, thereby enhancing their survival under stressful conditions.

Moreover, CGAs act as natural defenses against various biotic stresses. They exhibit antimicrobial activities against a wide range of pathogens, including bacteria, fungi, and viruses (Niggeweg, Michael, and Martin 2004; Sung and Lee 2010; Ding et al. 2017). In a study by Niggeweg, Michael, and Martin (2004), elevated CGA levels in tomato caused reduced cell death upon infection with *Pseudomonas syringae* var tomato T1. Additionally, smaller lesion sizes were observed on the tomato plants with higher CGA levels compared to the control plants, indicating a lesser extent of infection. The study indicated that higher CGA levels can contribute to enhanced resistance against bacterial infections by limiting cell death and reducing the spread of the infection (Niggeweg, Michael, and Martin 2004). CGA induces irreversible permeability changes in the bacterial cell membrane, leading to the loss of the cell's ability to maintain membrane potential and integrity (Lou et al. 2011). A study by Zhang et al. (2023) demonstrated that CGA was an effective substance for controlling gray mold in strawberries. They found that externally applied CGA inhibited the growth of *Botrytis cinerea*, a fungal pathogen, by inducing endoplasmic reticulum stress, triggering calcium release from the ER to mitochondria, leading to a burst of superoxide and subsequent oxidative damage that reduced cell viability. Chlorogenic acid employs its antiviral mechanisms primarily through its enzyme-inhibitory properties, specifically by binding to viral nucleoproteins (Ding et al. 2017).

The mode of action of chlorogenic acid differs when it acts as an anti-herbivore molecule compared to its action as an antimicrobial molecule. According to Felton et al. (2018), chlorogenic acid is oxidized by cellular peroxidases present in herbivorous insects. The resulting chlorogenoquinone molecules bind to free amino acids in the insect gut, specifically targeting nucleophilic centers in amino acids like cysteine, histidine, methionine, and lysine. This binding process reduces the availability of these amino acids and proteins for the feeding insects, ultimately leading to a lower nutritional value for the herbivores and causing growth reduction. The toxicity of chlorogenic acid in this context is directly proportional to the content of alkylatable amines (Felton et al. 1992). It was observed that the relative growth rate of first instar thrips larvae and their survival were significantly decreased when they were exposed to a medium containing 5% chlorogenic acid (Leiss et al. 2009). These findings suggest

that chlorogenic acid has a significant impact on inhibiting the growth and survival of thrips larvae. When a plant experiences an attack, it is common to observe a rise in CGA concentrations within the affected tissues. This increase aids in the creation of a physical and chemical barrier against the invading pest, providing an essential line of defense for the plant. This phenomenon has been studied by researchers such as Kundu and Vadassery (2018), demonstrating the crucial role CGAs play in plant defense mechanisms against biotic stress.

1.2.1.2. Potential Benefits of Chlorogenic Acid in Medicine

Chlorogenic acid CGA has been associated with a multitude of potential health benefits, showing promise for various medicinal applications. CGA is an antioxidant that can potentially reduce oxidative stress; lower chronic disease risk such as heart disease, cancer, and neurodegenerative disorders; regulate blood sugar levels and improve insulin sensitivity; aid in weight management by reducing fat absorption and improving metabolism; and exhibit antimicrobial properties.

The antioxidant activity of CGA is one of its most valuable properties from a medicinal perspective. As an antioxidant, CGA has the ability to counteract harmful free radicals that the body produces (Soviguidi et al. 2022). These free radicals can cause oxidative damage to cells, which contributes to the aging process and can lead to a variety of chronic diseases, including heart disease, diabetes, and cancer (Chatterjee et al. 2006; Valko et al. 2006). Several scientific studies indicated that CGA can effectively combat this oxidative stress, offering potential avenues for the management and prevention of these chronic diseases.

The scavenging effect of CGA is also useful for protecting the skin against UV light. In a study by Xue et al. (2022), it was discovered that CGA can reduce the breakdown of collagen caused by UVA light. They found that CGA has two ways of providing protection against collagen breakdown. Firstly, CGA reduces the production of reactive oxygen species (ROS), thereby, preventing DNA damage, promoting cell repair, and avoiding cell death. Secondly, CGA influences a signaling pathway called the TGF-beta/Smad pathway, which is important for collagen production. CGA

enhances the activity of this pathway in UVA-exposed cells, contributing to its positive effects on collagen. This suggests that CGA promotes the production and release of collagen from skin cells without interfering with their normal growth and function (Xue et al. 2022).

Chlorogenic acid also possesses anti-inflammatory properties, a characteristic that further amplifies its therapeutic potential. Inflammation is a biological response to injury, infection, or irritation; and while it is beneficial in moderation, chronic inflammation can contribute to various diseases, including heart disease, diabetes, cancer, arthritis, and even neurodegenerative conditions such as Alzheimer's disease (Akiyama et al. 2000). As an anti-inflammatory agent, CGA can help to modulate the body's inflammatory response, reducing the production of pro-inflammatory cytokines and inhibiting the activation of inflammatory pathways (Hwang et al. 2014). This means that CGA could potentially be used as a natural supplement or therapeutic agent to control chronic inflammation and help manage or prevent related diseases.

Several studies suggest that CGA could play a role in regulating blood glucose levels and improving insulin sensitivity. This could have significant implications for the management of type 2 diabetes and weight management (Tunnicliffe, Cowan, and Shearer 2015). CGA influences insulin and blood glucose regulation via several mechanisms. Firstly, CGA inhibits glucose absorption in the gut by blocking the sodium-dependent glucose transporter 1 (SGLT1), slowing glucose entry into the bloodstream thereby tempering the insulin response (Tunnicliffe, Cowan, and Shearer 2015). Secondly, CGA can alter the secretion of incretin hormones, such as Glucose-Dependent Insulinotrophic Polypeptide (GIP) and Glucagon-like Peptide-1 (GLP-1), which stimulate insulin secretion in response to nutrients in the gut (Mortensen et al. 2003). When glucose absorption is altered by CGA, this could result in changes in the release of these hormones and subsequently the insulin response (Van Dijk et al. 2009). Thirdly, CGA can inhibit glucose-6-phosphatase activity, a key enzyme in the final step of glucose production in the liver (Hemmerle et al. 1997; Henry-Vitrac et al. 2010). By doing so, CGA can reduce hepatic glucose output, which could influence insulin levels given the liver's crucial role in insulin regulation. Furthermore, CGA has the potential to alter the gut microbiome (Lozupone et al. 2012), with the microbiota capable of metabolizing CGA and increasing its bioavailability (Redeuil et al. 2011). The gut microbiota is known to influence host metabolic health, including glucose and insulin

regulation (Bäckhed et al. 2004). Finally, there is evidence suggesting that CGA may stimulate glucose uptake in skeletal muscle and adipocytes (Ong, Hsu, and Tan 2013), potentially through the activation of adenosine monophosphate-activated protein kinase (AMPK) (Tsuda et al. 2012), leading to an increase in GLUT-4 receptor expression. CGA has been identified as an active compound that may play a significant role in lipid metabolism. CGA has the potential to reduce cholesterol levels, an action that offers benefits since hyperlipidemia or high cholesterol is a known risk factor for cardiovascular disease (De Sotillo and Hadley 2002). Additionally, CGA is indicated to lower triglyceride levels in both plasma and liver. Consequently, the data suggest that CGA could be a valuable tool in regulating glucose and lipid metabolism.

Chlorogenic acid, a potent defense molecule in plants against various microbes, also has great potential for antimicrobial applications in human health. It exhibits a broad spectrum of in vitro activity against multiple bacterial, viral and fungal species. CGA has been found to inhibit specific viruses such as HBV (Wang et al. 2009). However, the available evidence regarding the antiviral effects of CHA against the H5N117 strain of the influenza virus, as well as its derivatives against the H3N227 strain, is limited (Karar et al. 2016). Further research is needed to better understand the antiviral effects of CHA and elucidate the potential mechanisms involved. The antiviral effect of CGA can also be applied in the food industry. A study by Abaidullah et al. (2021) showed that CGA might have an inhibitory effect on the Infectious Bronchitis Virus (IBV) which affects chickens, leading to severe financial losses in the poultry industry. In a study by Sung and Lee (2010), CGA exhibited antifungal properties against certain pathogenic fungi by disrupting their cell membrane structures, all while requiring no energy expenditure and causing no harm to human red blood cells. CGA demonstrates antifungal properties against certain pathogenic fungi by disrupting the structure of the fungal cell membrane. These findings make CGA a promising candidate for the development of alternative treatments for fungal infections, highlighting the valuable connection between plant defense mechanisms and potential human health benefits. Furthermore, chlorogenic acid's diverse antimicrobial properties, effectiveness in preventing lipid oxidation, protection of other beneficial compounds, and prebiotic function make it a valuable food additive (Santana-Gálvez, Cisneros-Zevallos, and Jacobo-Velázquez 2017). Further research, particularly large-scale clinical trials in humans, are needed to fully understand the potential of CGA in medicine and its safety

profile. It is also worth noting that the bioavailability and metabolism of CGA in the human body can affect its efficacy and potential for therapeutic uses. Despite these challenges, the versatile bioactivity of CGA presents a promising avenue for future medical and pharmaceutical research.

1.2.1.3. Biosynthesis of Chlorogenic Acid

The biosynthesis of CGAs such as 5-CQA (5-caffeoylquinic acid) in plants involves a series of enzymes in the phenylpropanoid pathway (Figure 1.2). The phenylpropanoid pathway is crucial for the biosynthesis of many secondary metabolites, such as flavonoids, lignin, and phenolic acids. The starting point of this pathway is the conversion of phenylalanine into p-coumaroyl-CoA, which is then converted to 5-CQA. Phenylalanine ammonia lyase (PAL) converts an amino acid called phenylalanine into cinnamic acid. There are different isomers of PAL depending on the plant and tissue type (Zhang and Liu 2015). Following this, another enzyme, cinnamate 4′-hydroxylase (C4H) then converts cinnamic acid into p-coumaric acid. Silencing the gene responsible for C4H in sweet sagewort (*Artemisia annua*) using RNAi technology resulted in the accumulation of cinnamic acid and notable decreases in p-coumaric acid, total phenolics, and anthocyanins, emphasizing the crucial role of this gene as a key regulator in the phenylpropanoid pathway (Kumar et al. 2016).

An enzyme called 4-cinnamoyl-CoA ligase (4CL) plays a pivotal role in phenylpropanoid biosynthesis by converting p-coumaric acid into p-coumaroyl-CoA, which serves as a crucial branch point (Clifford et al. 2017). This intermediate compound acts as a precursor for the synthesis of flavonoids and stilbenes, as well as being directed towards the production of methoxy guaiacyl- and syringyl-monolignols (Vogt 2010). The subcellular localization of 4CL isoforms can influence the direction of the pathway (Rigano et al. 2016).

Starting with p-coumaroyl-CoA, there are two pathways leading to 5-CQA. One involves the enzyme hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase (HQT), which produces 5-O-p-coumaroylquinic acid (5-pCoQA), and then the cytochrome P450 oxidase p-coumaroyl-3′-hydroxylase (C3H) hydroxylates 5-pCoQA to

create 5-CQA. The other pathway first involves C3H in hydroxylation of p-coumaroyl-CoA to form caffeoyl-CoA, which is then converted to 5-CQA by HQT (Clifford et al. 2017). Overexpression of HQT caused increases in CGA amount in tomato and tobacco plants (Niggeweg, Michael, and Martin 2004). The biosynthesis of other chlorogenic acid isomers is less well understood and is thought to occur via conversion from 5-CQA, although the specific isomerases involved have not been identified (Clifford et al. 2017).

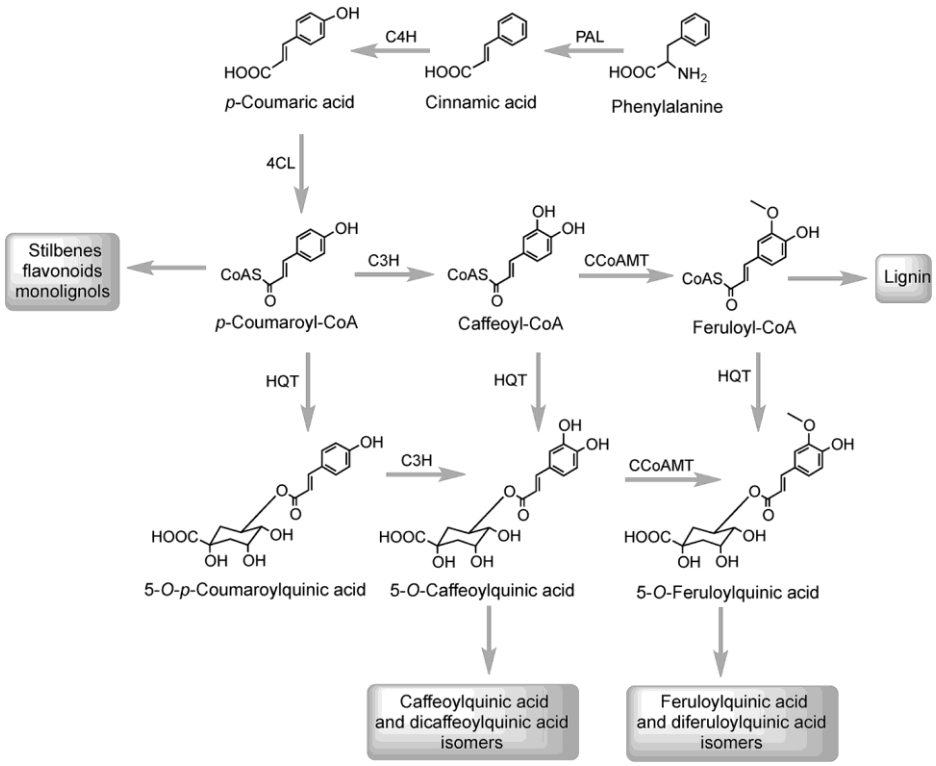

Figure 1.2. 5-O-caffeoylquinic acid biosynthesis pathways (Clifford et al. 2017). Enzymes: phenylalanine ammonia lyase (PAL), cinnamate 4′-hydroxylase (C4H), 4-cinnamoyl CoA ligase (4CL), hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase (HQT), p-coumaroyl-3′-hydroxylase (C3H), and caffeoyl-CoA-3-O-methyltransferase (CCoAMT).

Various plants, such as globe artichoke, switchgrass, and chicory, have a unique pathway to produce acyl-quinic acids (Sonnante et al. 2010) (Figure 1.3). This pathway also begins with p-coumaroyl-CoA, which is converted to 5-O-p-coumaroylshikimic acid by the enzyme hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (HCT1). The product is then hydroxylated by C3H to form 5-O-caffeoylshikimic acid (also known as dactylifric acid), which can be converted to caffeoyl-CoA by a reverse

action of HCT or a caffeoylshikimate esterase and a ligase. Caffeoyl-CoA can then be converted to 5-CQA via HQT. Alternatively a caffeoylshikimate esterase can release caffeic acid which is converted to caffeoyl-CoA by a ligase. Another enzyme, S-adenosyl-L-methionine: caffeoyl-CoA-3-O-methyltransferase (CCoAOMT), which is found in oats and other plants, can convert caffeoyl-CoA to feruloyl-CoA (Yang et al. 2004). This enzyme has multiple roles, contributing to the production of various compounds, including scopoletin, anthocyanins, and other flavonoids. It has been shown that CGA can also be converted to its cis isomer upon UV exposure (Clifford et al. 2008). Therefore, environmental factors may also influence the diversity of CGAs in plants.

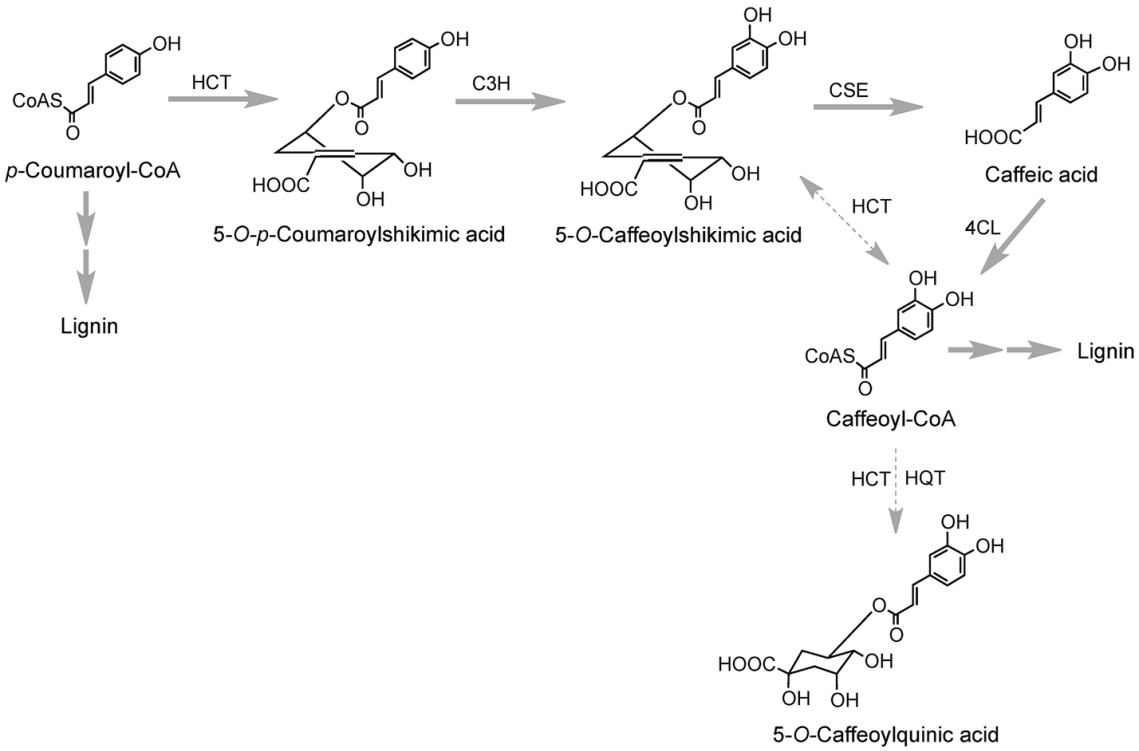

Figure 1.3. 5-O-caffeoylquinic acid biosynthesis by a shikimate shunt (Clifford et al. 2017). The most probable paths are shown with strong, bold arrows, while less common paths are represented with dotted arrows. Enzymes: hydroxycinnamoyl transferase (HCT), p-coumaroyl-3′-hydroxylase (C3H), caffeoylshikimate esterase (CSE), 4-cinnamoyl CoA ligase (4CL), hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase (HQT).

Overall, the regulation of these metabolic pathways is complex, involving multiple enzymes, each with their unique isoforms and subcellular localization. This

complexity contributes to the diverse outcomes in phenylpropanoid and CGA biosynthesis that are seen in different plant species and under varying environmental conditions.

1.2.1.4. Key Gene: *Myb1*

The *Myb1* gene, also commonly known as MYB1, encodes a transcription factor protein that serves an important role in eukaryotic organisms. Transcription factors control the rate of gene expression by binding to specific DNA sequences, consequently regulating various cellular functions. MYB proteins are part of a large and diverse MYB family, which is classified based on the number of repeats in the MYB domain, a region that is responsible for DNA binding. The R2R3-MYB transcription factors make up a specific subset within this larger MYB family, identifiable by two repeats, known as R2 and R3, in their DNA-binding domain (Dubos et al. 2010). Each of these repeats is approximately 52 amino acids in length and form three α-helices. The second and third helices establish a helix-turn-helix structure, which directly interacts with the DNA molecule (Dubos et al. 2010).

In plants, R2R3-MYB transcription factors have functions in primary and secondary metabolism, apoptosis, development, and response to both abiotic and biotic stresses (Dubos et al. 2010; Baldoni, Genga, and Cominelli 2015). The function of R2R3-MYB transcription factors varies depending on the specific tissue in plants. In *Arabidopsis thaliana*, several R2R3-MYBs are involved in regulating the biosynthesis of flavonoids. For example, some R2R3-MYBs control the production of flavonols in all tissues (Stracke et al. 2007) while other R2R3-MYBs are responsible for regulating anthocyanin biosynthesis in vegetative tissues (González et al. 2008). Additionally, there are R2R3-MYBs that specifically control the biosynthesis of proanthocyanidins in the seed coat of Arabidopsis (Lepiniec et al. 2006).

MYB1 falls within the category of R2R3-MYB class proteins. Structurally, MYB1 exhibits the characteristic feature of having two MYB repeats within its DNA-binding domain. The functions of the MYB1 transcription factor can vary across different plant species. For example, it has been reported to induce anthocyanin

production in onion (*Allium cepa*), radish (*Raphanus sativus*), mango (*Mangifera indica*), and apple (*Malus domestica*). However, in other plant species like strawberry (*Fragaria ananassa*) and lily (*Lilium* spp.), MYB1 may have a repressive role (Khusnutdinov et al., 2021). To improve plant phenolic content through plant breeding or genetic modification, it is crucial to elucidate the function of the *Myb1* gene, considering its role as an activator and repressor in different plant species.

It has been reported that in eggplant (*Solanum melongena*), the presence of SmMYB1 transcription factor can lead to increased accumulation of anthocyanins (Zhang et al. 2016) and CGA (Docimo et al. 2016). Zhang et al. (2016) discovered that expression of *SmMyb1* was correlated with that of phenylalanine ammonia-lyase (PAL), a crucial enzyme in the phenylpropanoid pathway that leads to the biosynthesis of anthocyanins, as well as chlorogenic acid. This correlation implies that SmMYB1 could have a regulatory role in the expression of PAL, potentially influencing the production of phenylpropanoid pathway metabolites, including CGA. The findings also suggest that the overexpression of *SmMyb1* could potentially enhance the production of chlorogenic acid in eggplant. A study by Docimo et al. (2016) showed that the C-terminal domain of SmMYB1 plays a role in regulating anthocyanin and CGA synthesis in eggplants. Docimo et al. (2016) also compared gene expression levels of key enzymes in the phenylpropanoid biosynthesis pathway in *Nicotina benthamiana* leaves transformed with *SmMyb1* and *SmMyb1Δ9*. They found that the expression of the HQT gene, which plays a role in the pathway, was similar regardless of whether the leaves had *SmMyb1* or *SmMyb1Δ9*. However, the genes *CHS*, *DFR*, and *ANS*, which are part of the anthocyanin synthesis pathway, exhibited higher expression in leaves with the *SmMyb1* gene compared to those containing the *SmMyb1Δ9* variant. This implies that the intact *SmMyb1* gene could be regulating the expression of these genes and therefore influencing the production of anthocyanin and CGA.

1.3. *Agrobacterium*-Mediated Plant Transformation

Agrobacterium tumefaciens is a bacterium used in genetic engineering to insert foreign DNA into plants (Newell 2000). It naturally injects a portion of its Ti plasmid into plant cells, causing the crown gall disease (Escobar and Dandekar 2003). The Ti

plasmid encompasses two critical regions: the transferred DNA (T-DNA), which is transported to the host plant cell as a single-stranded molecule, and the virulence (*vir*) genes that encode most of the bacterial protein machinery required for virulence. The T-DNA is defined by two border sequences, usually represented as LB (left border) and RB (right border) (Lacroix and Citovsky 2013). Scientists replace the disease-causing T-DNA genes with the gene of interest, and when the bacterium infects plant cells, it transfers the genetically engineered DNA into the plant genome. This results in the stable integration of the inserted gene, allowing it to be passed on to future generations. This technique is widely used in studying gene function in plants and the development of genetically modified crops with improved traits (Hamilton et al. 1996). There are also methods for transient transformation that involve the temporary expression of introduced DNA in the plant cell nucleus without integration into the host genome (Janssen and Gardner 1990). Transient expression is useful for quickly screening gene functions or promoter activities.

The plant transformation process is initiated when the *Agrobacterium* detects acetosyringone, a phenolic compound which was the first plant factor discovered to influence the virulence of *Agrobacterium* (Bolton, Nester, and Gordon 1986). Acetosyringone is released from wounded plant tissues. The presence of acetosyringone is detected by the VirA protein, a sensor kinase that forms part of the VirA/VirG two-component regulatory system (Raineri et al. 1993). Upon sensing this molecule or similar phenolic compounds, VirA becomes autophosphorylated and transfers a phosphate group to VirG (Brencic and Winans 2005). Phosphorylated VirG acts as an active transcription factor and triggers the expression of other virulence genes located on the Ti plasmid (Brencic and Winans 2005). These virulence genes are required for production of virulence proteins such as VirB, VirD and VirE. The VirD1 and VirD2 proteins introduce a nick in the T-DNA at the RB, and VirD2 remains covalently bound to the 5' end of the T-DNA (Lacroix and Citovsky 2013) (Figure 1.4). The T-strand (single-stranded form of T-DNA) is transported into the plant cell through a type IV secretion system (T4SS). The T4SS consists of a number of VirB and VirD proteins (Guo et al. 2007) which serve as the substrate receptor, as well as, ATPase and adhesins (Hwang, Yu, and Lai 2017). The T-DNA is coated by several Vir proteins. One such protein is VirD2 which has been suggested to play a role in protecting the T-DNA from degradation, aiding in its nuclear import, and possibly participating in its integration

into the plant genome (Gelvin 2012). Another Vir protein is VirE2 which is a ssDNA-binding protein that coats the T-strand, protecting it from nucleases and helping in its nuclear transport. VirE2 can interact with several plant proteins, some of which are thought to be involved in the nuclear import of the T-DNA (Sakalis, Van Heusden, and Hooykaas 2014). Both of these proteins have nuclear localization sequences (NLSs) (Gelvin 2012; Sakalis, Van Heusden, and Hooykaas 2014).

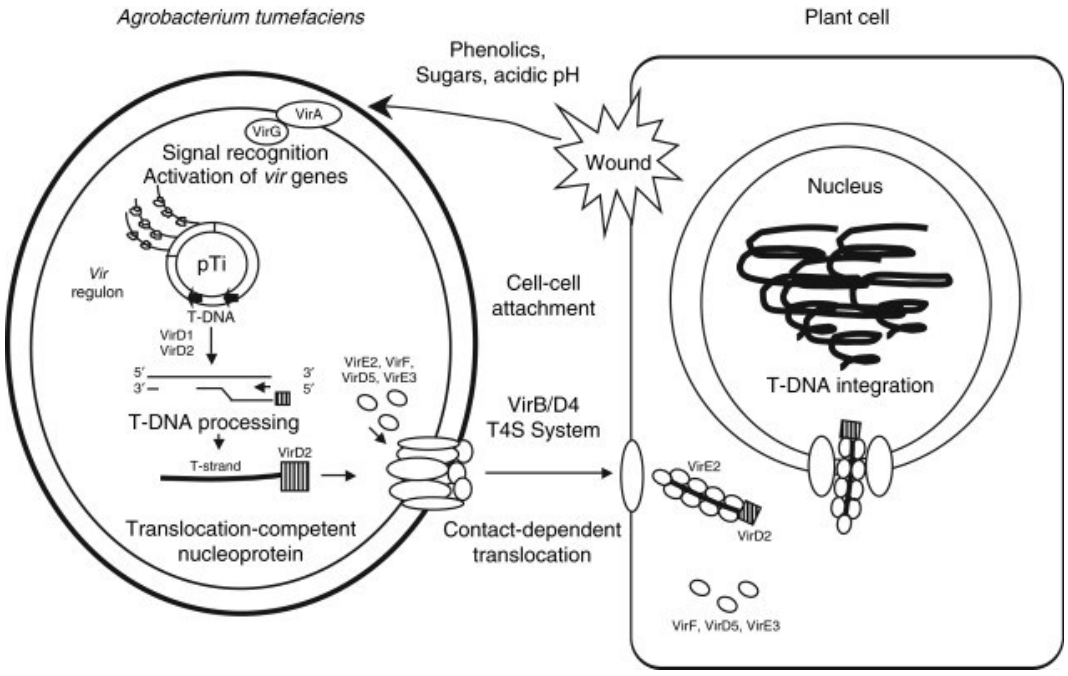

Figure 1.4. Schematic representation of the *Agrobacterium* T-DNA transfer process

The integration of T-DNA from *Agrobacterium tumefaciens* into the plant genome is generally a random process. The findings of the study by Kim, Veena, and Gelvin (2007) suggest that T-DNA can be integrated into both heterochromatin and euchromatin regions. The integration of T-DNA into the plant genome is not fully understood but is believed to utilize the plant's DNA repair machinery (Tzfira et al. 2004). After integration, the T-DNA is expected to be transcribed and translated by the plant's cellular machinery, resulting in the expression of the inserted genes. In the case of stable transformation, these genes are passed on to subsequent generations (Hamilton et al. 1996).

Agrobacterium tumefaciens-mediated transformation has been successfully applied across many plant species, including eggplants (Saini and Kaushik 2019). In

fact, the initial successful transformation of an eggplant using an *Agrobacterium*-mediated technique was achieved by Guri and Sink (1988). Since then, eggplants have been routinely transformed for a variety of purposes, one of the most significant being disease resistance. A prime target for these transformations has been enhancing resistance towards the Colorado potato beetle, a pest that causes major crop losses (Arpaia et al. 1997). Researchers have innovated and adapted various protocols to genetically modify eggplants. These methodologies employ different plant parts as explants, such as hypocotyls, epicotyls, node segments, cotyledon segments (Magioli and Mansur 2005), leaf disks (Singh Yadav and Venkat Rajam 1998), and roots (Franklin and Sita 2003).

One particularly successful method of transformation used a combination of cotyledons, hypocotyls, and leaves derived from two different eggplant genotypes, resulting in high transformation efficiencies (Davey et al. 2010). It is important to note, however, that the success of the genetic modification process can depend on a myriad of factors. These include the plant genotype, type of plant part or explant used, the specific binary vector employed, the density of *Agrobacterium* cells, the duration of infection, the length of pre- and post-infection culturing, and the selection and application of antibiotics used for controlling *Agrobacterium* overgrowth (Saini and Kaushik 2019). All these elements together determine the efficiency of the transformation procedure.

1.4. Aim of the Study

The aim of this study was to develop a transgenic eggplant with enhanced content of chlorogenic acid (CGA), a phenolic compound associated with various health benefits, including the potential to reduce the risk of diabetes and cancer. Despite being abundantly found in coffee, the geographical limitation of coffee cultivation necessitates an alternative, more globally accessible source of CGA. Eggplant is capable of thriving in diverse climates and is naturally rich in CGA; therefore, it is a promising candidate for engineering increased production of this valuable secondary metabolite. To increase the CGA content in eggplant, this study aimed to over-express the gene *Myb1* which plays crucial roles in CGA synthesis. *SmMyb1* encodes for a transcription factor activating the phenylpropanoid pathway. The gene was isolated, amplified, and

incorporated into the PRI910 vector system before being transformed into *Agrobacterium tumefaciens*, which was used to create the transgenic eggplants. Subsequently, transformation was verified using Polymerase Chain Reaction (PCR) analysis. Gene expression levels in transformed plants were investigated with real time-qPCR analysis. Furthermore, the study examined the total phenolic content of transgenic plants and the specific increase in CGA content using the Folin-Ciocalteu and HPLC methods, respectively.

Ultimately, the goal of the research was to provide foundational knowledge for understanding the CGA production pathway in eggplant, potentially establishing these transgenic plants as viable sources for the production of valuable CGAs. The potential application of CGA as a pharmaceutical and food additive underscores the potential economic benefits of these transgenic eggplant lines.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Biological Materials

Seeds of *Solanum melongena* cv. Kemer were obtained from Antalya Agriculture, Inc. (Antalya, Turkey). The seeds were germinated in MS medium and seedling hypocotyls were used for transformation. *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary vector PRI910, which carries the neomycin phosphotransferase II (*nptII*) gene for kanamycin resistance was used for transformation. The strain was grown on a yeast extract peptone (YEP) medium supplemented with appropriate antibiotics.

2.2. Methods

2.2.1. Preparation of Plasmid

The *SmMyb1* gene was first described by Zhang et al. (2014). The gene information documented in their research was employed to clone the gene from the cDNA of eggplant. The forward primer sequence was established as 5'ATGAATAATCCTCCTATAATCTGTACGTC'3, and the reverse primer sequence was set as 5'TTAATCAAGTAAATTCCATAAATCA'3. These primers were specifically chosen to ensure the successful amplification of the full coding sequence. Total mRNA was isolated from the fruit peel of the Kemer eggplant. Subsequently,

cDNA was synthesized from the isolated mRNA, which led to the successful cloning of the *SmMyb1* cds. The gene was amplified by PCR from the cDNA and cloned into the pTZ57R/T vector for amplification. From there it was transferred into the CaMV 35S cassette and incorporated into the pRI910 binary vector (Figure 2.1) at the SmaI restriction site. The pRI910 vector carries the kanamycin resistance gene, which serves as a selective marker for both bacteria and plants. Experiments for gene cloning and transfer of the gene to plant expression vector were conducted by Hatice Şelale.

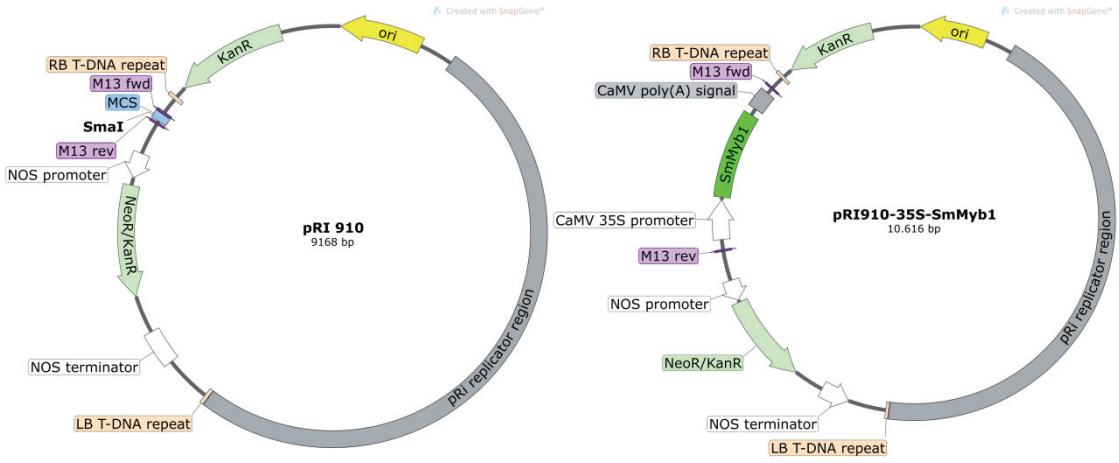

Figure 2.1. Schematic representation of pRI910 vector and its derivative pRI910-35S::*SmMyb1*. Kanamycin resistance (KanR)

2.2.2. Amplification of Cloned pRI910 Vector by *Escherichia coli*

The binary vector pRI910 containing the *SmMyb1* gene was introduced into *Escherichia coli* strain DH5α using the heat shock method as outlined by Hanahan, Jessee, and Bloom (1991). The bacterial colonies were grown on Luria-Bertani (LB) medium (10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, and 15 g/L bacto-agar) supplemented with 50 mg/L kanamycin to ensure selection of transformed bacteria. After the successful growth of bacterial colonies on the selective medium, individual colonies were inoculated into a 2 ml volume of liquid LB medium for plasmid extraction. For plasmid isolation the PureLink HiPure Plasmid Miniprep Kit (ThermoFisher) was used. To confirm the presence of the *SmMyb1* gene in the plasmid,

PCR was performed with *SmMyb1* specific KT727965_cds forward and reverse primers (5'ATGAATAATCCTCCTATAATCTGTACGTC'3 and 5'TTAATCAAGTAAATTCCATAAATCA'3, respectively). The PCR program for the reaction consisted of an initial step at 95°C for 5 minutes, followed by 30 cycles of 1 minute at 95°C, 1.5 minutes at 60°C, and 1 minute at 72°C, and a final step of 5 minutes at 72°C. The PCR products were visualized using 1% agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer (48.5 g Tris, 11.4 mL glacial acetic acid, and 0.5M EDTA at pH 8.0) and ethidium bromide staining to verify the correct amplification of the *SmMyb1* gene.

2.2.3. Preparation of Transgenic *Agrobacterium tumefaciens* Strain

The pRI910 binary vector harboring the *SmMyb1* gene was introduced into the *Agrobacterium tumefaciens* strain LBA4404 via the freeze-thaw method, as described by Jyothishwaran et al. (2007). Post-transfer, bacteria were grown on LB medium supplemented with 50 mg/L kanamycin and 15 mg/L rifampicin for selection. The presence of the desired gene within the *Agrobacterium tumefaciens* cells was then validated via PCR, using the *SmMyb1* specific KT727965_cds forward and reverse primers. PCR was carried out under the following conditions: an initial step at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 1.5 minutes, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. Verified bacteria containing the recombinant vector were subsequently preserved in a 10% glycerol solution at -80°C.

2.2.4. Seed Sterilization and Germination

The plant transformation began with the preparation of 400 Kemer seeds, which were soaked in double-distilled water overnight, followed by sterilization with 70% (v/v) ethanol for one minute and 50% (v/v) bleach containing 250 µl Tween 20 for 25 minutes. The seeds underwent three washes with sterilized distilled water to remove any

residual sterilization solution. The seeds were planted on hormone-free MS medium. In plant tissue culture all MS media contained the following concentrations of major and minor minerals: 1.9 g/L potassium nitrate, 0.17 g/L potassium monophosphate, 0.44 g/L calcium chloride dihydrate, 0.37 g/L magnesium sulfate heptahydrate, 1.65 g/L ammonium nitrate, 6.2 mg/L boric acid, 8.6 mg/L zinc sulfate heptahydrate, 0.83 mg/L potassium iodide, 22.3 mg/L manganese (II) sulfate tetrahydrate, 0.25 mg/L sodium molybdate dehydrate, 25 ug/L copper (II) sulfate pentahydrate, 25 ug/L cobalt (II) chloride hexahydrate, 43 mg/L ethylenediaminetetraacetic acid (EDTA) ferric-sodium salt, 0.1 g/L myo-inositol, and 8 g/L agar. MS medium for seed germination contained, 10 g/L sucrose, 2 mg/L thiamine, 0.5 mg/L nicotinic acid, 0.2 mg/L pyridoxine, at pH 5.6. Seeds were distributed across 500 ml jars containing MS medium with a seeding density of 20 seeds per jar. Five separate transformation experiments were conducted, each involving the planting of 400 seeds. Cultures were incubated in the dark for three days, before being exposed to light for 21 days at room temperature.

2.2.5. Plant Transformation

The methods for transformation were adapted from the protocols developed by Van Eck and Snyder (2006) and by García-Fortea et al. (2020). Preliminary research using Kemer cotyledons for the transformation resulted in a low yield of regenerated plants. To improve yield, hypocotyls from germinated seeds were chosen for the transformation.

To prepare the bacterial solution, *Agrobacterium* from a 10% glycerol stock was first streaked onto solid LB medium supplemented with antibiotics (kanamycin at 50 mg/L and rifampicin at 15 mg/L). The bacteria were then incubated at 28°C for two days. Following this, a single colony was selected and inoculated into liquid YEP medium. The YEP medium consisted of bacto-peptone (10 g/L), NaCl (5 g/L), and yeast extract (5 g/L) with the same antibiotic concentrations as the LB medium. The culture was then incubated overnight at 28°C and 120 rpm on an orbital shaker. To enhance the bacterial concentration, 200 µl of the overnight culture was transferred from the 10 ml liquid culture into a larger volume of fresh YEP medium, specifically scaling up to a 100 ml media stock. This 100 ml YEP medium contained the same

antibiotics as the previous medium, along with the addition of 100 μM acetosyringone. This culture was then left to incubate overnight at the same temperature of 28°C and 120 rpm on an orbital shaker. The culture was centrifuged at 4750 rpm for 10 minutes. The liquid YEP media was then carefully discarded, and the bacterial pellet was resuspended in liquid MS medium, composed of previously described major and minor minerals, and myo-inositol but without agar. In contrast to seed germination MS medium, this medium included 3 g/L sucrose, 0.25 mg/L folic acid, 2 mg/L glycine, 0.4 mg/L thiamine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, and 50 μg/L D-biotin at pH 5.6. Finally, to achieve an optimal optical density between 0.4 and 0.6 at 600 nm, the bacterial suspension was appropriately diluted in the medium. In a laminar flow hood, hypocotyls from three week old eggplants were aseptically and carefully cut into roughly two parts using a scalpel. The resulting explants were then soaked in an *Agrobacterium* solution for 10 minutes. Following this incubation, they were positioned in a pressure chamber and pressure was applied until -0.08 MPa was achieved. Once infiltrated with *Agrobacterium*, the explants were initially cultured on solid MS medium. This medium (pH 5.8), was composed of MS nutrients, 3 g/L sucrose, 0.25 mg/L folic acid, 2 mg/L glycine, 0.4 mg/L thiamine, 0.5 mg/L nicotinic acid, 0.4 mg/L pyridoxine, 50 μg/L D-biotin, 2 mg/L zeatin and 100 μM acetosyringone. After two days, the explants were moved to a selective MS medium containing 3 g/L sucrose, 0.25 mg/L folic acid, 2 mg/L glycine, 0.4 mg/L thiamine, 0.5 mg/L nicotinic acid, 0.4 mg/L pyridoxine, 50 μg/L D-biotin, 2 mg/Lzeatin, 25 mg/L kanamycin and 300 mg/L timentin. Explants were subcultured every 2 weeks. Throughout this entire process, the explant cultures were maintained at room temperature and exposed to 16 hr light : 8 hr dark conditions. During subcultures, the hypocotyls that regenerated shoots were transferred to a modified MS medium, which, along with the same quantities of zeatin and antibiotics as the prior medium, was supplemented with 0.75 mg/L gibberellic acid to stimulate shoot elongation. The gibberellic acid concentration was lowered to 0.5 mg/L after three subcultures, and the timentin was withdrawn from the medium. After elongation, the shoots from the hypocotyl explants were cut and placed in rooting medium (RM) that included MS nutrients, 1 g/L sucrose, 2 mg/L thiamine, 0.5 mg/L nicotinic acid, 0.4 mg/L pyridoxine, 2 mg/L IAA, and a pH of 5.6, to stimulate root growth. Sufficiently rooted plants were then transferred to sterilized soil and covered with a plastic bag to increase humidity and ease acclimatization. Plants were grown in room temperature under constant light. Within two weeks, the bag was progressively

opened to let the plant adapt to new environment. Two month old plants were transferred to the greenhouse.

2.2.6. DNA Isolation

For the DNA extraction process, a modified version of the Rezadoost, Kordrostami, and Kumleh (2016) protocol was used, with modifications tailored to reduce the impact of phenolic compounds. The method began with freezing 200 mg of leaf tissue sample using liquid nitrogen, a step crucial for preserving the integrity of the cellular structures and ensuring a successful DNA extraction. The sample was then homogenized using a tissue lyser. The homogenized tissue was treated with 1 ml of preheated CTAB buffer (Tris HCl 200 mM, EDTA 20 mM, NaCL 1.4 M, CTAB 2%) that was freshly supplemented with 0.2% PVP-40. The addition of PVP-40 to the CTAB buffer aimed to improve the yield and quality of the extracted DNA by preventing the binding of phenolic compounds to the DNA. This was followed by incubation at 65°C for 60 minutes and centrifugation at 11000 x g for 10 minutes. The supernatant was transferred to a new tube and mixed with 550 µl of 24:1 chloroform: isoamyl alcohol. After another round of vortexing and centrifugation at 11000 x g for 10 minutes, the upper phase was transferred to a clean tube. The samples were then treated with RNase A (100 µg/ml) and incubated at 35°C for 30 minutes to remove RNA. The next steps included the addition of a solution containing 50 mM Tris HCl and 0.2% beta-mercaptoethanol (half the volume of the sample) and subsequent 15-minute incubation at 35°C. This was followed by the addition of 4 M NaCl (half the volume of the sample), with the samples then placed on ice for 5 minutes. DNA precipitation was achieved by adding cold pure isopropanol at twice the volume of the sample, and incubating for an hour at 4°C. The samples were then centrifuged at 8000 rpm for 15 minutes, the supernatant discarded, and the pellet was washed with 75% (v/v) ethanol. This washing step was followed by another centrifugation for 2 minutes at 8000 rpm. The samples were dried using vacuum for 2 hours at room temperature and the pellet was resuspended in TE buffer. The concentration and purity of the isolated DNA were measured using a Nanodrop spectrophotometer. Finally, to ensure DNA quality, 200 ng

of each sample was run on a 1% agarose TAE gel followed by ethidium bromide staining.

2.2.7. Transformation Verification by PCR

The transformed plants were initially analyzed using PCR. PCR was performed with 35SMyb1 forward (5'CGCACAATCCCACTATCCTT3') and reverse primers (5'TCTTTGCAGCGTTCTTCCTT3'). The forward primer targets the CaMV 35S promoter region and the reverse primer targets the *SmMyb1 g*ene region. The PCR reaction had a total volume of 25 µl that included 0.4 mM of each dNTPs, 0.4 mM forward and reverse primers, 2.5 U of TAQ DNA polymerase and 2.5 µl of 10X PCR buffer. In addition to the 1.5 mM MgCl2 and 50 mM KCl found in 10X buffer, 3 mM MgCl2 and 40 mM KCL were added to each reaction with 300 ng of the DNA template (Lorenz 2012). The plasmid construct pRI910-SmMyb1 served as a positive control in these PCR tests. The PCR conditions included an initial 5-minute incubation at 95 °C, followed by 35 cycles consisting of 1 minute at 95 °C, 1 minute at 58 °C, 1.5 minute at 72 °C, and concluding with a 10 minute extension at 72 °C in a thermocycler. The resulting PCR products were run on a 1% TAE agarose gel stained with ethidium bromide and examined under UV light to identify the expected amplification product.

2.2.8. mRNA Expression Analysis

The process of mRNA expression level measurement employed Real-time Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted using the Plant/Fungi Total RNA Purification Kit from Norgen (Canada) following the kit's guidelines from plants confirmed as transgenic. RNA samples were treated with DNase from NEB (UK). RT-qPCR reactions were executed twice on each RNA sample, producing two technical replicates. For control group three non-transgenic plants were used. A Nanodrop spectrophotometer (MultiskanGO Microplate Spectrophotometer, Thermo Scientific, USA) was used to measure the RNA concentration. Per the

GoScript™ Reverse Transcription System from Promega's instructions, 2 µg of total RNA from each sample was reserved for cDNA synthesis. The isolated RNA was converted into cDNA using oligo(dT)18 primers. RT-qPCR was performed on a Light Cycler 480 with cDNA templates and the GoTaq® qPCR Master Mix from Promega. Along with the mix and cDNA, KT27965rt4 forward (5'GCAAAGAAATAACAAGTGACAAGCAAAC3') and reverse (5'TCTCCTTCAACAGCGTCGTCA3') primers, as well as Cyclophilin_RT forward (5'CGCGCGCTACACTGATGTATTCAA3') and reverse (5'ACAAAGGGCA GGGACGTAGTCAA3') primers were utilized. Each reaction was conducted in a 10 µl volume. The reaction commenced with initial denaturation via a 1 minute incubation at 95 °C, succeeded by amplification with 40 cycles at varying temperatures and times, concluding with a final cooling step of 10 seconds at 40 °C. The relative expression levels of each sample were determined using the Livak method (Livak and Schmittgen 2001). The ΔCT value was computed by subtracting the average Ct value of the reference gene, *Cyclophilin*, from the target gene, *SmMyb1* (Equation 1). The ΔΔCT value was derived by subtracting ΔCT of the transgenic plants from ΔCT of the non-transgenic plants (Equation 2). Finally, relative expression was calculated using Equation 3.

$\Delta\Delta Ct$ = Ct(Target gene, *SmMyb1*) - Ct(Reference gene, *Cyclophilin*) [Equation 1]

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

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

2.2.9. Total Phenolic Analysis

For the phenolic content analysis, the Folin Ciocalteau Method was applied (Siddiqui et al. 2017; Wu et al. 2006). For each sample, 25 mg lyophilized leaf tissue was mixed with 1 ml 80% methanol and subsequently subjected to shaking at a constant 100 rpm for 30 minutes. For the control group, three non-transgenic plants were used. Three replicates were performed for each sample. The samples were then centrifuged at 10,000 rpm for 20 minutes. The supernatant was collected and diluted to a quarter of its original volume. From this, a 50 µl aliquot was combined with 1.25 ml distilled water

and 50 µl 2 N Folin reagent. The samples were incubated at room temperature in the dark for 6 minutes. After this incubation period, 250 µl 20% sodium carbonate solution was added and the samples were incubated for an additional 30 minutes in the dark. Simultaneously, a standard curve was prepared using various concentrations of gallic acid (10 mg/L, 20 mg/L, 40 mg/L, 80 mg/L, 160 mg/L, and 320 mg/L). The absorbances of the samples and standards were then measured at 760 nm to quantify the phenolic content. After the calibration curve was established (Figure 2.2), the following formula was used to calculate mg of total phenolics per g of dry weight.

$$C=C_1*(V/m)$$

In this calculation, 'C' represents the total phenolic content in terms of mg/g, expressed in gallic acid equivalents (GAE). 'C_1' is the concentration of gallic acid determined from the calibration curve, given in mg/ml. 'V' is the volume of the plant extract in ml, and 'm' refers to the weight of the plant extract in g (Siddiqui et al. 2017).

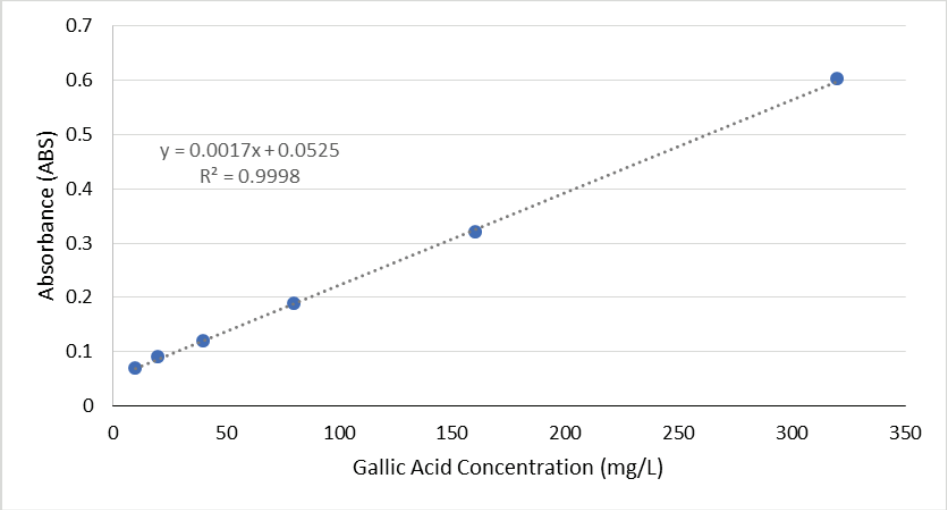

Figure 2.2. Standard curve of gallic acid

2.2.10. HPLC Analysis

In order to analyze chlorogenic acid extracts, a Shimadzu LC-20 AT model HPLC-PDA was utilized. The standard solutions were prepared using a 1:1 ratio (v:v)

of methanol and water. For the leaf samples, 25 mg of lyophilized leaf material was extracted using 1.8 ml of methanol. This extraction process involved an overnight incubation on an orbital shaker at 200 rpm. Samples were centrifuged for 5 minutes at 10,000 rpm and filtered using 0.45 µm PTFE filters. For the control group, three non-transgenic plants were used. Each 20 µL aliquot was injected into an Inertsil GL Sciences HPLC column (RP C18, 5 µm – 25 × 4.6 mm) kept at a temperature of 40 °C. The mobile phase used was a 50:50 (v:v) combination of methanol and an ammonium dihydrogen phosphate buffer ($NH_4H_2PO_4$, 5 mm, pH 2.5), maintaining a flow rate of 0.5 ml/min. The entire extraction and analysis procedure was carried out using three replicates.

2.2.11. Statistical Analysis

For the statistical analyses, the data were first organized and prepared in Microsoft Excel. Subsequently, R software was used to confirm that data from both the transgenic and non-transgenic control groups datasets followed a normal distribution by the Shapiro-Wilk test. The test's results allowed proceeding with Student's T-test. A p-value less than 0.01 was considered statistically significant.

Correlations between gene expression and metabolite analysis were calculated using Pearson correlation coefficient. The correlation coefficient (r) and the p-value were calculated by Excel's built-in statistical functions for correlation and t-distribution, respectively. The t-statistic was calculated using the following formula, where n is the number of data points and r is the correlation coefficient.

$$t\text{-statistic} = r * \sqrt{(n-2)/(1-r^2)}$$

The degrees of freedom were calculated as n-2. The two-tailed p-value was obtained using the t-distribution function in the R programming, with the calculated t-statistic and degrees of freedom as inputs. A p-value of less than 0.05 was considered statistically significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Verification of Plasmid in *Agrobacterium tumefaciens*

To transform *Agrobacterium* with the *SmMyb1* gene from eggplant, the pRI910 plasmid containing the *SmMyb1* gene was prepared and amplified in *E. coli*. This plasmid was subsequently introduced into *Agrobacterium*. The successful insertion of the *SmMyb1* gene was confirmed by PCR analysis of plasmids isolated from six selected *Agrobacterium* colonies. As expected, a distinct band corresponding to the 771 bp length of the coding sequence of *Myb1* was observed after agarose gel electrophoresis (Figure 3.1). The PCR was performed in two replicates.

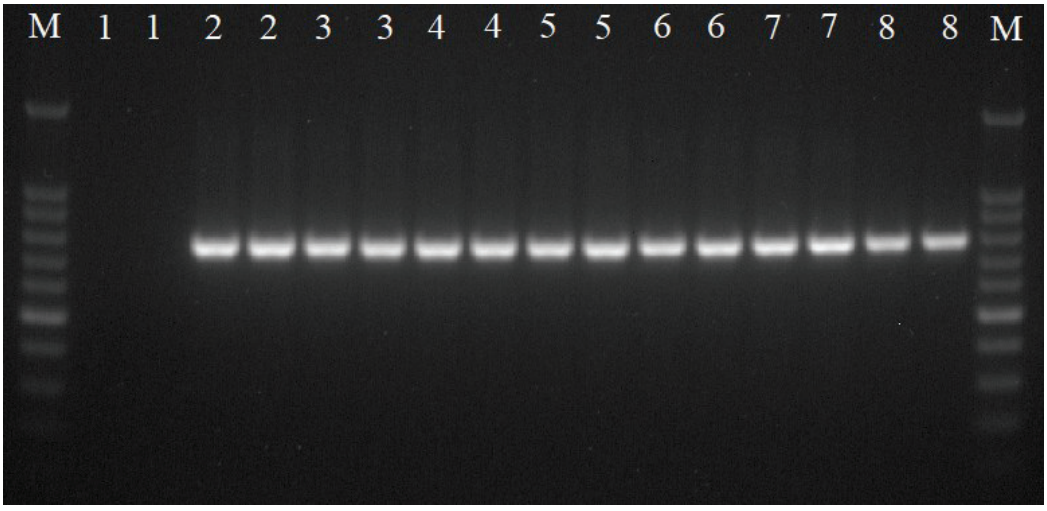

Figure 3.1. Confirmation of *Agrobacterium* transformation with the pRI910-*SmMyb1* plasmid by PCR. M-marker (100bp), 1-non-template negative control, 2-7 tested samples, 8-positive control for *SmMyb1* gene (771bp). Each PCR reaction was performed in duplicate.

This result not only confirms our initial hypothesis of successful transformation but also aligns with previous studies indicating the utility of the pRI910 plasmid in

genetic transformation via the freeze/thaw method (Wise, Liu, and Binns 2006). The amplified plasmid from *E. coli*, used as a positive control, produced the expected band at the same position, further supporting that transformation of *Agrobacterium* with the *SmMyb1* gene was successful. Conversely, the non-template negative control did not produce any band, providing reassurance about the absence of contamination.

3.2. Seed Sterilization and Germination

Five sets of transformations were performed. For each transformation set, hypocotyl explants from 400 seeds were used. The germination efficiency varied among the five experiments from 39.5 to 49.25% (Figure 3.2). The overall germination efficiency from all five experiments was 42.6% (SE = 0.041). Figure 3.3 shows the germinated aseptic seedlings. Generally, eggplant seeds are characterized by their relatively high germination rate. Low germination in the current work may have been the result of seeds which were a few years old. In these experiments, a low level of microbial contamination, around 1%, was observed among the seeds used, which had minimal impact on the overall procedure. This low contamination rate aligns with the successful sterilization practices observed in previous research studies on eggplant seed sterilization and germination (García-Fortea et al. 2020).

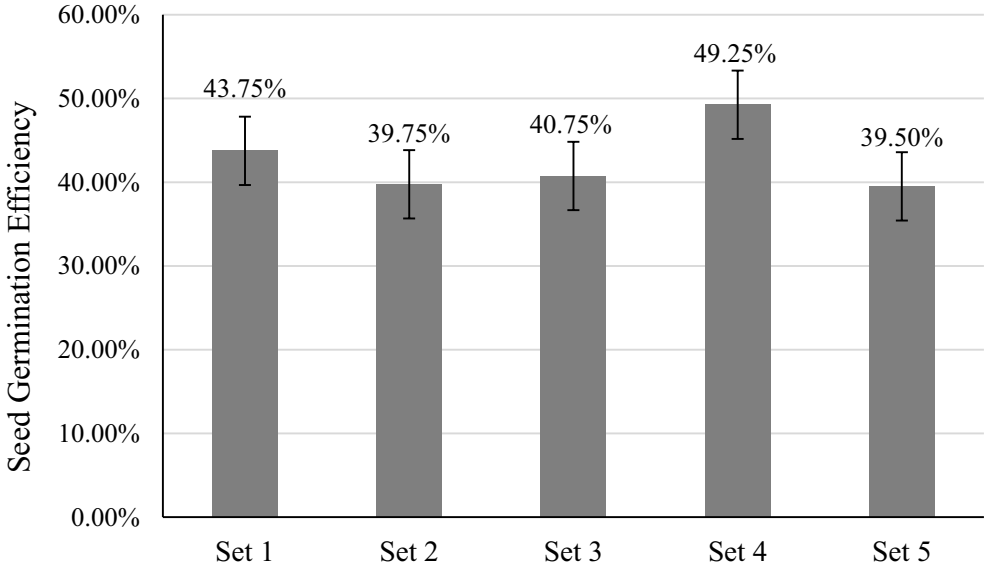

Figure 3.2. Seed germination efficiencies of each transformation set

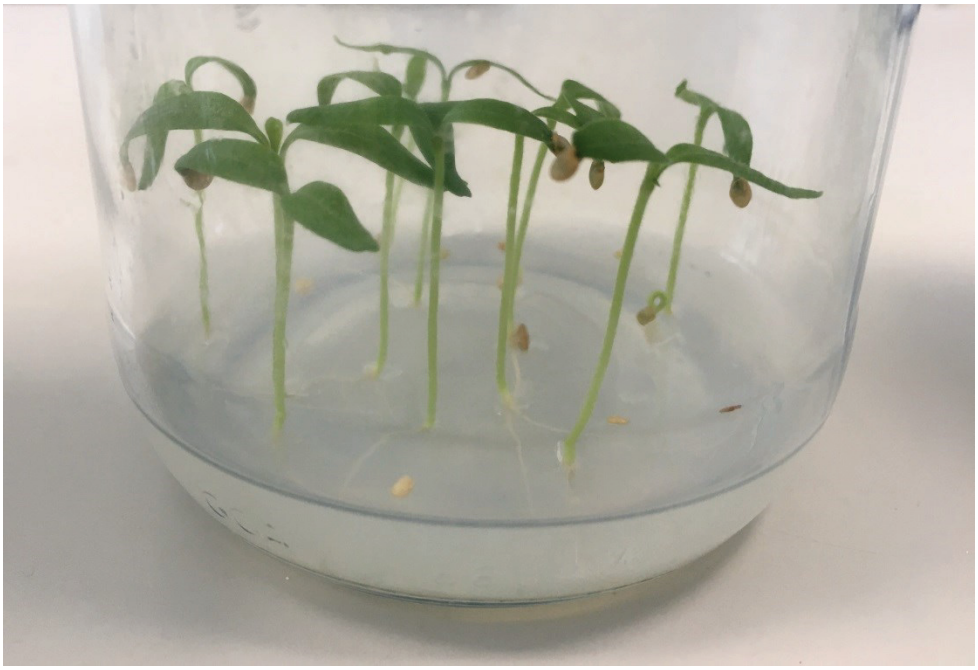

Figure 3.3. Sterile eggplant seedlings germinated in tissue culture

While seeds have an endosperm that assists germination, providing a suitable environment is critical in achieving high germination efficiency. For that reason, MS medium for seed germination was used to promote seedling growth following germination. In these experiments, seeds planted on MS medium were initially kept in the dark for three days before introducing them to light to mimic their natural germination conditions. After 21 days, the seeds reached the seedling stage, with the cotyledons fully emerged and ready for transformation. These findings agree with the results obtained in other studies, supporting the effectiveness of this protocol for seed germination (García-Fortea et al. 2020).

3.3. Plant Regeneration

Three weeks after planting seeds on MS medium, the seedlings' hypocotyls were sectioned under sterile conditions and incubated with transformed *Agrobacterium tumefaciens*. The explants were first placed on medium containing zeatin and acetosyringone. Acetosyringone, a phenolic compound, facilitates more effective

transformation by triggering the *Agrobacterium*'s *Vir* genes required for successful gene insertion. Zeatin, a type of cytokinin, was used to stimulate the explants to generate shoots. More specifically, trans-zeatin was preferred over other cytokinins in these experiments because it has demonstrated superior results in promoting eggplant regeneration (García-Fortea et al. 2020). After two days of *Agrobacterium* exposure, explants were transferred to a medium enriched with zeatin, kanamycin, and timentin. Timentin helped control bacterial growth and kanamycin enabled the selection of regenerated transformed cells. To ensure nutritional adequacy and optimal selection, the medium was replaced every two weeks. During the second week, the development of shoots on the hypocotyl explants could be observed. By the fourth week, these explants were transferred to medium containing gibberellic acid to stimulate shoot elongation. By the sixth week, some non-transgenic shoots began to yellow and wilt under the impact of the antibiotics.

Shoot regeneration peaked during the fourth week. The transformation sets had shoot regeneration efficiencies ranging from 27.84% to 58.33% as shown in Figure 3.4. On average, shoot regeneration efficiency was 41.64% (SE= 0.07). The fifth set yielded the highest regeneration efficiency. The difference between this experiment and the previous ones was that acetosyringone was added to the bacterial culture a day prior to transformation. In contrast, sets 1 to 4 had acetosyringone introduced on the day of transformation.

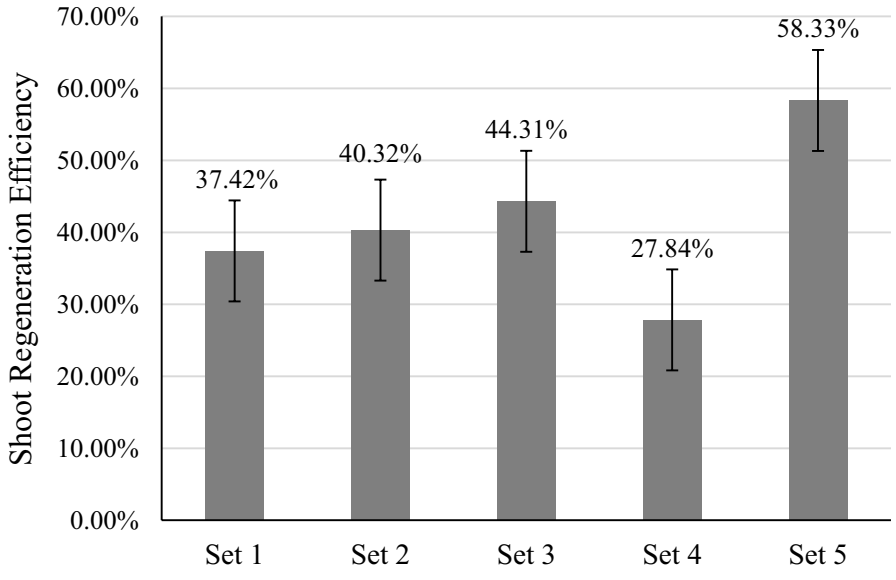

Figure 3.4. Shoot regeneration efficiencies of each transformation set

At the start of the eighth week, shoots that had grown to approximately 1 cm were excised and transferred to a rooting medium containing indole acetic acid (IAA), an auxin known to encourage root formation. By the 14th week, roots had formed, and the plantlets were ready for transfer to soil. Acclimatization was necessary due to the non-sterile environment and reduced humidity outside the plant tissue culture environment. To maintain humidity, the plants were covered with a plastic bag that was gradually opened and removed after two weeks. After 8-10 weeks, the plants were moved to larger pots to facilitate their continued growth. The regeneration process from explants to acclimated and fruiting plants within 40 weeks is illustrated in Figure 3.5.

The experiment, which started with 1692 explants, ended with the successful transfer of 53 plants to the soil. Thus, plant regeneration efficiency was calculated as the number of plant regenerated divided by total explant number, resulting in efficiencies of 2.15%, to 6.60%. The average efficiency for sets 1 to 4 for which acetosyringone was applied during incubation of explants with *Agrobacterium*, was 2.44% (SE= 0.0037). It was previously found that addition of 100 μM acetosyringone during infection and co-cultivation increased transformation efficiency by 2-3 fold in eggplant (Kumar and Rajam 2005). In the current work, addition of acetosyringone prior to infection further increased regeneration efficiency. Plant regeneration efficiency of set 5 was found to be 2.7 fold higher than average plant regeneration efficiency of sets 1 to 4. These findings underline the importance of incubation of *Agrobacterium* with acetosyringone prior to transformation thereby allowing activation of the bacterium's virulence genes.

In the literature, eggplant transformation efficiencies range from 7.6% to 30% (Khatun et al. 2022; Rotino and Gleddie 1990; Van Eck and Snyder 2006; Kumar and Rajam 2005). It is important to note that different cultivars of eggplant, tissue type, the binary vector used for transfomation, addition and concentrations of antibiotics, hormones and *vir* gene activating phenolic compounds all contribute to transformation efficiency. Because no previous publication used the Kemer cultivar for transformation experiments, our results cannot be directly compared with previous work.

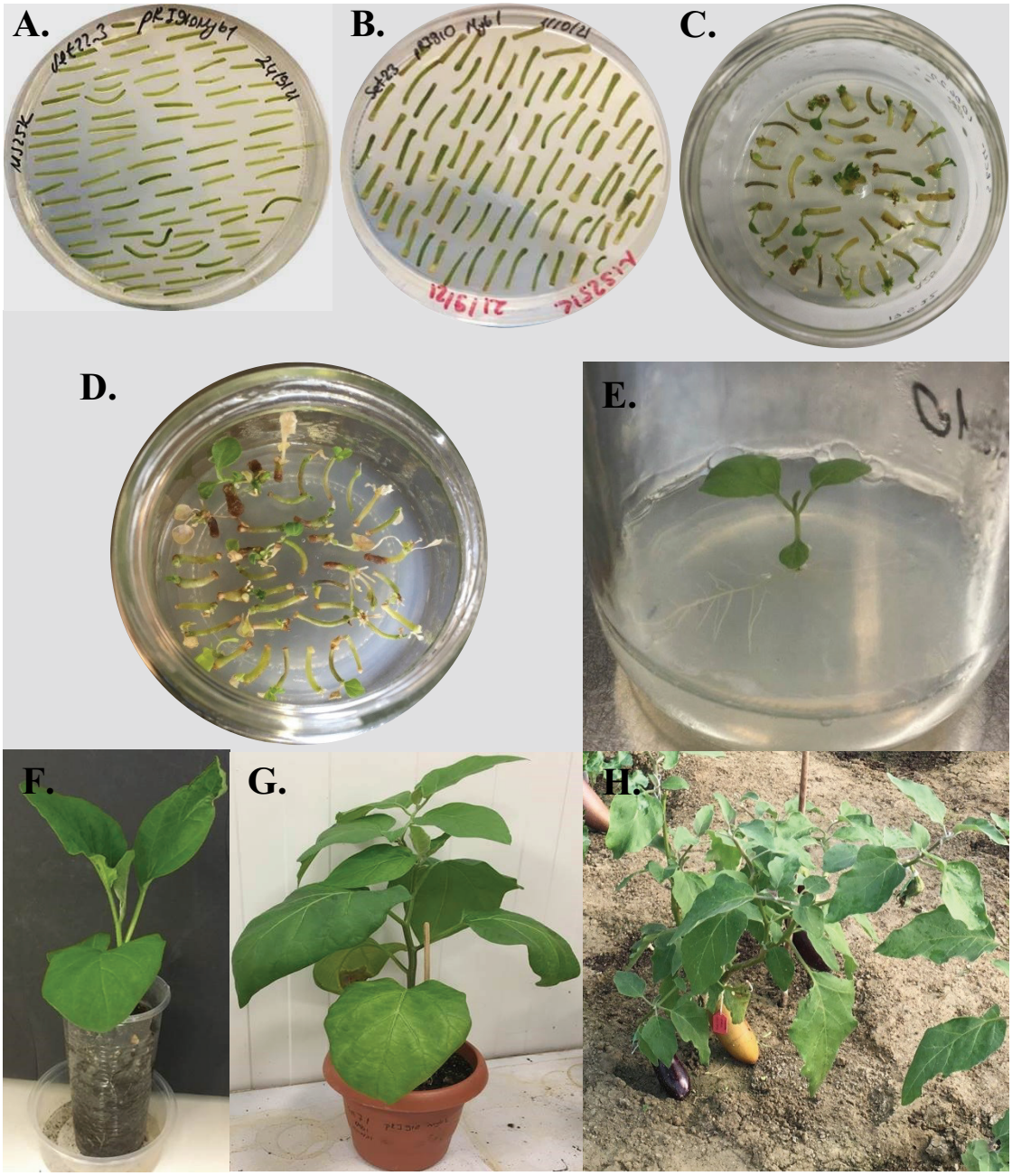

Figure 3.5. Plant regeneration from transformation within a 40-week period. (A) On the 3rd day, explants were incubated on antibiotic-free medium, (B) During the 1st week, explants were incubated on selectivemedium, (C) During the 4th week, shoots were visible, (D) During the 6th week, some shoots had grown large enough to transfer to RM, (E) During the 8th week, roots had formed, (F) By the 14th week, plants were transferred to soil, (G) During the 24th week, the plant was transferred to a larger pot, or to the greenhouse, (H) By the 40th week, fruit formation was observed.

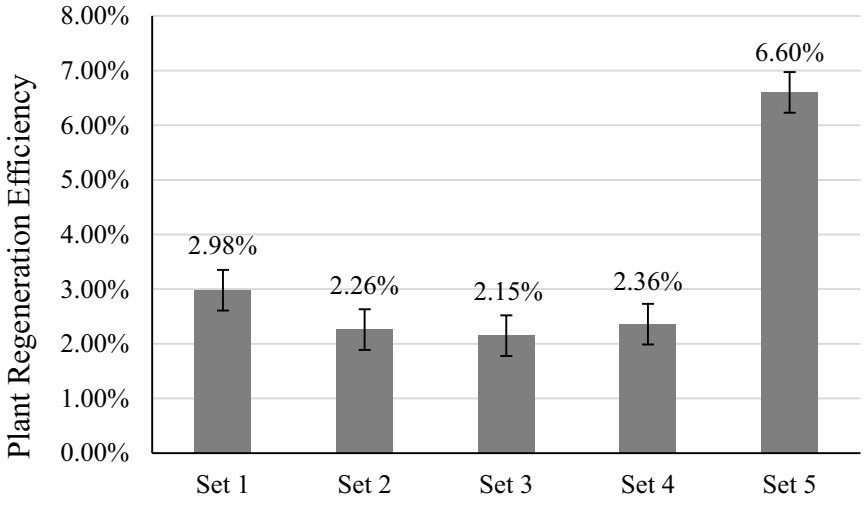

Figure 3.6. Whole plant regeneration efficiencies of each transformation set

3.4. Plant Transformation Verification

A PCR test was performed on DNA samples taken from 11 plants. The 35SMyb1 primers that amplified a 601 bp product spanning both the promoter region and *SmMyb1* gene were used for PCR screening. Seven of the 11 plants (63.6%) were verified as transgenic by PCR analysis as the expected bands were observed after agarose gel electrophoresis (Figure 3.7). The transgenic plants were those named S5T1, S5T3, S5T4, S5T6, S5T7, S5T 8, and S5T11. No band was observed in both non-template and non-transgenic plant negative controls, indicating the absence of contamination and the absence of amplification in non-specific regions by the primers. As positive control, pRI910 plasmid containing the *SmMyb1* gene was used. PCR products spanning 601 bp from the CaMV35S promoter and *Myb1* gene were sequenced by Sanger sequencing for two of the candidate transformants. This analysis also verified that plants are transgenic (Figure 3.8). However, a 6 bp insertion was observed in the transgenic plants in the CaMV35S promoter region when compared to the theoretical sequence generated by combining the known promoter and *Myb1* gene sequences. A possible explanation could be that the insertion of the *Myb1* gene into the CaMV35S promoter region during the cloning process resulted in some alterations in the surrounding sequence. Due to this discrepancy, future work should be done to sequence

the actual plasmid that was used in the transformation experiments to verify the source of this insertion.

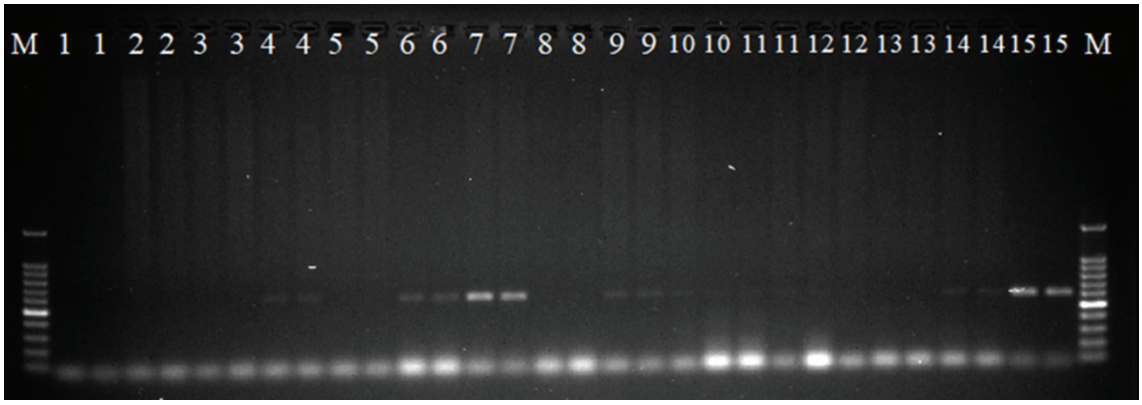

Figure 3.7. PCR verification of seven transgenic plants out of 11 regenerated plants for transformation set 5. M-marker (100bp), 1-non-template negative control, 2-3 non-transgenic plant samples, 4-14 tested plant samples named S5T1, S5T2, S5T3, S5T4, S5T5, S5T6, S5T7, S5T8 S5T9, S5T10, S5T11, 15-positive control for *SmMyb1* gene (601bp).

3.5. mRNA Expression Analysis

Expression analysis of the *Myb1* gene in young leaf samples, performed using RT-PCR and the Livak calculation method, demonstrated a range of mRNA expression levels across the seven transgenic plants (Figure 3.9). The relative mRNA expression levels of *Myb1* in transgenic plants were compared to non-transgenic plants, using the cyclophilin gene as a reference.

Myb1 mRNA expression levels of 2.37 (in plant S5T4), 1.81 (S5T6), and 2.53 (S5T11) are above 1, suggesting that the expression of *Myb1* in these transgenic plants is higher than in the non-transgenic plants. The high expression levels of the *Myb1* gene in these plants indicates successful integration and expression of the transgene. An additional plant, S5T8, had *Myb1* mRNA levels similar to the endogenous gene of non-transgenic control plants.

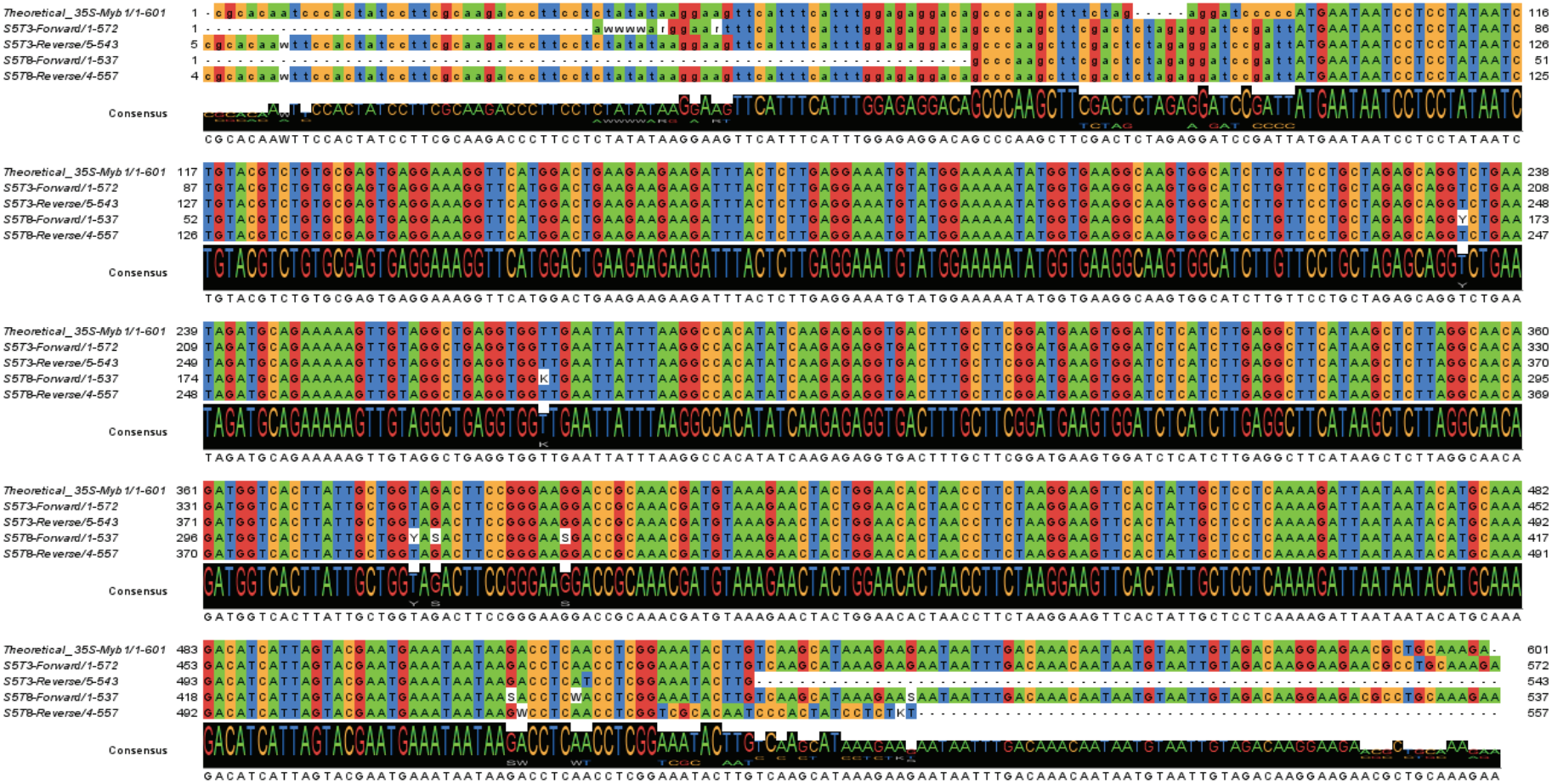

Figure 3.8. Sanger sequencing results for two transgenic plants using primers spanning the area from the CaMV35S promoter to the *Myb1* gene. The leftmost sample is the theoretical sequence based on the T-DNA construct. S5T3 and S5T8 are the transgenic plant samples. Lower case letters represent the CaMV 35S promoter region while upper case letters represent *Myb1* gene region. Image was generated by Jalview.

Three transgenic plants (S5T1, S5T3, and S5T7) demonstrated lower expression levels of the *Myb1* gene. This could potentially be due to gene silencing, a common phenomenon in transgenic plants which can occur as plant defense mechanisms against foreign DNA, including post-transcriptional gene silencing (PTGS) and DNA methylation. Post-transcriptional gene silencing is achieved by small interfering RNAs (siRNAs) that can target and degrade foreign mRNA sequences. Although Agrobacterium can counteract and inhibit siRNA synthesis, this does not necessarily prevent the limitation of gene expression levels (Dunoyer, Himber, and Voinnet 2006). Additionally, DNA methylation, a process where methyl groups are added to DNA, can suppress gene activity and could contribute to the reduced expression of *Myb1* in these lines (Gohlke et al. 2013). The probability of DNA methylation of an introduced gene depends on its location in the genome. This so-called 'position effect' is a result of the random integration of T-DNA into the plant genome (Matzke and Matzke 1998).

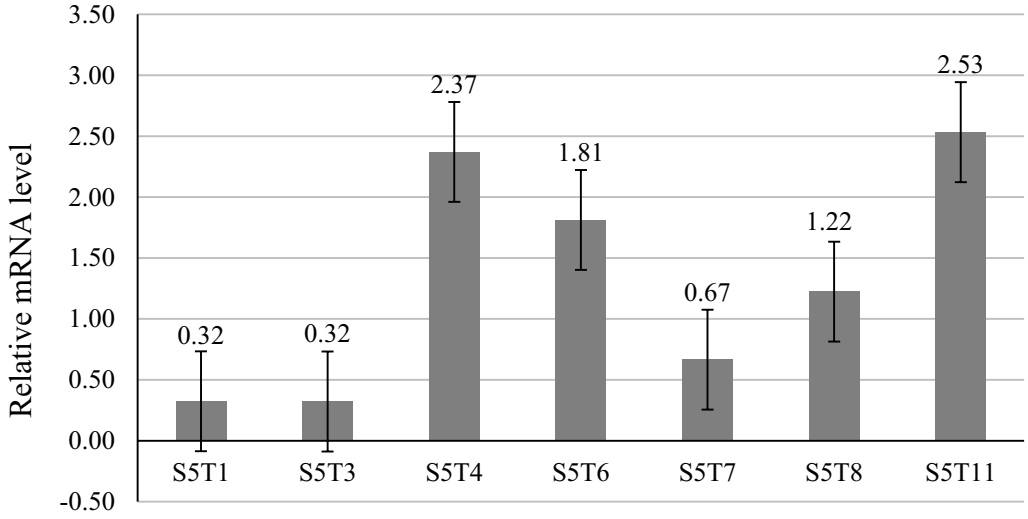

Figure 3.9. Relative mRNA expression levels of the *Myb1* gene in young leaves of the independent transgenic plants. Values are expressed relative to samples from untransformed plants

3.6. Total Phenolic Analysis

Young leaf samples were harvested from the transgenic plants during two seasons. The first samples were taken in the month of November and are called 'pre-

winter' samples. The second set was harvested in April and are called 'post-winter' samples. Total phenolic compound analysis by the Folin Ciocalteau method showed that leaves from the transgenic plants generally had higher total phenolic content compared to the non-transgenic plants, both pre and post-winter (Figure 3.10). Pre-winter phenolics content varied from 14.76 to 30.03 mg/g with all of the transgenic plants having significantly higher levels than the nontransgenic controls. Post-winter values were higher that pre-winter ones and ranged from 34.86 to 44.19 mg/g. During this season, six of the seven transgenic plants had significantly more total phenolic compounds than untransformed Kemer leaves. These results indicate that the introduction of the *SmMyb1* gene in the transgenic plants positively influenced the biosynthesis of phenolic compounds.

Correlation analysis was performed to determine the association between *Myb1* mRNA levels and total phenolics content. No significant correlation was observed across all seven transgenic plants. However, significant increases in total phenolic content were observed for the three plants with the greatest gene expression: S5T4, S5T6, and S5T11. These results suggest that the introduction of the *Myb1* gene enhanced phenolic compound synthesis. Despite the lower mRNA levels of plants S5T1, S5T3, and S5T7 their leaves also displayed statistically significant increases in total phenolic content post-winter. This could be due to several reasons. For one, gene expression does not always directly correlate with phenotypic outcomes due to the complexity of biological systems. Additionally, other regulatory mechanisms could be influencing the synthesis of phenolic compounds in plants. Phenolic content can be influenced by various environmental factors, including stress, which might not necessarily correlate with mRNA levels. Moreover, post-transcriptional regulation could be contributing to the synthesis of phenolic compounds in these plants.

The data suggest that the transgenic modification of eggplants with the *SmMyb1* gene enhanced the synthesis of phenolic compounds. In addition, the total phenolic content in all plants increased post-winter. The substantial increase in phenolic content in transgenic plants post-winter compared to pre-winter indicates a possible seasonality effect on the biosynthesis of phenolic compounds. Environmental stressors such as low temperatures during winter could induce plants to produce more phenolic compounds as part of their defense mechanism (Swigonska et al. 2014). In agreement with this, Garcia-Salas (2014) found that the phenolics contents of eggplants harvested in spring

were higher than those harvested in summer. The winter season appeared to further enhance the production of phenolic compounds triggered by introduction of the transgene. This could suggest a potential role of the *SmMyb1* gene in enhancing the plant's response to environmental stress, although this needs further investigation.

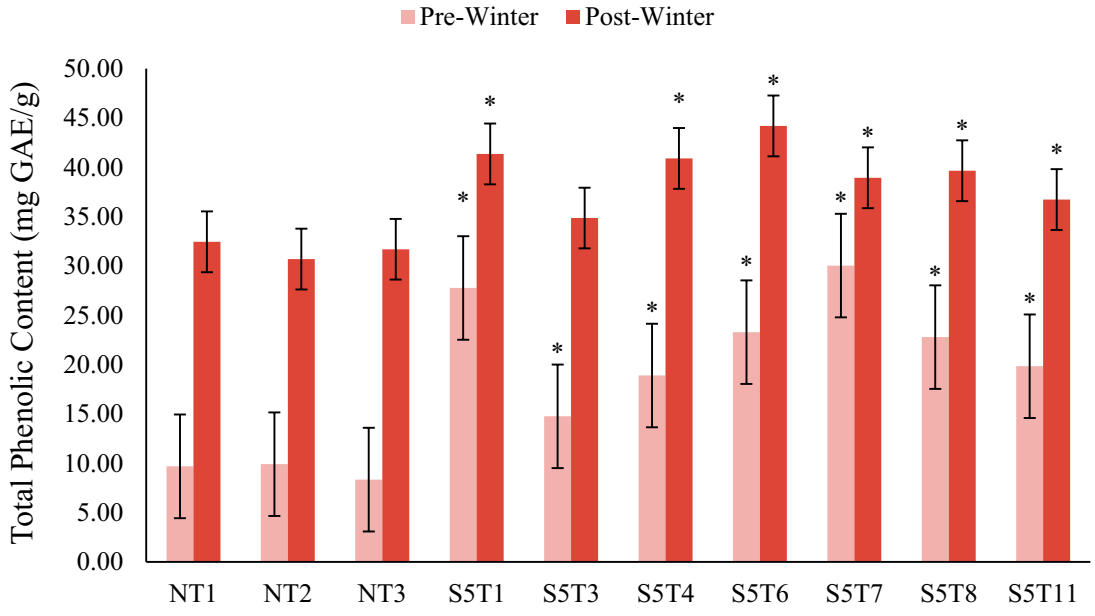

Figure 3.10. Seasonal variation in total phenolic content (GA equivalent) in non-transgenic (NT) and transgenic eggplants. Statistical significance as compared to non-transgenic plants was determined using a Student's t-test at $p < 0.01$.

3.7. HPLC Analysis

The results from the HPLC analysis of chlorogenic acid (3-CQA) were significantly and positively correlated (0.73) with the expression levels of the *Myb1* gene, demonstrating the effect of the *Myb1* gene on the biosynthesis of this phenolic compound.

The majority (71%) of the transgenic plants showed a statistically significant increase in 3-CQA content (Figure 3.11). Two-fold increases in *Myb1* expression of three plants (S5T4, S5T6, S5T11) corresponded with two-fold increases in 3-CQA

content. This strong association between *Myb1* expression and 3-CQA content further validates the role of the *Myb1* gene in regulating the biosynthesis of chlorogenic acid in eggplant.

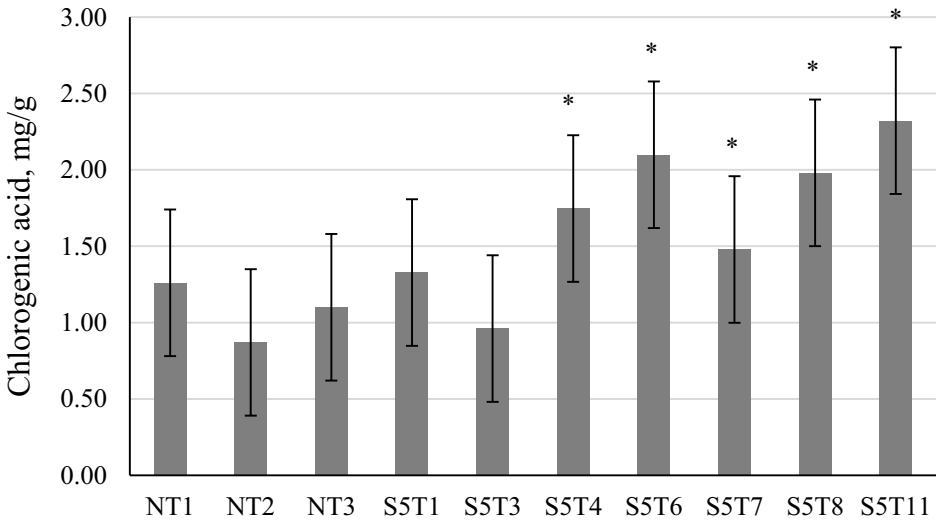

Figure 3.11. Post-winter variation in chlorogenic acid content (3-CQA equivalent) in non-transgenic (NT) and transgenic eggplants. Statistical significance was determined using a Student's t-test at $p < 0.01$.

It is important to mention that the standard used in this analysis, 3-CQA, is not the most abundant form of chlorogenic acid found in eggplant, which is 5-CQA (Stommel and Whitaker 2003; Meinhart et al. 2019). Due to unavailability of 5-CQA standard, 3-CQA was used instead. Hence the concentration of total chlorogenic acid may not be fully represented in our result. Further analysis with 5-CQA standard should be performed to confirm these results.

CHAPTER 4

CONCLUSION

The aim of this research was to enhance the production of chlorogenic acid (CGA) in eggplants through genetic modification. This was achieved by *Agrobacterium*-mediated transformation using the *Myb1* gene, which regulates CGA synthesis in eggplants. The *Myb1* gene which has a critical role in the phenylpropanoid pathway, was inserted into the PRI910 vector system. This vector was then successfully transformed into *Agrobacterium tumefaciens*. Among the 53 regenerated eggplants, 11 were initially screened using PCR. Out of these, seven plants were verified as being transgenic. When the mRNA expression levels in young leaves from the seven transgenic eggplants were analyzed, a two-fold increase was observed in three plants. Furthermore, the total phenolic content showed a significant increase in six plants, and five displayed a significant increase in 3-CGA content measured by HPLC analysis. Importantly, the *Myb1* expression level correlated with the CGA content. In three transformed plants, a two-fold increase in *Myb1* expression corresponded with a two-fold increase in 3-CGA content. This research indicates the potential of genetic modifications to improve the production of phenolic compounds such as CGA in eggplants. Given the wide climatic range where eggplants can be cultivated, they could serve as an alternative to coffee for the production of CGA, especially in non-tropical regions. Several important topics for future research have been identified. Further work should examine the CGA content in different parts of the fruit, such as the flesh and peel, using 5-CGA equivalent measurement. Additionally, next-generation T_1 plants need to be studied to confirm the stability of the transformation. In conclusion, this thesis presents a crucial first step towards understanding the utility of eggplant as a reliable source of CGA production. However, it also underlines the necessity for further research to study the implications and potential applications of this genetic transformation.

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